Repression of the pro-apoptotic *bik* gene by the Epstein-Barr virus.

A dissertation submitted for the degree of Ph.D.

By

Eva Campion B.Sc. (Hons) Biotechnology

Under the supervision of Dr. Dermot Walls

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School of Biotechnology, Dublin City University, Dublin 9, Ireland.

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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I dedicate this thesis to Mum and Dad,
For their never ending wisdom and love,
I am forever grateful.

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Abstract

Repression of the pro-apoptotic *bik* gene by the Epstein-Barr virus.

Eva Campion

The Epstein-Barr virus, or EBV, is a B-lymphotrophic herpesvirus, which is prevalent in over 95% of the population. EBV, the first human cancer virus to be discovered, is currently associated with numerous lymphoid and solid tissue malignancies in both immuno-competent and immuno-compromised individuals. Inhibition of B-cell apoptosis is central to the EBV strategy and may play an important role in the development of EBV-related diseases. The Bcl-2 family of proteins constitutes a critical checkpoint in the apoptotic cascade and it is now generally accepted that pro-apoptotic members of the Bcl-2 family are essential for the initiation of those pathways to apoptosis that are regulated by their anti-apoptotic relatives. The so-called BH3-only proteins, which share only the short BH3 domain with the other Bcl-2 family members, bind via the alpha-helical BH3 to pro-survival Bcl-2 proteins and this interaction is required for their ability to kill cells. Bik, the founding member of the BH3-alone group is a potent inducer of apoptosis, and the loss of Bik expression has been implicated in the development of cancers and the sensitisation of tumour cells to various therapeutic agents.

Here it is shown for the first time that that loss of Bik protein appears to be a general feature of cells expressing the EBV growth programme, in which the full spectrum of viral latent proteins are expressed, but not the more restricted EBV latency programme. Introduction of ectopic Bik led to apoptosis by a mechanism that is dependent on its BH3 domain. The data presented here demonstrates for the first time that the *bik* gene is activated through a transcriptional mechanism driven by EBNA2, and that c-Myc can also drive *bik* repression independently of EBNA2 during the EBV growth programme. It is also shown for the first time that inactivation of the EBV growth programme leads to the de-repression of *bik*. The findings indicate that EBNA2 may down-regulate *bik* by decreasing transcription from the *bik* promoter and that neither the Notch pathway associated protein CBF1 nor the EBNA2-CBF1 interaction are required for repression. The EBV latent membrane protein LMP1,

itself a major effector of phenotypic change in B-cells, did not affect *bik* expression. Together, these results indicate for the first time that down-regulation of the pro-apoptotic *bik* gene promotes B-cell survival during the EBV growth programme. It is concluded that this host-virus interaction contributes to the survival of EBV-infected B-cells proliferating on the viral growth programme, thus implicating a contributory role for Bik in setting the elevated threshold of resistance of these EBV-infected cells. Mechanistic studies of the contribution of Bik to cell survival will provide important information about both normal B-cell development and potential routes to B-cell and non-B-cell malignancy.

Table of Contents:

List of Figures

List of Tables

Abbreviations

A	Absorbance
A_{260}/ A_{280}	Absorbance at 260nm/ Absorbance at 280nm
aa	Amino acid
AAD	Amino-actinomycin D
ABI	Applied Biosystems
ACT	Activation Domain
AIDS	Acquired immuno-deficiency syndrome
Ank	Ankyrin
AML	Acute Myeloid Leukaemia
AP	Alkaline phosphatase
Apaf	Apoptotic protease activating factor 1
APS	Ammonium persulphate
ArgGly	Arginine–glycine
ATP	Adenosine tri-phosphate
BART	*Bam*HI A rightward transcript
BATF	B-cell specific transcription factor
BCA	Bicinchoninic acid
B-CLL	B-cell chronic lymphocytic leukaemia
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BCR	B-cell receptor
BCS	Bathocuproine disulfonic Acid
BD	Becton Dickinson Biosciences
BH	Bcl-2 homology
Bim-EL	Bim Isoform
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
BTM	Basal transcription machinery
Bu	Busulfan
C	Cytosine
Ca^{2+}	Calcium

CAD	Caspase Activated DNase
CAG	Chicken-β-actin
CAGp	Chicken-β-actin promoter
CAT	Chloroamphenicol acetyl transferase
CBF	C promoter binding factor
CBP	CREB Binding Protein
cdk	Cyclin dependent kinase
CDKI	Cyclin dependent kinase Inhibitor
cDNA	Complementary DNA
c-IAP	Cellular inhibitor of apoptosis
CIP	Calf intestinal phosphatase
CIR	Co-repressor of CBF1
CKII	Casein kinase II
CML	Chronic Myeloid Leukaemia
CMV	Cytomegalovirus
CO_2	Carbon dioxide
Cp	C promoter
CpG	Cytosine and Guanine
CR	Conserved region
CSL	CBF1/Su(H)/Lag-1/RBP-Jκ/KBF2
CST	Complementary-strand transcript
C_T	Threshold Cycle
CTAR	C-terminal activating region
CTL	Cytotoxic T Lymphocyte
C-terminal	Carboxy terminal
DD	Death domain
DEAE	Diethyl aminoethyl
DEPC	Diethylpyro-carbonate
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNMT	DNA Methyltransferase
dNTP	Deoxyribonucleotide

DOSPA	2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,Ndimethyl-1-propanaminium trifluoroacetate
DOPE	Dioleoyl phosphatidylethanolamine
DRP	Dynamin-related protein
DS-DNA	Double stranded DNA
DSL	Delta, Serrate and LAG-2
E. coli	*Escherichia coli*
EA	Early antigen
EA-D	Diffuse early antigen
EA-R	Restricted early antigen
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen-leader protein
EBV	Epstein-Barr virus
EBV-1/A	EBV Type I/A
EBV-2/B	EBV Type II/B
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
ERE	EBNA2-responsive element
ER/EBNA2	Estrogen-responisve EBNA2
EtBr	Ethidium bromide
ETOH	Ethanol
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain
FAM	6-carboxyfluorescein
FAS	Fatty acid synthase
FBS	Foetal bovine serum
FSC	Forward light scatter
G	Growth Phase
Gal	Galactosidase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Germinal centre

GFP	Green fluorescent protein
Gly	Glycine
Gly-Ala	Glycine Alanine
gp	Glycoprotein
HA	Hemagglutinin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDAC i	Histone deacetylase Inhibitor
HL	Hodgkin's lymphoma
HP1α	Heterochromatin protein 1
HRP	Horseradish peroxidase
H/RS	Hodgkin/Reed-Sternberg
HRT	Hairy-related transcription factor
HSCT	Haematopoietic stem cell transplantation
HSV	Herpes simplex virus
IAP	Inhibitor of Apoptosis Protein
IC	Intracellular
ICAD	Inhibitor of Capase activated DNase
ICAM	Intercellular cell adhesion molecule
Ig	Immunoglobulin
IgK	Immunoglobulin K
IgG	Immunoglobulin G
IgH	Immunoglobulin H
IgL	Immunoglobulin L
IgM	Immunoglobulin M
IκB	Inhibitor of κB
IKK	IκB kinase complex
IL	Interleukin
IM	Infectious mononucleosis
IR	Internal repeat
IRES	Internal ribosomal entry site
JAK	Janus kinase
JNK	c-Jun NH_2 terminal kinase
Lat I	Latency I

Lat III	Latency III
LB	Luria-Bertani broth
LCL	Lymphoblastoid cell line
LFA	Lymphocyte-function-associated antigen
LMP	Latent membrane protein
Luc	Luciferase
M	Mitosis
M/MW	Molecular weight marker
MA	Membrane antigen
mAb	Monoclonal antibody
Mam	Mastermind
MAML	Mastermind in Drosophila
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MCS	Multiple cloning site
MEF	Mouse Embryonic Fibroblast
MEK	MAP/ERK kinase
MEKK	MAPK/ERK kinase
MG132	Z-Leu-Leu-Leu-al
MHC	Major histocompatability complex
M-MLV RT	Moloney Murine leukemia virus reverse transcriptase
MMP	Mitochondrial Membrane Potential
MOMP	Mitochondrial Outer Membrane Potential
mRNA	messenger RNA
myrBid	myristoylated Bid
MZ	Mantle Zone
NaB	Sodium Butyrate
NEB	New England Biolabs
NF-κB	Nuclear factor κB
NHL	Non-Hodgkin lymphomas
NIK	NF-κB inducing kinase
NK	Natural killer cell

NLS	Nuclear localisation signal
NotchIC	Notch intracellular domain
NPC	Nasopharyngeal carcinoma
Nt	Nucleotide
N-terminal	Amino terminal
O.D.	Optical density
ObsCpG/ExpCpG	Observed CpG content/Expected CpG content
OHL	Oral Hairy leukoplakia
OMM	Outer Mitochondrial Membrane
ONPG	*o*-nitrophenyl-*β*-D-galactopuranoside
ORF	Open reading frame
oriP	Origin of replication
oriLyt	Lytic Origin of replication
p53	Protein 53
p38/MAPK	p38/mitogen activated protein kinase
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP ribose) polymerase
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PE	Phycoerythrin
pH	Power of the hydrogen ion
PI	Propidium Iodide
PI3-K	Phosphatidylinositol 3-kinase
PML NB	Promyelocytic leukaemia nuclear bodies
PMSF	Phenylmethanesulphonylfluoride
pRb	Retinoblastoma protein
PS	Phosphatidyl serine
PTK	Protein tyrosine kinase
PTLD	Post transplant lymphoproliferative disorder
PTP	Permeability transition pore
Qp	Q promoter
QPCR	Quantitative PCR
R	Region

RAM	RBP-Jκ-associated molecule
$RbCl_2$	Rubidium chloride
RBP-Jκ	Jκ-recombinant-binding protein
RBS	Ribosome binding site
RCC	Renal Cell Carcinoma
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNase	Ribonuclease
ROX	6-carboxyl-X-rhodamine
RPA	Ribonuclease protection assay
RPMI	Roswell Park Memorial Institute
RSK	Ribosomal S6 kinase
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
S	Synthetic Phase
SAHA	Suberoylanilide hyrdoxamic acid
SAM	S-adenosylmethionine
SAP30	SIN3-associated protein 30
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
sIgG	Surface immunoglobulin G
Sin3A	SIN3 homolog A
SKIP	Ski-interacting protein
SMRT	Silencing mediator of retinoid and thyroid hormone receptors
SSC	Side light scatter
STAT	Signal transducers and activators of transcription
SuH	Supressor of hairless
SV40	Simian virus 40
+/- T	Plus/minus tetracycline
TACE	Tumour necrosis factor α-converting enzyme
TAD	Trans-activation domain
TAE	Tris acetate ethylenediamine tetraacetic acid
TAMRA	Tetramethyl-6-carboxyrhodamine

tBid	Truncated Bid
TBS	Tris buffered saline
TBS-T	Tris buffered saline + Tween 20
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tet	Tetracycline
TESS	Transcription Element Search Software
TFB	Transformation buffer
TGF	Transforming growth factor
T_m	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TR	Terminal repeat
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factors
TRAIL	TNF-related apoptosis inducing ligand
TSA	Trichostatin A
tTA	Tetracycline Trans-activator
upH_20	Ultra pure water
UV	Ultra violet
UTR	Untranslated Region
UP	Undecylprodigiosin
VCA	Viral capsid antigen
VDAC	Voltage dependent anion channel
v-IL	Viral Interleukin
v/v	Volume per volume
Wp	W promoter
wt	Wild type
w/v	Weight per volume
XIAP protein	X-linked mammalian inhibitor of apoptosis
zVAD-fmk	*N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone

β-gal	Beta-galactosidase
ΔBH3	BH3 domain deleted
$\Delta\Delta C_T$	Delta Delta C_T
ΔCBF1	CBF1 deleted
ΔTK	Tk promoter deleted
+1	Transcriptional Start Site
3D	Three dimensional
5azaC	5-Azacytidine

Units

%	Percentage
˚C	Degrees Celsius
bp	Base pairs
cm	Centimetre
g	Grams
H	hours
Kb	Kilobase pairs
kDa	Kilo Dalton
Kg	Kilogram
L	Litres
lb/sq	Pounds per square inch
M	Molar
mA	Milliamperes
mg	Milligrams
ml	Millilitres
mM	Millimolar
ng	Nanograms
nm	Nanometres
nt	Nucleotides
pmole	Picomoles
rpm	Revolutions per minute
U	Enzyme units
V	Volts
x g	Relative centrifugal force
W	Watts
µF	Micro Faraday
µg	Micrograms
µl	Microlitre
µM	Micromolar
µm	Micrometer/ micron

Publications

Manuscripts

- **Campion, E.M**., Loughran, S.T**.,** D'Souza, B.N., Phelan, S., Kempkes, B., Bornkamm, G., Hayward, D. and Walls, D. Downregulation of the Pro-apoptotic Nbk/Bik Gene During the EBV Growth Programme. (Manuscript in preparation, May 2008).

- Loughran, S.T., **Campion, E.M.,** D'Souza, B., Murray, P., Bornkamm, G., Walls, D. *Bfl-1* is a crucial pro-survival Nuclear factor kappa B target gene in Hodgkin/Reed-Sternberg cells (Manuscript in preparation, May 2008).

- McMahon, K.J., Minihan, D., **Campion, E.M**., Loughran, S.T., Allan, G., McNeilly, F. and Walls, D. (2006). Infection of pigs in Ireland with lymphotropic [gamma]-herpesviruses and relationship to postweaning multisystemic wasting syndrome. *Veterinary Microbiology.,* 116, 60-68.

Presentations: Posters

- **Campion, E.M**., Loughran, S.T**.,** D'Souza, B.N., Phelan, S., Kempkes, B., Bornkamm, G., Hayward, D. and Walls, D. Downregulation of the Pro-apoptotic Nbk/Bik Gene During the EBV Growth Programme. Royal Academy of Medicine in Ireland, Winter Meeting, Dublin City University. Dublin, January 11th 2007 and the Tumour Associated Herpes Viruses 13th International Symposium on Epstein Barr Virus and associated malignant Disease. Boston, USA, 8th – 12th July 2006.

- Loughran, S.T**, Campion, E.M,** D'Souza, B.N, Murray, PG, Bornkamm G, Walls D. Irish Association for Cancer Research Conference 2008. Down, 29th February-1st March 2008. *Bfl-1* is a crucial pro-survival Nuclear factor kappa B target gene in Hodgkin/Reed-Sternberg cells of Hodgkin's Lymphoma.

- Loughran, S.T., D'Souza, B.N., Smith, S.M., **Campion, E.M**., McGee, S., Tam, L., and Walls, D. Irish Association for Cancer Research Conference 2005. Kilkenny, 11th-12th March 2005. The cellular anti-apoptotic *bfl-1* gene is highly expressed in cultured Hodgkin/Reed Sternberg cells from Hodgkin's Lymphoma and is regulated by NF-κB.
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- Loughran, S.T., D'Souza, B.N., Smith, S.M., **Campion, E.M.,** McGee, S., Tam, L., and Walls, D. The cellular anti-apoptotic *bfl-1* gene

is highly expressed in cultured Hodgkin/Reed Sternberg cells from Hodgkin's Lymphoma and is regulated by NF-κB**.** Irish Association for Cancer Research Conference 2005. Kilkenny, 11th-12th March 2005.

Presentations: Oral

- **E.M Campion**. Role and regulation of the cellular *bik* gene during EBV-infection and associated malignancies**.** Transfer from MSc to Ph.D presentation, 25th August 2005.

CHAPTER 1

Introduction

1.1 The discovery of the Epstein Barr Virus

The Epstein Barr Virus (EBV) was first identified in 1964 in cell cultures derived from a Burkitt's lymphoma (BL) biopsy (Epstein *et al*, 1964b). Denis Burkitt, first described this common aggressive B-cell lymphoma primarily affecting children in specific regions of Africa in the late 1950s and early 1960s (Burkitt and O'Conor, 1961; Burkitt, 1962a). Burkitt suspected a virus might be responsible for the cancer, given the climatic and geographic distribution of the lymphoma as well as its increased incidence in African children, but was unsuccessful in identifying a candidate agent (Burkitt, 1962b; Burkitt and Wright, 1966). EBV was not successfully identified until 1964 when Anthony Epstein along with colleagues Yvonne Barr and Bert Achong succeeded in culturing lymphoma cells from BL patients and discerned herpesvirus particles in these cells by electron microscopy (Epstein and Barr, 1964a; Epstein *et al*, 1964b) (As reviewed in Kutok and Wang, 2006). Further studies revealed that sera from patients with the lymphoma that Burkitt had described had much higher antibody titres to EBV than did the controls. Subsequently EBV DNA was detected in BL and the experimental production of lymphomas in cotton-top marmosets and owl monkeys linked this infection with Burkitt's lymphomas and identified EBV as the first virus directly associated with a human cancer (Thompson and Kurzrock, 2004). Later, epidemiological studies made it clear that BL is a rare neoplasm relative to the large pool of EBV-infected individuals. EBV is present in all populations, infecting more than 95% of human beings within the first decades of life (Thompson and Kurzrock, 2004).

1.2 Classification

Humans are the exclusive natural host for EBV/ HHV4 (Middeldorp *et al*, 2003; Thompson and Kurzrock, 2004), which is classed as a herpes virus based on its structural properties (IARC Monographs, 1997). EBV is one of the most extensively characterised members of the gamma subfamily of herpesviruses. This subfamily includes two genera, the gamma-1 herpesviruses, or lymphocryptoviridae (LCV), and the gamma-2 herpesviruses, or rhadinoviridae (RV). EBV belongs to the former. LCV are found only in primates, with EBV representing the only LCV to infect humans

(Kutok and Wang, 2006). The gamma-herpes viruses are classified on the basis of their biological properties rather than their genomic organisation (Kieff, 1996). Common features of these viruses are their tropism for lymphoid cells and their capacity to induce cell proliferation *in vivo* and cell immortalisation *in vitro* (Middeldorp *et al*, 2003).

1.3 Structure of the virion.

EBV is a 172 kilo-base pair (kbp) linear double-stranded DNA genome encoding more than 85 genes. The viral genome consists of a series of 0.5kb terminal direct repeats at either end, and internal repeat sequences that serve to divide the genome into short and long unique sequence domains that harbour most of the coding capacity (as reviewed in Young and Murray, 2003; Thompson and Kurzrock, 2004). Similar to other herpesviruses, EBV has a toroid-shaped protein core wrapped with double-stranded DNA (Figure 1.3.1). This DNA wrapped protein core is contained within a nucleocapsid with 162 capsomeres approximately 100 nanometres (nm) in diameter. A protein tegument surrounds the nucleocapsid and this is enclosed by the outer envelope, which has external glycoprotein spikes, the most abundant of which is Gp350/220 (Middeldorp *et al*, 2003; Thompson and Kurzrock, 2004).

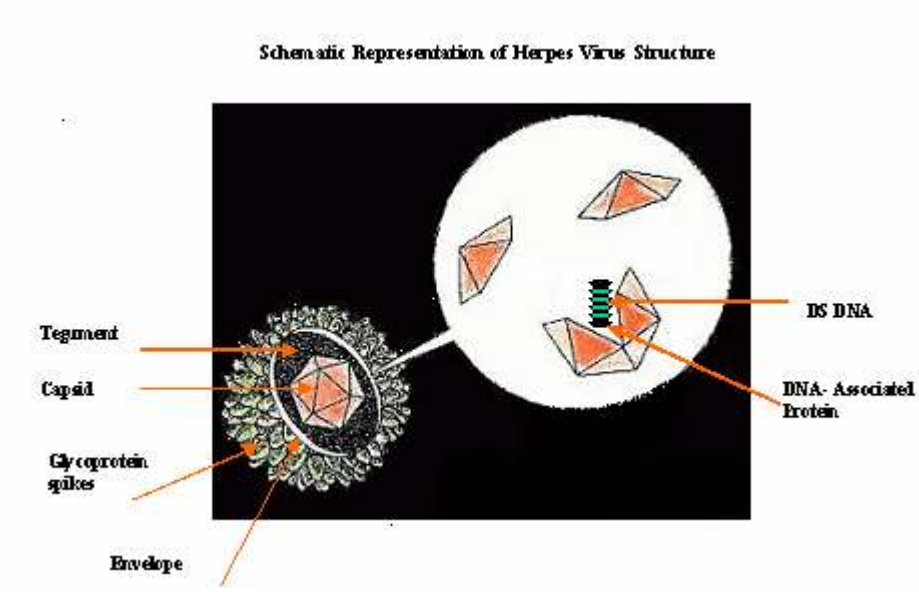


Figure 1.3.1: A Schematic representation of the herpes virus structure.

EBV consists of a toroid protein core (illustrated in black) that is wrapped with double-stranded DNA (DS DNA, green). The DNA wrapped protein core is contained within a nuclecapsid (red). A protein tegument surrounds the nucleocapsid and is enclosed by an outer envelope with external glycoprotein spikes.

1.4 EBV Strains

EBV strains are categorised into two distinct types; EBV type I and EBV type II (or EBV-A and EBV-B respectively). EBV-1 and EBV-2 differ in the organisation of their EBV nuclear antigen (EBNA)2 genes, and in their EBNA3A, EBNA3B, and EBNA3C genes (Dambaugh *et al*, 1984b; Sample *et al*, 1990). Minor 'sub-strain' variations in the EBNA1, latent membrane protein 1 (LMP1), and BZLF1 have also been reported (Middeldorp *et al*, 2003). EBV-2 is known to transform B-cells less efficiently than EBV-1 *in vitro* and the viability of EBV-2 lymphoblastoid cell lines (LCLs) is also less than that of EBV-1 cell lines (Buisson *et al*, 1994; Middeldorp *et al*, 2003). The difference in transforming efficiency of the EBV subtypes is thought to relate to a variation in the EBNA2 sequences (Rickinson *et al*, 1987; Cohen *et al*, 1989 and also reviewed in Thompson and Kurzrock, 2004).

1.5 Epidemiology of EBV

EBV-1 and EBV-2 show considerable geographical variation (Thompson and Kurzrock, 2004). However, there is no apparent correlation between genotype and the specific type of neoplasma found, nor is there a relationship between genotype and healthy individuals or neoplasma patients (Khanim *et al*, 1996). Type 1 is detected worldwide, generally in Central Africa, Papua New Guinea, North America and Asia, whereas type 2 is less ubiquitous, detected mainly in southern countries such as Central Africa, Reunion Island and Papua New Guinea (reviewed in Thompson and Kurzrock, 2004; Serraino *et al*, 2005).

1.6 EBV Associated Malignancies

While EBV infection is generally benign without any detectable clinical symptoms in the majority of carriers, the link connecting EBV infection and human tumours is irrefutable. The presence of EBV in various stages of B-cell development, and its ability to infect certain epithelial cells, can have pathogenic consequences, and can contribute to the development of a diverse group of lymphomas and carcinomas in a small percentage of individuals (Thompson and Kurzrock, 2004; Kutok and Wang, 2006). In this regard EBV has been detected in tissues from patients with Nasopharyngeal carcinoma (NPC), non-Hodgkin lymphoma (NHL), and T-cell and Hodgkin lymphomas (HL) (as reviewed in Kutok and Wang, 2006) and is currently associated with numerous lymphoid and solid tissue malignancies in both immuno-competent and immuno-compromised individuals (Thompson and Kurzrock, 2004; Kutok and Wang, 2006). Known EBV-associated diseases are listed in Table 1.6-1.

Table 1.6-1: The spectrum of EBV-associated disease.

Disease State	Host Status	Specific Site Of Infection	Associated Malignancy
Non-malignant			Oral hairy leukoplakia, Chronic active infection, Infectious mononucleosis (IM)
Malignant	**Immuno-compromised**	**B-cell**	AIDS-associated B-cell lymphomas, Post-transplantation lymphoproliferative disorder (PTLD), Lymphomatoid granulomatosis, Methotrexate-associated B-cell lymphoma Severe combined immuno-deficiency–associated B-cell lymphomas, Wiskott-Aldrich syndrome–associated B-cell lymphomas, X-linked lymphoproliferative disorder–associated B-cell lymphomas
		Mesenchymal	Leiomyosarcoma
	Immuno-competent	**B-cell**	Burkitt lymphoma, Classical Hodgkin lymphoma
		T-cell	Extranodal NK/T-cell lymphoma, (nasal type), Virus-associated hemophagocytic syndrome T-cell lymphomas
		Epithelial	Nonglandular nasopharyngeal carcinoma, Lymphoepithelioma-like carcinoma (salivary, thymus, lungs, stomach), Breast carcinoma, Hepatocellular carcinoma
		Mesenchymal	Follicular dendritic cell sarcoma

Adapted from Kutok and Wang, 2006.

1.7 EBV infection and persistence *in vivo*.

EBV is transmitted from host to host via salivary contact. Upon infection, the individual remains a lifelong carrier of the virus. Primary infections occurring during the first few years of life are generally silent or asymptomatic (Thompson and Kurzrock, 2004). In developed countries, primary infection can be delayed until late adolescence or early adulthood and may result in the self-limiting non-malignant lymphoproliferative syndrome infectious mononucleosis (IM), which is also referred to as glandular fever or 'kissing disease' (Gerber *et al*, 1972; IARC Monographs, 1997; Kuppers, 2003). Infection that is delayed and associated with IM is characterised by a variety of symptoms, including fever, malaise, lymphadenopathy (obvious swelling of peripheral lymphoid tissues) and the presence of atypical dividing lymphocytes in the peripheral blood (Kutok and Wang, 2006). The development of an EBV-specific immune response controls primary infection, but it is unable to eliminate the infection completely from the body since the virus can hide within long-lived memory B-cells. Thus, EBV infection persists asymptomatically during the host's life, maintaining a perpetual equilibrium between the immune response and a stealthy virus infection. A low level of active viral replication continues asymptomatically in EBV carriers, leading to virus shedding via oral secretions and transmission of EBV from one individual to another (Kutok and Wang, 2006).

EBV has two distinct life cycles in the human host; a lytic form of infection that describes the full cycle of infection, leading to the production of new infectious virions, and eventual lysis of the infected cell, and a latent form of infection that allows the virus to persist in a dormant state for the lifetime of the host in circulating memory B-cells (Thorley-Lawson, 2001). Incoming virus establishes a primary focus of lytic replication at the oropharyngeal epithelium. Oropharyngeal infection is followed by a latent infection of B-cells as they pass in close proximity to the infected epithelial cells (Figure 1.7.1), resulting in B-cell proliferation and viral spread throughout the B-cell compartment (Young and Rickinson, 2004).

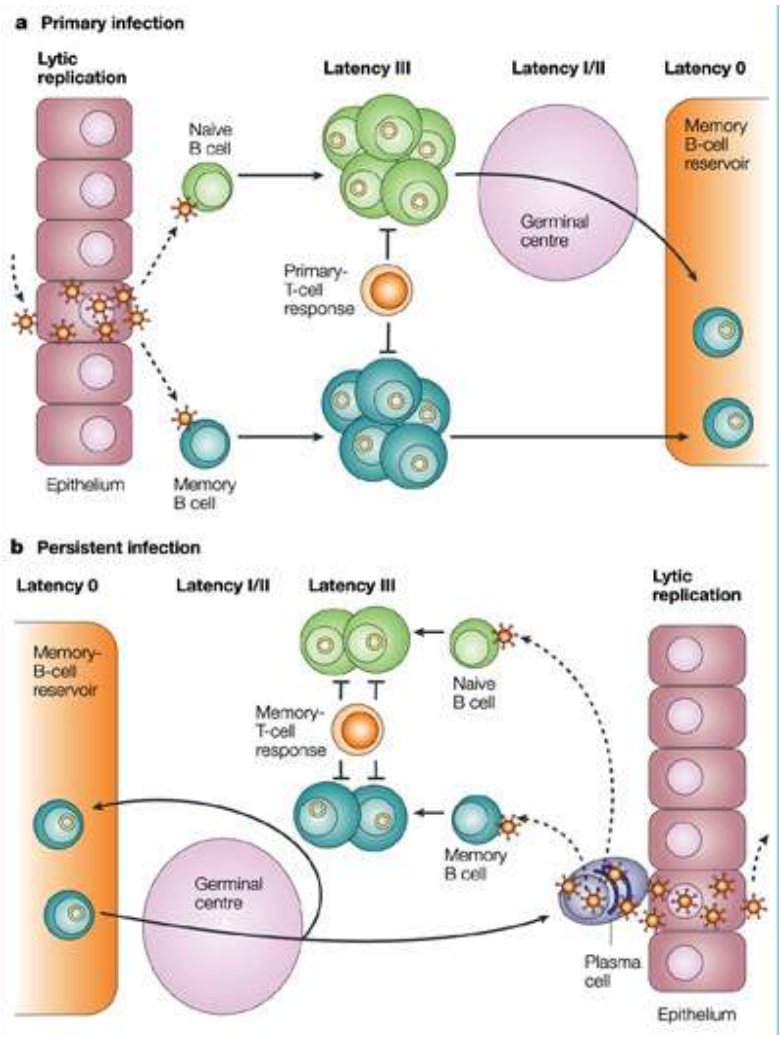


Figure 1.7.1: Putative *in vivo* interactions between Epstein-Barr virus and host cells

(A) Primary EBV infection. Different views of these events are described in the text and are illustrated above. (B) Persistent EBV infection. (From Young and Rickinson, 2004).

EBV infection of B-cells is instigated with the attachment of the gp 350/220 viral membrane glycoprotein to the CD21 molecule on lymphocytes (Middeldorp *et al*, 2003). After-attachment CD21 becomes cross-linked, which triggers an initial activating signal that is thought to prepare the cell for EBV infection. EBV binding to CD21 immediately activates tyrosine kinase *lck* and mobilises calcium (Gordon *et al*, 1986; Cheung and Dosch, 1991). This is followed by an increase in mRNA synthesis,

blast transformation, homotypic cell adhesion, surface CD23 expression (a characteristic surface marker for activated B-cells), and interleukin (IL)-6 production (as reviewed in Thompson and Kurzrock, 2004). The viral genome is then uncoated and delivered to the nucleus where it immediately becomes a circular episome. Circularisation and W (EBNA) promoter expression launch an ordered sequence of events that leads to the expression of all of the EBNA proteins and the three latent membrane proteins (LMPs) (Young and Rickinson, 2004). EBV does not encode an RNA polymerase, and uses host cell RNA polymerase II for transcription of viral RNAs (Kieff, 1996). The EBV nuclear antigen leader protein (EBNA-LP) and EBNA2 proteins are the first proteins to be detected upon EBV infection (Middeldorp *et al*, 2003). A promoter shift occurs 24 to 48 hours after infection where the C promoter (Cp) is used in favour of the initial W promoter to drive EBNA expression (reviewed in Rowe, 1999). Many transcripts now pass the polyladenylation site downstream of EBNA2 and extend to the EBNAs 1, 3A, 3B and 3C (Kieff, 1996) open reading frames (ORFs) located downstream (Figure 1.7.2). The different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long "rightward" primary transcript expressed from either Cp or Wp which are located in close proximity to each other in the *Bam*HI C and W regions of the genome (Murray and Young, 2001; Middeldorp *et al*, 2003) (Figure 1.7.2). The processing of the transcripts is then determined by their polyadenylation sites (Kieff, 1996). All of the EBNA transcriptional products are involved in transcriptional control and participate in the activation of the expression of the viral LMP-encoding genes (LMP1 and LMP-2) and several cellular genes (as reviewed in Thompson and Kurzrock, 2004). The combined action of these viral and cellular proteins serves to initiate cellular S-phase 24 to 48 hours after infection (Rowe, 1999).

By 32 hours post-infection, all of the EBNAs and the LMPs are expressed. The LMP transcripts are expressed from separate promoters in the *Bam*HI N region of the EBV genome, with the "leftward" LMP1 and "rightward" LMP2B mRNAs apparently controlled by the same bi-directional promoter. EBV encoded RNA (EBER) expression follows at approximately 24 hours and does not reach substantial levels until approximately 70 hours after-infection (Murray and Young, 2001). A diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome is shown in Figure 1.7.3.

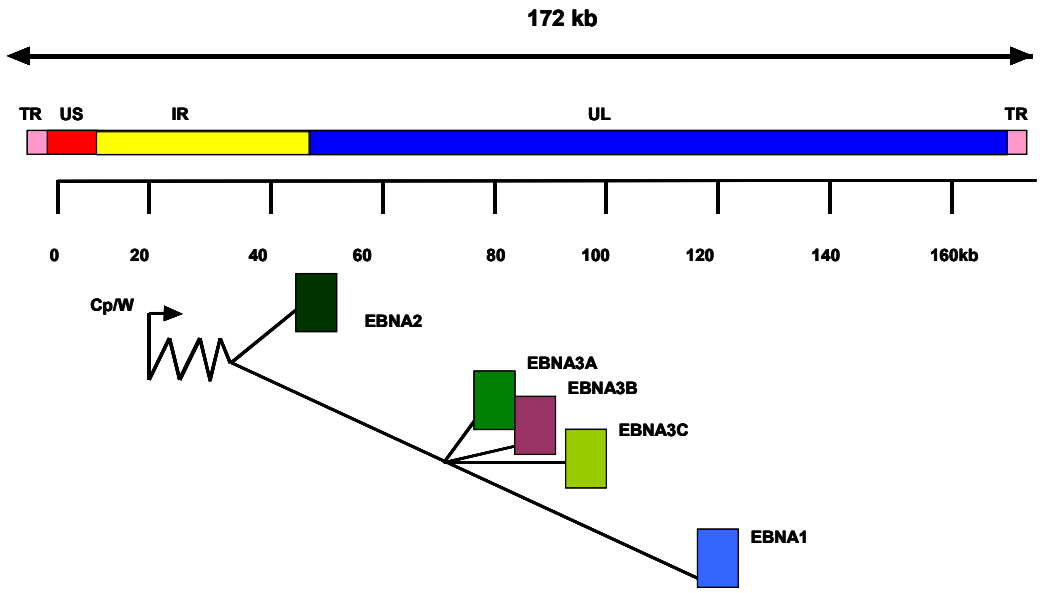


Figure 1.7.2: A simplified outline of the splicing of the EBV nuclear antigen coding mRNAs.

All the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs generated by differential splicing and alternate polyadenylation.

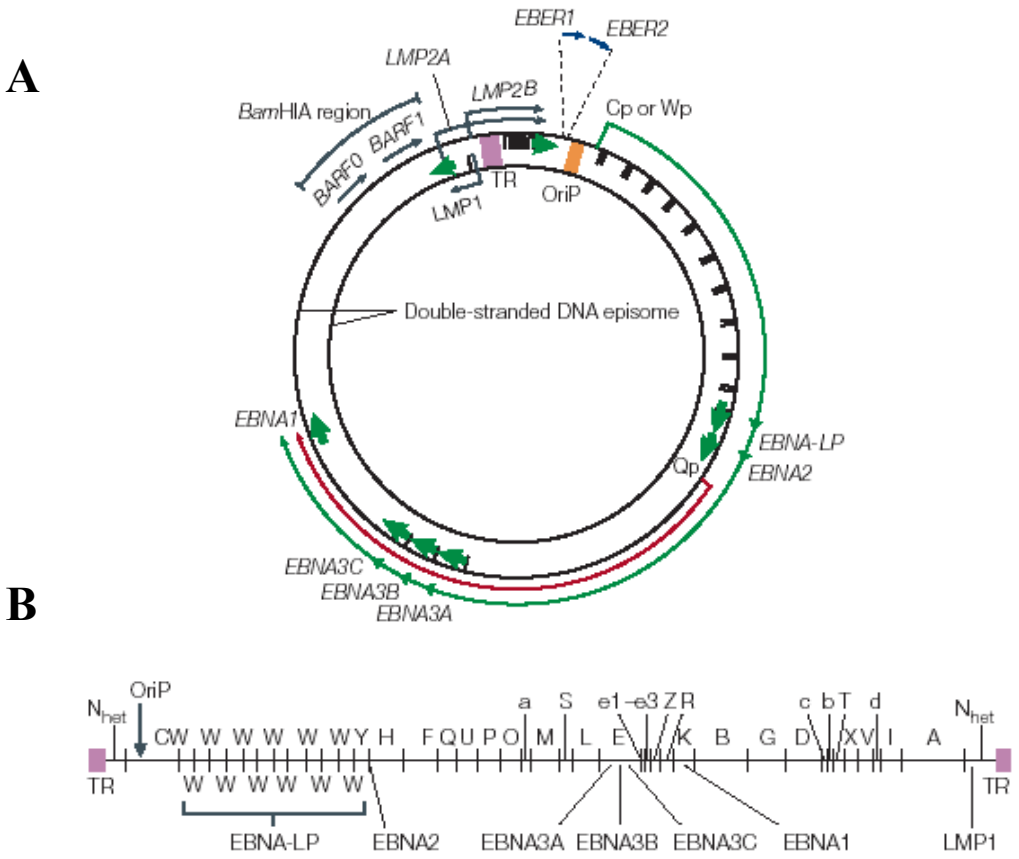


Figure 1.7.3: EBV latent genes.

(A) A schematic representation indicating the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The origin of replication (OriP) is shown in orange. The large green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which the genes are transcribed. The terminal repeat (TR) region, formed during the circularisation of the linear DNA to produce the viral episome is shown in purple. The blue arrows at the top represent *EBER1* and *EBER2*. The long outer green arrow represents EBV transcription during type III latency, in which all the EBNAs are transcribed from either the Cp or Wp promoter. The inner, shorter red arrow represents the *EBNA1* transcript, which originates from the Qp promoter during latency type I and latency type II. The locations of the BARF-0 and BARF-1 coding regions are also illustrated. (B) Locations of the open reading frame (ORF) for the EBV latent proteins on the *Bam* HI restriction endonuclease map of the prototype B95-8 genome (From Young and Rickinson, 2004).

In addition to its recognised B-cell tropism, EBV infection may also target epithelial cells, T-cells, and cells of the macrocytic, granulocytic, and natural killer lineages (Thompson and Kurzrock, 2004). It is thought that these cells may be infected by mechanisms other than the CD21-mediated pathway used in B-cells, given that epithelial cells generally do not express CD21 (Thompson and Kurzrock, 2004). Various human epithelial cells can be infected *in vitro* either by direct contact with high-titre virus supernatant or by co-cultivation with EBV-producing B-cells, such as AKATA (Imai *et al*, 1998). This suggests an *in vivo* model of EBV infection whereby epithelial tissues might be infected by virtue of their close proximity to (i) infectious virus present in saliva or (ii) lytically infected B-cells resident near or within epithelial tissues, for example adjacent to the sub-epithelial sinus in tonsil or within nasopharyngeal mucosa (Murray and Young, 2001).

After initial infection, the EBV genome persists in a latent form (latency III), circulating in a division of the resting memory B-cells in healthy individuals at a low frequency of approximately 1 in 1 X 10^5 to 1 X 10^6 cells. Immuno-suppressive states allow spontaneous replication of the episomal virus in circulating B-cells (as observed in acute IM). Immuno-competent carriers control latent EBV infection via their cytotoxtic T lymphocyte (CTL) response. Some of these proliferating cells avoid detection by down-regulating antigen expression and establishing a stable reservoir of resting EBV-positive memory B-cells, in which viral antigen expression is mostly suppressed (latency 0). EBNA3A, EBNA3B, and EBNA3C all elicit specific CTL responses and the loss of the EBV-specific CTL may play an important role in EBV-associated malignancies (Young and Rickinson, 2004).

EBV is persists in the peripheral blood within the IgD^- memory B-cell pool, with a limited EBV gene expression of latent membrane protein 2A (LMP2A) and EBNA1 (Babcock *et al*, 1998). The exact route of entry to the memory B-cell pool is still a matter of much contention since *in vitro* studies indicate that both naïve and memory B lymphocytes seem equally susceptible to EBV infection (Young and Rickinson, 2004). One view is that naïve B-cells are the main target of incoming EBV infections *in vivo*. In this model, viral transformation drives naïve cells into memory by mimicking the physiological process of antigen-driven memory-cell development, a

process involving somatic immuno-globulin-gene hypermutation during transit through a germinal centre (Figure 1.7.1). However, the finding that EBV-infected B-cells in tonsils from patients with IM are known to localise to extrafollicular areas and not to the germinal centres, and in addition show no evidence of hypermutation within expanding clones, makes this hypothesis difficult to reconcile (Young and Rickinson, 2004). This strategy gives the virus the opportunity to recruit infected cells into germinal centres, activating their latency programmes, resulting in progeny that either return to the circulating memory pool or differentiate to become plasma cells, which may migrate to mucosal sites in the oropharynx (Young and Rickinson, 2004). Alternatively it is thought that infection of pre-existing memory cells may provide direct entry into the memory compartment (Figure 1.7.1). This view is concurrent with the above observations in IM tonsils, but still leaves in question the apparent disappearance of the infected naïve cell pool (Young and Rickinson, 2004).

The reservoir of EBV-infected memory B-cells is stably maintained and befalls to the same physiological regulation that governs memory B-cell migration and differentiation (Laichalk *et al*, 2002). Occasionally, these EBV-infected cells might be recruited into germinal-centre reactions, involving the activation of different latency programmes, after which they might either return to the reservoir as memory cells or differentiate to become plasma cells, which may migrate to mucosal sites in the oropharynx, in the process, activating the viral lytic cycle. Progeny produced at these sites might initiate foci of lytic replication in receptive epithelial cells, allowing low-level shedding of infectious virus in the oropharynx, and might also initiate new latency III infections of naïve and/or memory B-cells. New infections may possibly replenish the B-cell reservoir, but are more likely to be efficiently removed by the CTL response (Young and Rickinson, 2004). The interactions between EBV and host cells are summarised Figure 1.7.1.

EBV has evolved a life cycle that mimics the natural differentiation pathway of antigen-activated B-cells, giving the virus access to its site of latent infection, the resting memory B-cell. By guiding infected cells through the stages of lymphocyte differentiation, EBV takes advantage of a cell type suitable for long-term latent persistence and periodic reactivation (Pattle and Farrell, 2006).

1.8 The Genes of the Lytic and Latent Cycles of EBV

The uncontrolled replication of a virus, which is harmful to the host is also detrimental to the virus (Shen and Shenk, 1995). Most viruses are unable to compete with the complex defence mechanisms of the host immune system and as a result become eliminated during the course of infection. Consequently, only the time between the initial infection and the elimination remains for these viruses to replicate and spread (Shen and Shenk, 1995). EBV however, has developed a strategy for persisting long-term, usually asymptomatically, concealed from the immune system while also producing infectious progeny periodically. This strategy depends on a separation of latency and lytic replication (Schwarzmann *et al*, 1998).

1.9 EBV Lytic Genes

Infection of epithelial cells by EBV *in vitro* results in active replication, leading to production of virus progeny and ultimately lysis of the infected cell (reviewed in Cohen, 2000). EBV encodes lytic cycle proteins that are important during viral replication for (1) regulating the expression of viral genes, (2) replicating viral DNA, (3) forming structural components of the virion, and (4) modulating the hosts immune response (Cohen, 2000).

1.9.1 Immediate Early, Early and Late Lytic Cycle Genes

Not unlike several other herpesviruses, EBV encodes lytic cycle genes that can prevent or delay death of the host cell, resulting from apoptosis triggered by the cell recognising the replication of the virus as DNA damage (Wensing and Farrell, 2000). The genes of the viral lytic cycle are expressed in a regimented cascade and are directly responsible for viral DNA replication (Lu and Chen, 2006). Some of these genes also encode structural proteins or proteins that modify the infected cells to facilitate viral envelopment (IARC Monographs, 1997; Cohen, 2000).

Lytic cycle gene expression follows a temporal and sequential order (Amon and Farrell, 2005; Lu and Chen, 2006) (Figure 1.9.1). Some viral genes are expressed independently of new protein synthesis early after lytic cycle induction, and are classified as immediate early genes (Amon and Farrell, 2005). These include the BZLF1, BRLF1 and BI'LF4 encoded proteins which are potent *trans*-activators of early EBV lytic genes (Takada and Ono, 1989; Kieff, 1996 and Young and Murray, 2003). Viral immediate early genes are induced directly by signal transduction from the B-cell receptor (BCR), independent of the expression of other proteins (Amon and Farrell, 2005). These immediate early genes are also involved in the transition from latent to lytic infection, cellular differentiation and cell cycle progression (Sinclair *et al*, 1992; Zacny *et al*, 1998; Swenson *et al*, 1999; Young and Murray, 2003). BZLF1 is expressed as early as 1.5 hours after EBV infection (Wen *et al*, 2007), and expression of this protein alone is sufficient to trigger the entire lytic cascade (Amon and Farrell, 2005). BZLF1 is a transcription factor which has sequence homology to c-Fos and harbours binding sites in several viral early gene promoters and promoters of cellular genes, as well as the lytic origins of replication. BZLF1 also further activates its own expression by binding to its promoter, an event that also activates the adjacent gene BRLF1, which is also a transcription factor. Together BZLF1 and BRLF1 activate most of the early genes in the next phase of the lytic cycle (Amon and Farrell, 2005). The early lytic viral genes are expressed slightly later and there are at least 30 EBV mRNAs classified as early gene products (Hummel and Kieff, 1982). Two of the most abundant early proteins are the BALF2 protein which is homologous to a HSV DNA binding protein and is important in DNA replication (Hummel and Kieff, 1982) and the BHRF1 protein, which shows partial (25%) sequence homology to the human *bcl-2* proto-oncogene, both of which protect human B lymphocytes from apoptosis (Henderson *et al*, 1993; Young and Murray, 2003). The late proteins are formally categorised as late based on a marked reduction in expression after inhibition of viral DNA synthesis (Kieff, 1996) and are those that encode structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress (IARC Monographs, 1997). Amoung the non-glycoproteins, BCLF1, BNRF1 and BXRF1 encode the major nucleocapsid protein, the major external non-glycoprotein of the virion and the basic core protein respectively. The genes encoding the EBV glycoproteins include BCRF1, which shows 84% sequence homology to the human IL-10 (Vieira *et al*, 1991). EBV-derived IL-10 is thought to

play a role in the establishment of latent infection by suppression of the host immune system (Rousset *et al*, 1992; Kurilla *et al*, 1993; Helminen *et al*, 1999; Young and Murray, 2003). An overview of the EBV lytic cycle is illustrated in Figure 1.9.1.

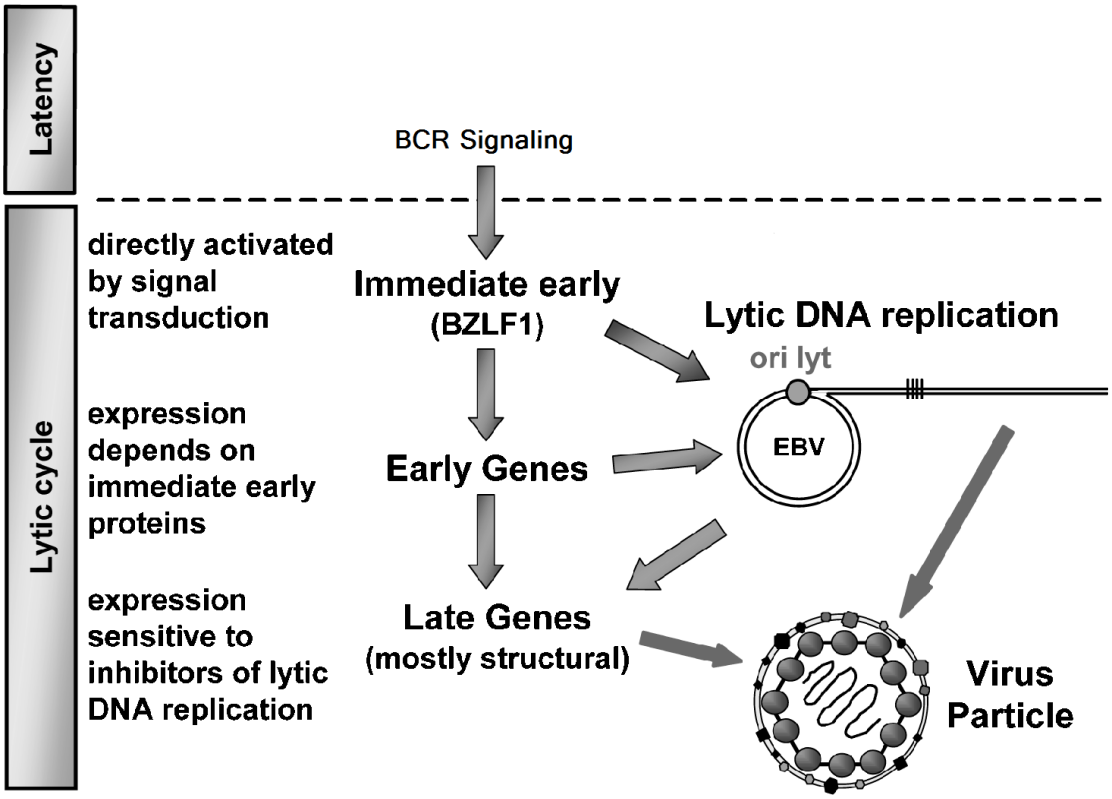


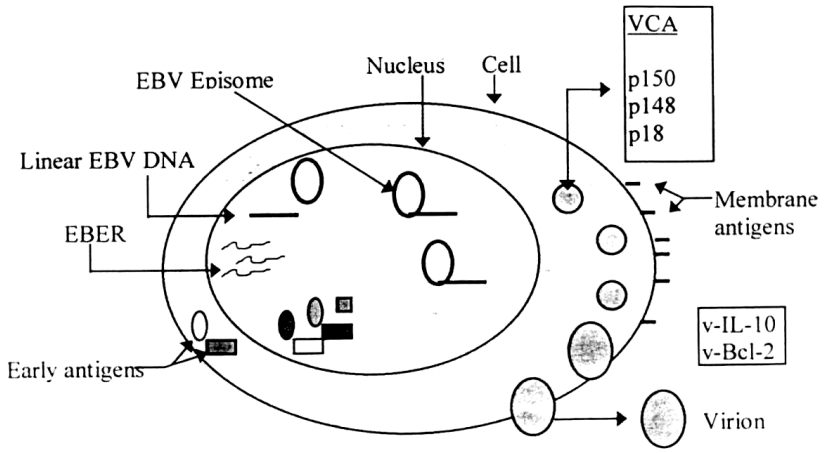
Figure 1.9.1: Overview of EBV lytic cycle.

Immediate early, early and late genes are expressed in sequential order. These genes are responsible for viral DNA replication and some genes also encode structural proteins or proteins that modify the infected cells to facilitate viral envelopment Adapted from Amon and Farrell, 2005.

1.9.2 Early, Membrane, and Viral Capsid Antigens.

The EBV lytic cycle is the replicative stage of the virus life cycle and is crucial for the production of infectious virus, which can then be spread to EBV naïve hosts through oral secretions into the saliva. The replicative proteins of the EBV lytic cycle have been classified as early antigens (EA), membrane antigens (MA), and viral capsid antigens (VCA) (Figure 1.9.2). The early antigens have been further subdivided into

diffuse early antigens (EA-D) and restricted early antigens (EA-R) (Kieff, 1996). Following induction of the lytic cycle, cells that have become permissive to viral replication, undergo cytopathic changes characteristic of herpesviruses, including margination of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (Kieff, 1996). Viral replication is spontaneously activated in only a small percentage of latently infected B-cells, and in these cells the viral DNA is amplified several hundred fold by a lytic origin of replication oriLyt (Hiraki *et al*, 2001). A schematic diagram illustrating early and late EBV gene expression is seen in Figure 1.9.2.



EA	ORF
EBV DNA polymerase	**BALF5**
Thymidine Kinase	**BXLF1**
Ribonucleotide reductase	**BORF2 & BARF1**
Alkaline Dnase	**BGLF5**
DNA binding protein p188	**BALF2**

MA	ORF
gp350/220	**BLLF1**
gp150	**BDLF3**
gp110	**BALF4**
gp85	**BXLF2**
gp78	**BILF2**
gp42	**BZLF2**
gp25	**BKRF2**

Figure 1.9.2: A schematic representation of early and late EBV gene expression

The VCA, the MA and the EA are illustrated and their ORF are written in bold.

1.10 EBV Latent Genes

Infection of B-cells by EBV *in vitro* results in a latent infection, with immortalisation of the cells (Cohen, 2000). The transformation of B-cells by EBV involves the synchronised action of several latent gene products (Young *et al*, 2000). EBV uses its viral proteins, the actions of which mimic several growth factors, transcription factors and apoptotic factors, to usurp control of cellular pathways that regulate diverse homeostatic cellular functions, allowing both cellular transformation and the establishment of a latent infection in the memory B-cell compartment.

1.10.1 The EBV-encoded nuclear antigens

1.10.1.1 EBNA1

EBV nuclear antigen 1 (EBNA1), a 641 amino acid protein, is the only viral protein consistently expressed in all EBV-associated malignancies and EBV-proliferating cells in healthy carriers (Knecht *et al*, 1997; Young and Murray, 2003; Young and Rickinson, 2004). EBNA1 is essential for lifelong viral persistence and is required for maintenance and replication of the EBV genome, through specific binding of EBNA1 to the EBV-origin of replication (oriP) (reviewed in Young and Murray, 2003). EBNA1 can also activate expression of critical cellular genes affecting cellular growth control and is known to possess oncogenic properties (Wilson and Levine, 1992 and Young and Murray, 2003). This ~80kDa protein consists of a short amino-terminal region, a 20kDa-40kDa glycine-alanine (Gly-Ala) repetitive sequence flanked by arginine rich sequences and a highly charged acidic carboxy-terminal sequence (Hennessy and Kieff, 1983; Young and Murray, 2003; Young and Rickinson, 2004). The presence of the Gly-Ala repeat domain in EBNA1 prevents processing by the proteasome (Dantuma *et al*, 2002), and has also been found to inhibit *EBNA1* mRNA translation. In this manner EBNA1 greatly reduces its own recognition by cytotoxic T-cells, enabling infected cells to evade the immune response (Yin *et al*, 2003).

1.10.1.2 EBNA2

EBNA2 is an 83 kDa transcription factor that localises to large nuclear granules and is associated with nucleoplasmic chromatin and nuclear matrix fractions (Petti *et al*, 1990). EBNA2 plays a critical role in the immortalisation of primary B lymphocytes. EBNA2, together with EBNA-LP, is the first protein expressed after infection of resting B-cells, underscoring its function as a transcriptional activator of cellular and viral gene expression (Middeldorp *et al*, 2003). The inability of the P3HR-1 EBV strain, (which carries a deletion of the gene that encodes EBNA2 and the last two exons of that for EBNA-LP) to transform B-cells *in vitro* was the first indication of the crucial role of this protein in the transformation process. Restoration of the EBNA2 gene in P3HR-1 has indisputably confirmed the importance of EBNA2 in B-cell transformation (Dambaugh *et al*, 1984a; Hennessy and Kieff, 1985; Mueller-Lantzsch *et al*, 1985 and as reviewed in Young and Rickinson, 2004). Since this time experiments have shown that EBNA2 is not only essential for initiation but also for maintenance of B-cell immortalisation (Kempkes *et al*, 1995a). Sequencing of the EBNA2 gene in different EBV strains has revealed two alleic isoforms of EBNA2, namely EBNA2A and EBNA2B, which are found in type 1 and type 2 EBV isolates respectively (Aitken *et al*, 1994). These proteins share only about 50% sequence homology with each other (Adldinger *et al*, 1985). While both genes are essential for immortalisation of EBV infected cell lines, EBNA2B displays a significantly reduced ability to transform B-lymphocytes relative to the EBNA2A gene (Dambaugh *et al*, 1984c; Cohen *et al*, 1989; Hammerschmidt and Sugden, 1989) and accordingly these genes are thought to be responsible for the biological difference that enables the type 1 strains to transform B lymphocytes more efficiently than type 2 (Rickinson *et al*, 1987 and reviewed in Zimber-Strobl and Strobl, 2001). EBNA2A, identified from the B95.8 strain of EBV consists of 483 amino acids, whereas EBNA2B represented by the AG876 cell line consists of 455 amino acids.

1.10.1.2.1 Structure of EBNA2

The EBNA2 protein consists of: (i) a negatively charged region at the amino-terminus, which is reported to participate in homo-dimerisation (ii) a polyproline stretch, consisting of 10–40 successive prolines depending on the virus strain; (iii) a diversity region at the centre of the protein, where very low homology between EBNA2A and EBNA2B exists; (iv) a domain responsible for the interaction with the DNA-binding protein CBF1; (v) an arginine–glycine rich stretch of around 18 amino acids; (vi) a negatively charged region, which contains a *trans*-activation domain; and (vii) a nuclear localisation signal at the carboxy-terminus (Figure 1.10.1). (Zimber-Strobl and Strobl, 2001).

Regions of the protein fundamental to the transforming ability of EBNA2 have been charted by extensive mutational analysis (as discussed in Zimber-Strobl and Strobl, 2001). Several stretches of the protein could be deleted without abrogating the immortalisation function of the protein. At the amino-terminus, the first 230 amino acids excluding the amino acids 94–110 which encompass seven prolines were found to be unnecessary for B-cell transformation. Two further regions were established as crucial for B-cell immortalisation; the area harbouring the amino acids 280–337 which is necessary for mediating the indirect contact of EBNA2 with DNA through the Cp Binding Factor 1(CBF1) transcription factor (see below) and the region containing the amino acids 425–462; which contains an acidic *trans*-activation domain (Cohen and Kieff, 1991; Ling *et al*, 1993a; Zimber-Strobl and Strobl, 2001).

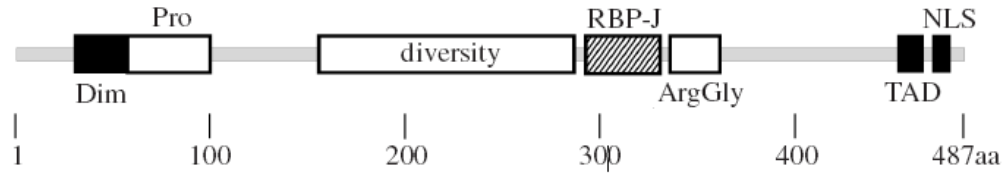


Figure 1.10.1: Structure of the EBNA2A protein of the B95.8 strain of EBV.

Characteristic parts of the EBNA2 protein: a negatively charged region at the amino-terminus, (Dim), a polyproline stretch (Pro), a diversity region, a domain responsible for the interaction with RBP-Jκ (RBP-J), an arginine–glycine rich stretch (ArgGly), a *trans*-activation domain (TAD) and a nuclear localisation signal (NLS) at the carboxy-terminus. (From Zimber-Strobl and Strobl, 2001).

1.10.1.2.2 Function of EBNA2

EBNA2 is a specific *trans*-activator of latent viral genes and is known to regulate the expression of all other viral proteins expressed in LCLs through the *trans*-activation of (i) the BamHI-C-promoter (Cp) from which transcription of all EBNA genes is regulated (Woisetschlaeger *et al*, 1990; Sung *et al*, 1991); and (ii) the promoters of the latent membrane proteins LMP1, LMP2A and LMP2B (Abbot *et al*, 1990; Fahraeus *et al*, 1990; Wang *et al*, 1990; Zimber-Strobl *et al*, 1991; Laux *et al*, 1994b). In addition to this, EBNA2 *trans*-activates various cellular genes (*CD23, CD21, CCR7, hes-1, c-fgr, AML-2, BATF , bcl-2 and bfl-1, c-myc, FcRH5* and *EBI1/BLR2*) (Wang *et al*, 1987; Cordier *et al*, 1990; Knutson, 1990; Patel *et al*, 1990; Finke *et al*, 1992; Burgstahler *et al*, 1995; Sakai *et al*, 1998; Kaiser *et al*, 1999; Spender *et al*, 2001; Spender *et al*, 2002; Johansen *et al*, 2003; Maier *et al*, 2005; Mohan *et al*, 2006; Pegman *et al*, 2006) and to date is known to transcriptionally repress only one cellular gene, the immuno-globulin heavy chain locus (IgM) (Jochner *et al*, 1996). Many of these genes are in turn involved in the immortalisation process. Furthermore, it has been shown that EBNA2 and EBNA-LP co-operate to induce the transition of the infected cells from G_0 to G_1 (Sinclair *et al*, 1994).

1.10.1.2.3 Mechanism of promoter targeting by EBNA2

Although EBNA2 does not bind to DNA directly, it achieves promoter specificity of interaction through contact with other sequence specific DNA binding proteins (Zimber-Strobl and Strobl, 2001; Young and Murray, 2003). The recruitment of EBNA2 to DNA can occur via interactions with the recombinant signal-binding protein Jκ (RBP-Jκ)/Cp Binding Factor 1 (CBF1) (Figure 1.10.2), the Ets family protein Spi-1/PU.1, ATF/CRE, the CREB-binding protein CBP and the SWI/SNF chromatin remodeling complex (Zimber-Strobl *et al*, 1993; Henkel *et al*, 1994; Laux *et al*, 1994a; Ling *et al*, 1994; Waltzer *et al*, 1994; Johannsen *et al*, 1995; Wu *et al*, 1996; Sjoblom *et al*, 1998; Wang *et al*, 2000; Wu *et al*, 2000; Pegman *et al*, 2006). EBNA2 can also interact through its acidic *trans*-activation domain with components of the basal transcription machinery (BTM) including TFIIH, TFIIE, TFIIB, TAF40, and RPA70 to regulate promoter activity (Tong *et al*, 1995a; Tong *et al*, 1995b; Tong *et al*, 1995c). In addition to this there is also an established association of EBNA2 and

the DEAD-box protein, DP103, (Grundhoff *et al*, 1999), which has an established role in transcriptional repression (Yan *et al*, 2003). Furthermore, *trans*-activation is augmented by binding to the EBV encoded EBNA-LP protein (Harada and Kieff, 1997; Nitsche *et al*, 1997; Young and Murray, 2003; Young and Rickinson, 2004). Of particular interest, CBF1 is ubiquitously expressed and highly conserved during evolution (Zimber-Strobl and Strobl, 2001). The cognate DNA sequence element to which CBF1 binds is 5'-GTGGGAA-3' (Zimber-Strobl *et al*, 1993). The first indication of this cellular protein mediating binding of EBNA2 to its response element was described in a study on the *LMP2A* promoter (Zimber-Strobl *et al*, 1993). Following this, the promoters of several EBV latent genes and also several cellular genes were also shown to contain functional CBF1-binding motifs and to be targets of EBNA2-CBF1 signaling including *EBNA Cp, LMP1, LMP-2B, CD21, CD23, CCR7, FcRH5* and *bfl-1* (Ling *et al*, 1993a; Zimber-Strobl *et al*, 1993; Ling *et al*, 1994; Johannsen *et al*, 1995; Maier *et al*, 2005; Mohan *et al*, 2006).

1.10.1.2.4 CBF1 as a link between EBNA2 and the cellular Notch pathway

CBF1 is established as a transcriptional repressor, and can mediate this effect (i) through directly binding with elements of the BTM, disturbing the TFIIA-TFIID interaction, which is a requisite for the initiation of transcription and also (ii) as a result of histone deacetylation, which results in chromatin remodelling and the consequential loss of transcription factor access to the nucleosome-associated promoter sequences. The CBF1 repressor complex (Figure 1.10.2) includes the proteins SMRT/NcoR, HDAC1, HDAC2, SIN3-associated protein 30 (SAP30), the CBFI-interacting co-repressor (CIR) and SKIP (Ski interacting protein).

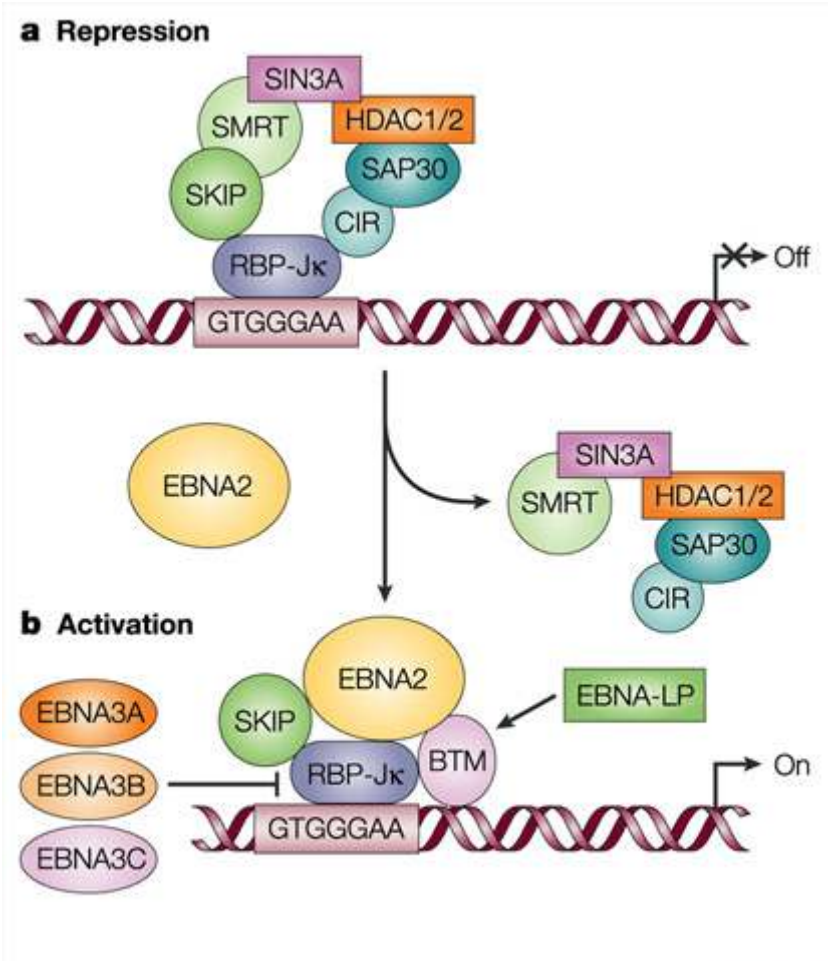


Figure 1.10.2: Model for EBNA2 Activation of CBF1 Repressed Promoters.

(A) The CBF1 (RBP-Jκ above) repressor complex includes the proteins SMRT/NcoR, HDAC1, HDAC2, SAP30, CIR and SKIP. (B) EBNA2 abolishes CBF1 mediated repression by competing for the SMRT–HDAC co-repressor complex through binding to both CBF1 and SKIP, followed by recruitment of the BTM to activate transcription. From Young and Rickinson, 2004.

EBNA2 *trans*-activates promoters by (i) binding to the repression domain of CBF1 to alleviate repression and (ii) by presenting a strong transcriptional activation domain to the promoter (Hsieh and Hayward, 1995). During EBNA2 displacement of the CBF1 co-repressor complex, neighbouring domains make contact with CBF1 and SKIP to dislodge SMRT (Figure 1.10.2). Direct competition for binding to SKIP has been demonstrated for SMRT and EBNA2 (Zhou *et al*, 2000a). Conserved region (CR) 5 of EBNA2 binds SKIP and conserved region 6 (CR6) binds to CBF1. The positions of CR5 and CR6 on the EBNA2 protein can be seen in Figure 1.10.3 below. A mutation within CR6 that abolishes EBNA2 binding to CBF1 (ww323sr) also entirely eliminates EBNA2-associtated *trans*-activation (Ling *et al*, 1993a; Ling and Hayward,

1995). In addition, when incorporated into the virus, this mutation results in a non-immortalising EBV (Yalamanchili *et al*, 1994). Mutations within CR5, such as the II307 mutant, which are impaired for SKIP interaction, are equally detrimental to the *trans*-activating function of EBNA2 (Zhou *et al*, 2000a). Furthermore, the introduction of deletions that span CR5 into the EBV genome led to a mutant virus that either failed to immortalise B-cells *in vitro* or resulted in B-cell colonies that grew more slowly than the wild type virus-immortalised controls (Harada *et al*, 1998). Overall, this data indicates that EBNA2 requires interaction with both SKIP and CBF1 for effective activation of CBF1 responsive promoters.

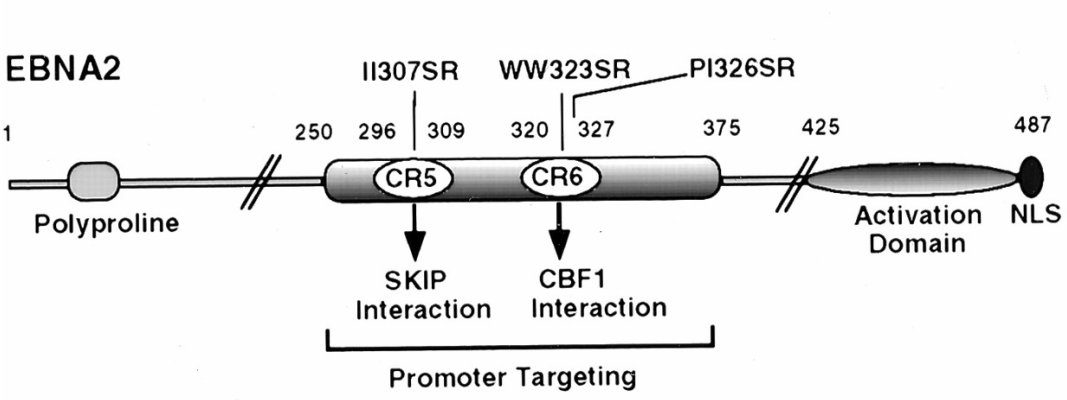


Figure 1.10.3 : The locations of the SKIP and CBF1 interaction regions on the EBNA2 protein.

A schematic representation of the EBNA2 protein illustrating the relative locations of characterised functional domains and the position of the ww323sr mutation. The amino acid numbers are indicated. CR5, CR6 and a nuclear localisation signal (NLS) are also indicated. From Zhou *et al*, 2000a.

The CBF1 homologue in *Drosophila* (suppressor of hairless, (SuH) is involved in signal transduction from the Notch receptor. The Notch pathway is an essential cell-cell interaction mechanism, which controls processes such as cell proliferation, cell fate decisions, differentiation and stem cell maintenance (Pinnix and Herlyn, 2007).

In mammals four Notch receptor-coding genes have been described (Notch1, Notch2, Notch3 and Notch4). *Notch1* encodes an evolutionarily conserved 300kDa *trans*-membrane receptor, which is translated as a full-length protein but is subsequently cleaved in the trans-golgi network into two subunits by furin-like convertases

(Blaumueller *et al*, 1997; Logeat *et al*, 1998; Pinnix and Herlyn, 2007). Following transport to the membrane, the subunits form the hetero-dimeric Notch receptor through calcium-dependent, non-covalent interactions. The Notch signaling cascade is instigated upon binding of membrane-bound ligand (DSL family members Jagged 1,2 and DSL 1,3,4) to one of four hetero-dimeric *trans*-membrane Notch receptors (Notch1–4) in mammalian cells. Upon ligand binding two sequential proteolytic events occur to liberate active Intracellular Notch (NotchIC). The first cleavage is mediated by a metalloprotease, TNF-α-converting enzyme (TACE) (Brou *et al*, 2000; Hartmann *et al*, 2002) and the second and final cleavage is performed by a γ-secretase complex (Edbauer *et al*, 2003; Edbauer *et al*, 2003).

NotchIC is then targeted to the nucleus by its nuclear localisation signal (NLS), where it binds to the CSL family of transcription factors (CBF1/RBP-Jκ, Suppressor of Hairless Su(H) or Lag-1) where it forms a large transcriptional activation complex together with the human homolog of Drosophila Mastermind MAML1 protein (Pinnix and Herlyn, 2007). CBF1 is a constitutive repressor of Notch target genes (Kao *et al*, 1998; Taniguchi *et al*, 1998; Hsieh *et al*, 1999; Zhou *et al*, 2000b; Oswald *et al*, 2002). Upon entering the nucleus, NotchIC displaces co-repressors and their associated chromatin-modifying proteins from CBF1 and forms a transcription-activating complex (Fortini and Artavanis-Tsakonas, 1994; Tamura *et al*, 1995; Kato *et al*, 1997; Tani *et al*, 2001). NotchIC interacts via its RAM (RBPJκ-associated molecule) domain and ankyrin repeats with CBF1 (Jarriault *et al*, 1995).

Several target genes have been described, which are induced through the interaction of NotchIC with CBF1, for example the hairy enhancer of split complex related genes *Hes-1 and Hes-5, Hey-1, Hey-2 and Hey-L* (Jarriault *et al*, 1995; Nishimura *et al*, 1998; Maier and Gessler, 2000), *ERBB-2* (Chen *et al*, 1997), *NF-κB2* (Oswald *et al*, 1998) and the hairy-related transcription factor (*HRT*) (Nakagawa *et al*, 2000). In addition, alpha-actin, calponin, myosin, and smoothelin expression are down-regulated by Notch-mediated CBF-1-dependent interaction in vascular smooth muscle cells (Morrow *et al*, 2005). However, regulation by Notch is context dependent. The activation of NotchIC as a result of ligand binding is summarised in Figure 1.10.4.

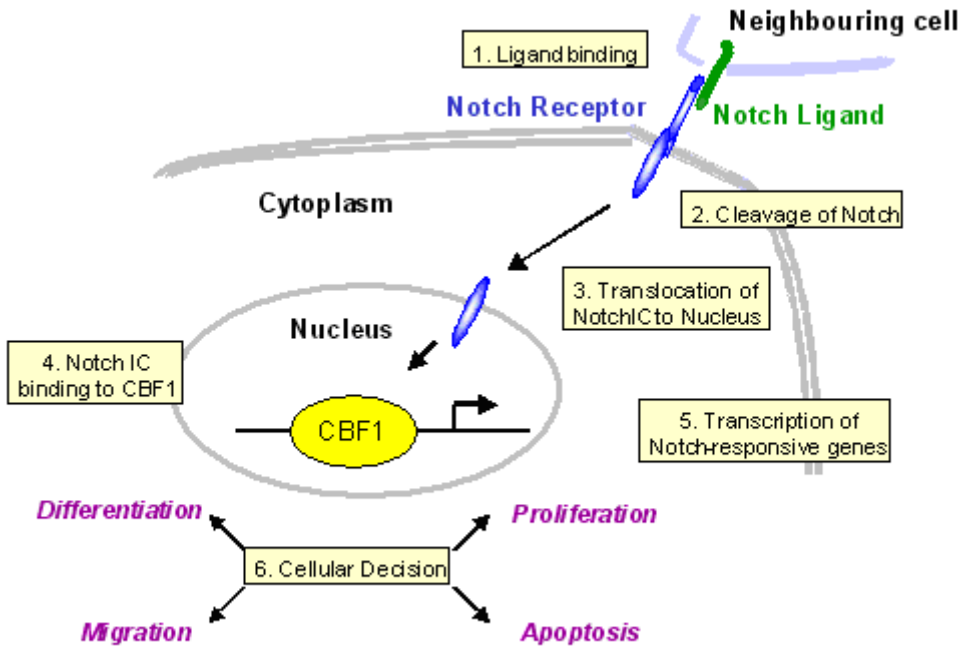


Figure 1.10.4: The Notch signaling pathway.

(1) Ligand on a neighbouring cell binds to the Notch receptor. (2) The ligand-receptor interaction results in a series of cleavages of the Notch receptor, which releases NotchIC. (3) NotchIC is translocated to the nucleus, where it interacts with CBF1. (5) Binding of NotchIC to CBF1 displaces the co-repressor complex, allowing transcriptional activation of Notch responsive genes. (6) Expression of Notch responsive genes determines the fate of the cell. From Smith, 2005.

1.10.1.2.5 EBNA2 and NotchIC overlap in their functions and in their target genes

Since EBNA2 associates with the CBF1 repression complex in order to relieve repression and subsequently *trans*-activate target genes, EBNA2 can be regarded as a functional homologue of the activated Notch receptor (Hsieh *et al*, 1996 and Hayward, 2004). Although there is no obvious sequence homology between these two proteins, it is clear that both EBNA2 and NotchIC *trans*-activate genes by interacting with CBF1, thereby relieving repression by replacing the HDAC co-repressor complex with their *trans*-activation domains.

The mechanism of NotchIC and EBNA2 displacement of the CBF1 co-repressor complex appears to be identical in that in each case adjacent domains make contact with CBF1 and SKIP to displace SMRT (Figure 1.10.5). The RAM domain of NotchIC is the major contact with CBF1 (Tamura *et al*, 1995; Hsieh *et al*, 1996; Hsieh *et al*, 1997) while the ankyrin repeats bind SKIP (Zhou *et al*, 2000b). CR5 of EBNA2 binds SKIP and CR6 binds CBF1. Thus it is clear that both EBNA2 and NotchIC each require contacts with SKIP as well as CBF1 for effective activation of promoters containing CBF1-binding sites.

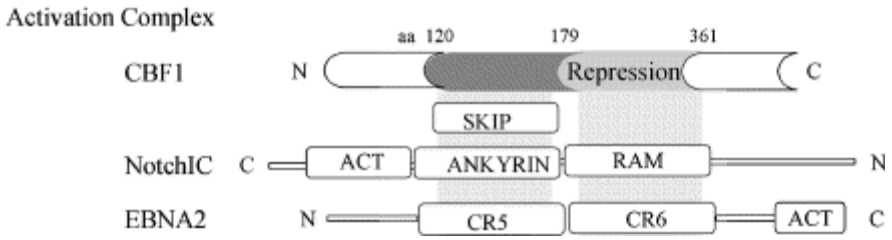


Figure 1.10.5: Mechanism of NotchIC and EBNA2 displacement of the CBF1 co-repressor complex

Both EBNA2 and NotchIC require contacts with SKIP as well as CBF1 for effective modulation of promoters containing CBF1-binding sites. The RAM domain of NotchIC is the major contact with CBF1 while the ankyrin repeats bind SKIP. CR5 of EBNA2 binds SKIP and CR6 binds CBF1. Transcriptional activation of target genes occurs through the combination of loss of repression and the positive effects of the activation domain (ACT). N, amino terminus; C, carboxy terminus. From Hayward, 2004.

There are additional similarities between EBNA2 and NotchIC in the manner in which transcriptional activation through CBF1 is effected. The activation domains of both EBNA2 and NotchIC bind co-activating molecules that modify chromatin structure through histone acetylase activity and make contact with the BTM. In this regard EBNA2 is known to recruit p300, PCAF and CBP (Wang *et al*, 2000) a co-activator, p100 that interacts with TFIIE (Tong *et al*, 1995c) and the NotchIC activation domain binds GCN4, PCAF and p300. Indeed, both proteins have a co-activator molecules in common (PCAF and p300). Further to this, EBNA2 *trans*-activation is potentiated by the EBNA-LP protein (Harada and Kieff, 1997; Nitsche *et al*, 1997) and also through interactions with the SWI/SNF complex (Wu *et al*, 2000)

and the DEAD-box protein DP103 (Yan *et al*, 2003), while full transcriptional activation by NotchIC involves MAML1 (Pinnix and Herlyn, 2007).

Responses to Notch and EBNA2 are highly concentration-dependent and negative regulation of the activity of the CBF1 complex occurs through modulation of CBF1 DNA binding. During EBV infection, EBNA3A, 3B and 3C can compete with EBNA2 for binding to CBF1 (reviewed in Hayward, 2004) whilst NotchIC activity is also regulated by loss of CBF1 binding due to interference from the cellular proteins KyoT2 and MINT/SHARP (Hayward, 2004).

Since both EBNA2 and NotchIC proteins share a common mechanism of regulation through CBF1 and SKIP, it not surprising then that they also overlap in the range of functional activities and in the genes that they regulate. In this regard both molecules have been implicated in the regulation of *Hes-1*, *LMP2A*, *BATF*, *CD21* and *IgH* expression in B-cells (Zimber-Strobl and Strobl, 2001). Additionally EBNA2 and Notch-IC have been shown to protect cells from apoptosis by binding to the cellular *Nur77* (*TR3*/*NGF1B*) transcription factor (Jehn *et al*, 1999; Lee *et al*, 2002). In contrast only EBNA2, but not NotchIC, can induce *LMP1, CD23* and *c-myc* (reviewed in Zimber-Strobl and Strobl, 2001).

Although the role of Notch in the B-cell compartment remains to be fully elucidated, it has been postulated that the EBNA2 mimicry of Notch function may facilitate a sustained life-long EBV infection in the host (Hayward, 2004). In support of this, there is evidence that Notch signaling increases the formation of hematopoietic stem cells. EBNA2-driven B-cell proliferation may thus be recapitulating a precursor stem cell response. This proliferation is thought to be important in expanding the infected cell population after primary EBV infection to permit establishment of a life-long latency in the B-cell compartment. Additionally, the fact that EBNA2 is selectively expressed in the same compartment of the germinal centre where Notch2 expression is essential for B-cell development further strengthens the concept that EBV is using EBNA2 as a virally controlled, constitutive Notch surrogate (Hayward, 2004).

1.10.1.3 The EBNA3 family (EBNA3A, EBNA3B and EBNA3C)

The EBNA3 family consists of 3 nuclear proteins, EBNA3A, -3B and -3C (previously referred to as EBNA3, EBNA4, and EBNA6 respectively). The nuclear proteins are encoded by alternatively spliced transcripts initiated at the Cp promoter (Kieff, 1996) and are composed of 944, 937 and 992 amino-acid residues, respectively (Petti *et al*, 1990). While EBNA3A and -3C are essential for the growth transformation of lymphocytes *in vitro*, EBNA3B is not required for B-cell immortalisation under these conditions (Young and Murray, 2003). EBNA3C can induce the up-regulation of both cellular and viral gene expression, and is also known to repress the Cp promoter (as reviewed in West, 2006). EBNA3C interacts with the retinoblastoma protein, pRb, to promote cell transformation (Parker *et al*, 1996). In addition, EBNA3A and EBNA3C co-operate as the main determinants of drug resistance in BL cells and in the down-regulation of the pro-apoptotic Bcl-2-family member Bim (Anderton *et al*, 2007). EBNA3B has been shown to induce expression of vimentin and CD40 (Silins and Sculley, 1995). All three EBNA3 proteins can also bind CBF1, negatively regulating gene expression in the Notch pathway. This results in repression of EBNA2 *trans*-activation, which in turn regulates the synthesis of the EBNA3 proteins themselves (Robertson *et al*, 1995; Waltzer *et al*, 1995; Robertson, 1997). Thus the EBNA3 proteins counterbalance and fine-tune the action of EBNA2, precisely regulating CBF1 activity, thereby regulating the expression of cellular and viral promoters containing CBF1 binding sites (Cludts and Farrell, 1998; Young and Rickinson, 2004).

1.10.1.4 EBNA-LP

The EBNA Leader Protein (EBNA-LP/EBNA5) is one of the first viral proteins produced during EBV infection of B-lymphocytes (Kieff, 1996). EBNA-LP is encoded by the first ORF (leader sequence) of the extensively spliced primary transcript, originating from either the Wp or Cp promoter, and is composed a multi-repeat domain (W1W2) and a unique carboxyl-terminal domain (Y1Y2) (Figure 1.10.6). EBNA-LP encodes a protein of variable size (20-130kDa) depending on the number of *Bam*HI W repeats contained by a particular EBV isolate. While EBNA-LP

is known to localise primarily to the nucleus, the distribution of EBNA-LP within the nucleus is variable. During early infection in B-cells and following expression of EBNA-LP in type I BL cell lines (Kieff, E. and Rickinson, A.B, 2001), EBNA-LP is distributed throughout the nucleus (Szekely *et al*, 1995). Whereas in LCLs, EBNA-LP localises to promyelocytic leukemia nuclear bodies (PML NBs) (Szekely *et al*, 1995; Szekely *et al*, 1996). Although not essential for transformation, EBNA-LP enhances the efficiency of the process (Hammerschmidt and Sugden, 1989).

EBNA-LP is known principally as a transcriptional co-activator of EBNA2. It has previously been shown that EBNA-LP co-operates with EBNA2 in the up-regulation of LMP1/LMP2 and EBV BamHI C latency promoters in B-cells (Harada and Kieff, 1997; Nitsche *et al*, 1997; Peng *et al*, 2005) and that EBNA-LP and EBNA2 co-operative to stimulate expression of cyclin D2 in resting B-cells in addition to mediating G_0 to G_1 transition during immortalisation (Sinclair *et al*, 1994). Furthermore, studies in the laboratory indicate that co-transfection of EBNA-LP also considerably potentiates EBNA2 *trans*-activation of the *bfl-1* promoter (Pegman, P.M. unpublished data).

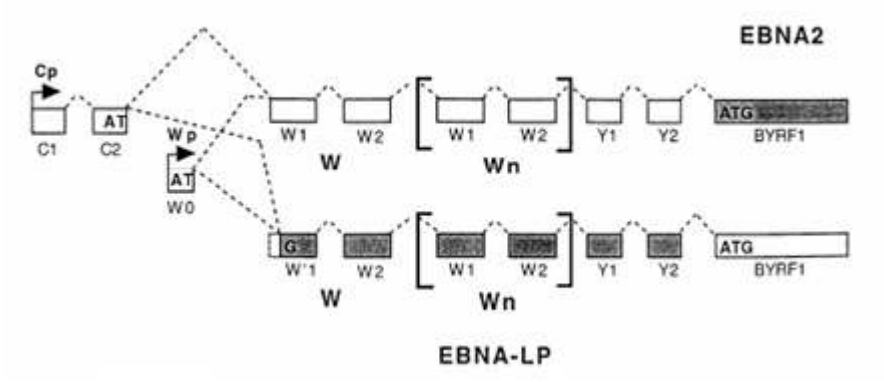


Figure 1.10.6: Detailed representation of how EBNA2 and EBNA-LP mRNAs are generated from a unique RNA precursor by facultative splicing.

Boxes represent exon sequences and broken lines represent intron sequences. The exons coding for EBNA2 or EBNA-LP respectively, are indicated by the grey boxes. The EBNA-LP protein AUG is created following the use of an alternative acceptor splice site, five bases into the W1 exon. This alternate splice provides the G of the EBNA-LP initiation codon. In latency III, only the Cp promoter is active (From Manet *et al*, 1998).

EBNA-LP co-activates EBNA2 through interacting with the promyelocytic leukemia nuclear body (PML NB)-associated protein Sp100 and displacing Sp100 and heterochromatin protein 1 α (HP1α) from PML NBs (Ling *et al*, 2005). Numerous other potential co-factors that mediate EBNA-LP activity have been identified including pRb, p53, hsp72/hsc73, hsp27, Hax-1, ERR1, p14ARF, DNA-Pkcs, α-tubulin, β-tubulin, prolyl-4-hydroxylase, and HA95 (Ling *et al*, 2005 and the references therein). However, to date, no correlation has been made between the association with these factors and their ability to co-operate with EBNA2.

1.10.2 The EBV-encoded latent membrane proteins

1.10.2.1 LMP1

Latent Membrane Protein 1 (LMP1) is a major transforming protein of EBV. Several studies have shown that this protein functions as a classic oncogene and is essential for EBV-induced B-cell transformation *in vitro* (reviewed in Li and Chang, 2003; Young and Rickinson, 2004). LMP1 has pleiotropic effects when it is expressed in BL-derived cells, resulting in many of the phenotypic changes observed during EBV-infection, including induction of cell adhesion molecules and activation markers (Kieff, E. and Rickinson, A.B, 2001), and the up-regulation of anti-apoptotic proteins Bcl-2, Mcl-1, Bfl-1, A20 and c-IAPs (Henderson *et al*, 1991; Laherty *et al*, 1992; Fries *et al*, 1996; Wang *et al*, 1996; D'Souza *et al*, 2000; Hong *et al*, 2000). This 63kDa integral membrane phosphoprotein functions as a constitutively activated receptor (Gires *et al*, 1997; Li and Chang, 2003) and is a member of the tumour necrosis factor receptor (TNFR) superfamily, activating several signaling pathways in a ligand-independent manner (as reviewed in Young and Rickinson, 2004). Functionally, LMP1 mimics the cellular growth signal that normally results from the binding of CD40 ligand (another member of the TNFR superfamily) (Thompson and Kurzrock, 2004) and can partially substitute for CD40 *in vivo*, providing both growth and differentiation signals to B-cells (Uchida *et al*, 1999).

The LMP1 protein can be divided into three domains (Figure 1.10.7). Firstly, an amino-terminal cytoplasmic tail (amino acids 1-23), which tethers LMP1 to the

plasma membrane and orientates the protein. Secondly, a *trans*-membrane region, consisting of six hydrophobic *trans*-membrane loops, which are involved in self-aggregation and oligomerisation (amino acids 24-186). Third, a long carboxy-terminal cytoplasmic region (amino acids 187-386), which possesses most of the signaling activity of the molecule (Young and Rickinson, 2004). LMP1 signals mainly from the intracellular compartments (Lam and Sugden, 2003). Both oligomerisation and localisation within glycosphingolipid-rich membrane rafts are essential for the initiation of signaling (Clausse *et al*, 1997; Eliopoulos and Young, 2001; Higuchi *et al*, 2001) which results in the activation of several signaling pathways that contribute to the many phenotypic consequences of LMP1 expression. At least four signaling pathways have been implicated in the function of LMP1, namely nuclear factor κB (NF-κB), c-Jun NH_2 terminal kinase (JNK), p38/mitogen activated protein kinase (p38/MAPK) and janus kinase/signal transducers and activators of transcription (JAK/STAT) (Murray and Young, 2001). It is the cytoplasmic C-terminus of LMP1 that is responsible for the transduction of signaling cascades that result in primary B-cell transformation and phenotypic changes. Within the C-terminus of LMP1 there are at least two major activating domains (Figure 1.10.7), C-terminal activating region 1 (CTAR1) and CTAR2 (Young and Rickinson, 2004). CTAR1 (also known as transformation effector site 1, TES1) is located proximal to the membrane (amino acids 186-231), binds TNFR-associated factors (TRAFs) (Huen *et al*, 1995; Devergne *et al*, 1996), and is essential for EBV-mediated B-cell immortalisation (Kaye *et al*, 1993; Izumi and Kieff, 1997). CTAR2/TES2, which is located near the C-terminus (amino acids 351-386), supports the long-term growth of immortalised B-cells (Izumi and Kieff, 1997) and recruits the TNFR-associated death domain (TRADD) protein and receptor-interacting protein (RIP) (Huen *et al*, 1995; Eliopoulos *et al*, 1999a). The activities of CTAR1 and CTAR2 affect diverse signaling cascades and provide the basis for the molecular explanation of the transforming properties of LMP1. A diagram illustrating the structure and function of LMP1 is shown in Figure 1.10.7.

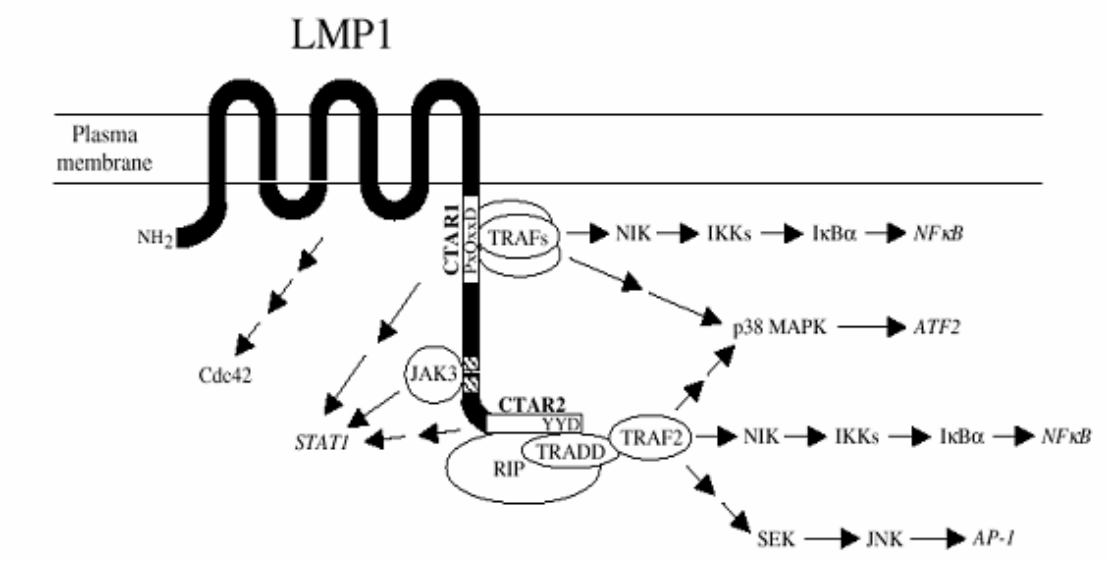


Figure 1.10.7: Structure and Function of LMP1.

The C-terminus of LMP1 contains two activating regions, the C-terminus activation regions 1 and 2 (CTAR1 and CTAR2). CTAR1, is essential for EBV-mediated B-cell immortalisation, and binds TRAF1, TRAF2, TRAF3 and TRAF5 activating the NF-κB and p38 signaling pathways. CTAR2 supports the long-term growth of immortalised B-cells and recruits TRADD to activated downstream signals, such as NF-κB, JNK, and p38. Both of the C-terminal domains also mediate the activation of the JAK/STAT pathway (From Eliopoulos and Young, 2001).

1.10.2.2 LMP2A and LMP2B

Transcription of *LMP2* is regulated by two separate promoters 3kb apart in the viral DNA (Sample *et al*, 1989). Two mRNAs that have unique 5' exons followed by eight common exons encode two distinct proteins of 2.0 and 1.7kb in length, namely LMP2A and LMP2B, respectively (Laux *et al*, 1989; Sample *et al*, 1989). Thus the structures of LMP2A and LMP2B are similar; LMP2A contains an N-terminal cytoplasmic domain of 119 amino acids with eight tyrosines that are phosphorylated in LCLs, 12 *trans*-membrane domains, and a C-terminal domain of 12 amino acids. LMP2B, however, lacks the entire N-terminal cytoplasmic domain (Kieff, 1996 and Young and Rickinson, 2004).

LMP2A is known to block BCR signal transduction through specific phosphotyrosine motifs in its N-terminal domain, prohibit induction of lytic EBV infection and promote B-cell survival (Longnecker *et al*, 1991; Miller *et al*, 1994; Fruehling *et al*,

1996; Fruehling and Longnecker, 1997; Babcock and Thorley-Lawson, 2000a; Babcock *et al*, 2000b; Swart *et al*, 2000). Neither LMP2A nor LMP2B are essential for EBV-induced B-cell transformation *in vitro* (Longnecker, 2000). While most of the research to date has focused upon LMP2A, LMP2B has been shown to colocalise with LMP2A in the membrane where the C terminus of both splice variants can interact and regulate the activity of one another (Lynch *et al*, 2002). LMP2B was shown to negatively regulate LMP2A activity by interfering with its aggregation (Rovedo and Longnecker, 2007). Furthermore, LMP2B has recently been found to regulate susceptibility to induction of lytic EBV infection (Rechsteiner *et al*, 2008). Taken together, these data support a role for LMP2A in modifying the normal programme of B-cell development to favour the maintenance of EBV latency and to prevent inappropriate activation of the EBV lytic cycle. It is also evident that LMP2B is involved in the regulation of switching from latent to lytic EBV in B-cells harbouring functional EBV through impacting the activity of LMP2A (Rechsteiner *et al*, 2008).

1.10.3 Other EBV latent transcripts

1.10.3.1 EBER1 and EBER2

EBV encodes two small non-polyadenylated RNAs termed Epstein-Barr virus-encoded RNAs 1 and 2 (EBER 1 and EBER 2), the most abundant viral transcripts in latently EBV infected cells with the exception of oral hairy leukoplakia lesions from AIDS patients and some hepatocellular carcinomas (Sugawara *et al*, 1999). In addition to the latent proteins, EBER1 and EBER2 are expressed in all forms of latency. EBERs 1 and 2 are uncapped, non-coding RNAs of 167 and 172 nucleotides respectively (Thompson and Kurzrock, 2004). The EBERs are not essential for the EBV-induced transformation of primary B lymphocytes, since recombinant EBV harouring EBER gene deletions can transform lymphocytes (Swaminathan *et al*, 1991). Expression of the EBERs in BL cell lines has been found to increase tumourigenicity, promote cell survival and induce IL-10 expression (Kitagawa *et al*, 2000; Ruf *et al*, 2000; Nanbo and Takada, 2002) and it has been suggested that EBER-mediated inhibition of PKR (a latent, IFN-inducible Ser/Thr kinase) function might be important for viral persistence (Nanbo and Takada, 2002). Furthermore,

transfection of the EBER genes into the EBV-negative AKATA cell line restored the oncogenic potential that was originally present in the EBV-positive AKATA cells but was lost in the EBV-negative subclones (Komano *et al*, 1999).

1.10.3.2 BARTs/ CSTs

The EBV *Bam* HI A rightward transcripts (BARTs) or complementary-strand transcripts (CSTs) (Karran *et al*, 1992; Smith *et al*, 2000) are a group of abundantly expressed RNAs that are encoded by the *Bam* HI A region of the EBV genome and were originally identified in NPC tumour tissues (Hitt *et al*, 1989), but were subsequently found to be expressed in other EBV-associated malignancies, such as BL, HL and nasal T-cell lymphoma, as well as in the peripheral blood of healthy individuals (Young and Rickinson, 2004). The protein products of these highly spliced transcripts remain to be conclusively identified. Another transcript that is generated from the *Bam* HI A region is *BARF1*, which encodes a 31 kDa protein that was originally identified as an early antigen expressed on induction of the EBV lytic cycle (Young and Rickinson, 2004). The BARTs are often expressed in circumstances in which the EBNA2 and EBNA3 proteins are not synthesised, such as epithelial cell infection and in EBV-associated malignancies in immuno-competent individuals (Hayward, 2004).

1.10.3.3 EBV microRNAs (miRNAs)

EBV also encodes at least 22 miRNAs arranged within three clusters (Pfeffer *et al*, 2004; Cai *et al*, 2006; Grundhoff *et al*, 2006). A total of 14 of these miRNAs are located within the region deleted in the B95-8 strain of EBV and accordingly this transformation competent strain is known to encode only eight miRNA genes [miR-BHRF1-1-, -2, -3, miR-BART-1, -2, -3, -4 and the partially deleted miR-BART5] (Barth *et al*, 2008). The coding potential of the majority of these transcripts is unknown and indeed, their potential to serve as pri-miRNAs remains uncertain (Grundhoff *et al*, 2006). It has however been established that the mature EBV-encoded miR-BHRF1-1 and -2 are down-regulated upon TPA induction of the two EBV-infected BL cell lines Daudi and Mutu I (Cai *et al*, 2006). In addition, miR-BART2 associates with Ago2 directing the sequence-specific cleavage of the BALF5

mRNA. Upon induction of the lytic cycle, miR-BART2 expression is down-regulated which results in reduced cleavage of BALF5 mRNA (Barth *et al*, 2008). Recent reports have also identified EBV BHRF1-3 miRNA and findings indicated that changes in BHRF1-3 miRNA levels in a pair of isogenic type I/III BL cell lines were inversely correlated with *CXCL-11/I-TAC* levels. Furthermore, antisense delivery of the BHRF1-3 miRNA could relieve BHRF1-3 miRNA repression of *CXCL-11/I-TAC* (Xia *et al*, 2008). It has been broadly speculated that the viral miRNAs function to establish and maintain latency (Barth *et al*, 2008). In keeping with this induction of lytic EBV replication was found to enhance the expression of many, although not all, of the EBV miRNAs. Indeed the BART miRNAs are highly expressed in latently infected epithelial cells and at lower levels in B-cells. In contrast to this, the BHRF1 miRNAs are found at high levels in B-cells undergoing stage III latency but are undetectable in B-cells or epithelial cells in stage I or II latency (Cai *et al*, 2006).

1.11 EBV Latency Programmes.

Phenotypically distinct human B-cell lines and EBV tumour carrying biopsies display three transcriptionally distinct forms of EBV latency frequently described as latency I, II and III (Young *et al*, 2000). The different types of EBV latency are summarised in Table 1.11-1. These modes of viral latency were suggested on the basis of differential expression of viral latent genes and promoter usage in EBV associated tumours and immortalised B-cell lines (Kerr *et al*, 1992).

Table 1.11-1: Distinct latency programs of the EBV virus

Type of Latency	Viral Genes Expressed	Associated Malignancies
Type I	EBNA1, EBERs, BARTs	Burkitts Lymphoma, Gastric Carcinoma
Type II	EBNA1, EBERs, BARTs, , BARF0 LMP1,LMP2A, LMP2B	Hodgkins Disease, Nasopharyngeal Carcinoma, Peripheral T/NK lymphoma
Type III	All EBV Latent Genes	AIDS-associated lymphomas PTLD*, IM*
Other	EBNA1, 2, LMP1	T-cell Lymphomas
Other	EBERs, EBNA1,2	Smooth Muscle tumors
Other	EBNA1, 2, LP, LMP1	OHL*

*PTLD-Post Transplant Lymphoproliferative Disorder, IM- Infectious mononucleosis, OHL- Oral Hairy Leukoplakia. Adapted from Young *et al*, 2000.

Specifically in EBV-positive BL latency programme I, the EBNA1-only programme is expressed, permitting only the synthesis of EBNA1 transcripts clustered around one other open reading frame; BARF-0, as well as large copy numbers of EBER1 and EBER2 (Young *et al*, 2000). In most other EBV-containing malignant tumours (NPC, EBV-positive HL and gastric cancers), latency programme II is expressed. In these cells both LMP1 and LMP2A/B are expressed in addition to EBNA1 and the EBER RNAs (Young *et al*, 2000). Finally, latency programme III is observed in EBV-induced lymphoblastoid proliferation and in EBV-positive B-cell lymphomas arising under conditions of immuno-suppression and in some B-cell blasts in patients with IM (Kerr *et al*, 1992; Young *et al*, 2000). Latency III (also known as the growth programme) is designed to drive the limited proliferation of B-cells prior to differentiation and the establishment of persistent infection in memory B-cells. EBV preferentially infects B lymphocytes and possesses the unique ability to transform resting B-cells into permanent, latently infected LCLs *in vitro* (Young and Rickinson,

2004). B-cell transformation by EBV, resulting in the establishment of LCLs, therefore remains the dominant *in vitro* model of infection (Young and Rickinson, 2004). In EBV-transformed LCLs, every cell carries multiple extra-chromosomal copies of the viral episome and is driven to proliferate by the EBV growth programme (Lat III) In addition LCLs display transcripts from the *Bam* HI A region of the viral genome (BART transcripts) and abundant expression of *EBER1* and *EBER2*. (Murray and Young, 2001).

EBV-infected LCLs show high levels of expression of the B-cell activation markers CD23, CD30, CD39 and CD70, and of the cell adhesion molecules lymphocyte-function-associated antigen 1 (LFA1; also known as CD11a/18), LFA3 (also known as CD58) and intercellular cell adhesion molecule 1 (ICAM1; also known as CD54) (Young and Rickinson, 2004). These markers are usually absent or expressed at low levels on resting B-cells, but are transiently induced to high levels when these cells are activated into short term growth by antigenic or mitogenic stimulation, indicating that EBV-induced immortalisation can be elicited through the constitutive activation of the same cellular pathways that drive physiological B-cell proliferation (Young and Rickinson, 2004). The ability of EBNA2, EBNA3C and LMP1 to induce LCL-like phenotypic changes when expressed individually in human B-cell lines indicates that these viral proteins are key effectors of the immortalisation process (Wang *et al*, 1990). The role of EBV latent genes in the *in vitro* transformation of B-cells has been confirmed by the generation of recombinant forms of EBV that lack individual latent genes. Studies using such viruses have confirmed the absolute requirement for EBNA2 and LMP1 in the transformation process, and have also highlighted a crucial role for EBNA1, EBNA-LP, EBNA3A and EBNA3C (Kieff, E. and Rickinson, A.B, 2001).

Other less well-characterised patterns of EBV gene expression have been identified in further EBV associated diseases. For example it had been proposed that EBV may be associated with some breast and hepatic cancers where EBNA1 but not EBER expression has been observed; however other studies indicate that breast and hepatic cancers are not EBV associated diseases (reviewed by Herrmann and Niedobitek, 2003).

1.12 EBV-mediated regulation of cell growth and survival

Suppression of the cellular apoptotic program by EBV is central to both the establishment of latent infection and the development of EBV-associated malignancies. When EBV infects resting B lymphocytes it primarily encounters resting B-cells in the oropharyngeal mucosal lymphoid tissue (Young and Rickinson, 2004). It drives the infected cells into cycle and maintains cell division, increasing the population of infected cells and allowing them to progress, via the B-cell maturation pathways, to the site of latent persistence of the virus, in resting memory B-cells (Young and Rickinson, 2004). The complex mechanisms by which EBV affects cell growth and can prevent cell death are a result of a combination of EBV-induced pathways that affect both cell growth and apoptosis, and the co-operative roles of the latent genes, especially EBNA2, EBNA3C and LMP1 in these processes has long been established (Wang *et al*, 1990).

1.12.1 EBV affects Cell Cycle Progression

Cells typically progress through the cell cycle in a orderly fashion through a growth phase (G_0/G_1) to a synthetic phase in which the DNA is copied (S), to a second growth phase (G_2) prior to cell division or mitosis (M) and back to a quiescent cell arrest phase (G_0/G_1) following cell division (Flaitz and Hicks, 1998) (Figure 1.12.1). This cell cycle is designed to have several checkpoints to assess cells harbouring damaged or mutated DNA, which if not repaired or eliminated from the cell pool could potentially result in the development of a malignancy (Park and Lee, 2003). Progression through the cell cycle is regulated by many different proteins, such as tumour suppressor gene products, p53 and pRb (retinoblastoma gene product), which act within the cell cycle facilitating the repair of damaged or mutated DNA within single cells, or alternatively, induce these injured cells to undergo apoptosis (Flaitz and Hicks, 1998; Park and Lee, 2003). EBV exploits normal cellular pathways to regulate cell cycle progression. Indeed EBV genes can manipulate the proliferation machinery of the infected cell through the deregulation of the function of several oncogenes and tumour suppressor genes implicated in the cell cycle such as c-myc,

p53 and pRb, thus inhibiting the ability of these proteins to redirect cells with damaged or mutated DNA toward a DNA repair pathway or apoptosis. As a consequence, host cells with latent viral infection are allowed to proliferate without regulation. Although rare events, these virally-infected cells may then develop mutations induced by 'DNA-damaging' events, including tobacco, alcohol, carcinogens, toxins, ionizing radiation, prolonged ultraviolet light/sun exposure, chemical insult, or even other viral infections which may result in the development of a malignant tumour (Flaitz and Hicks, 1998).

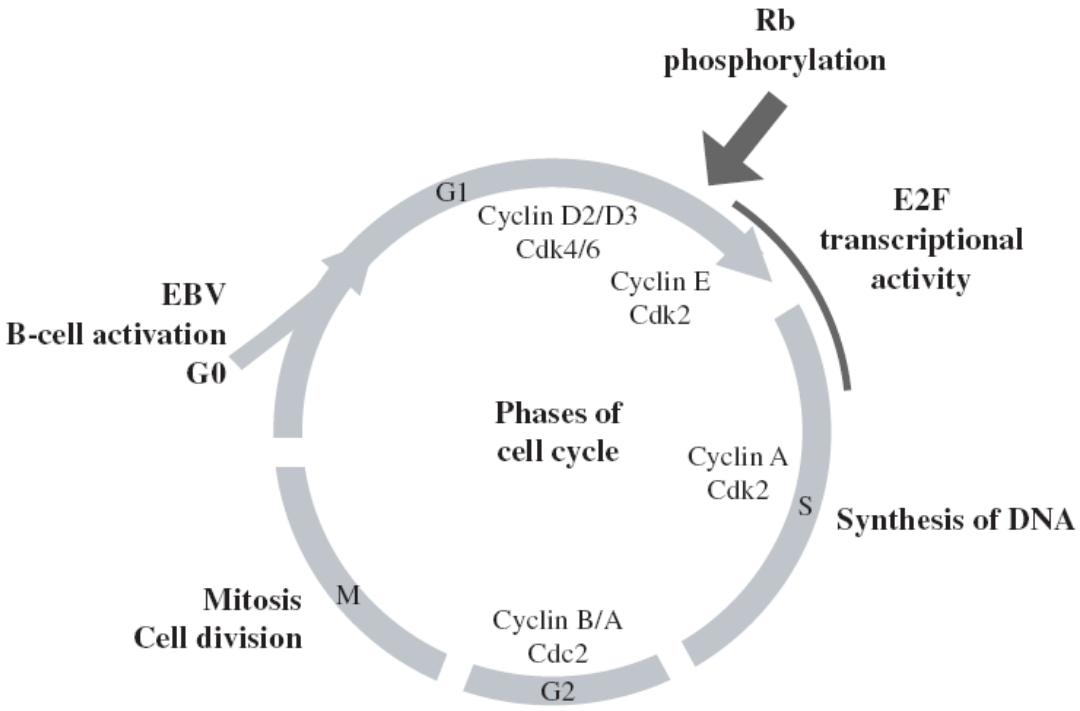


Figure 1.12.1: Phases of lymphocyte cell cycle

The cell cycle is regulated in four distinct phases; G1, S-phase, G2 and M-phase. The first biochemical event observed, in early G1 phase, is the induction of the D-type cyclins and their partners, cyclin dependent kinases (cdks), cdk4 and cdk6. One of either cdk4 or cdk6 partners a D- type cyclin, in the absence of cyclin dependent kinase inhibitors (CDKIs), to become catalytically active. The induction of D-type cyclins is paralleled by a loss in CDKIs. The most important substrate of the cyclin D/cdk4/6 complex is the family of pocket proteins, including pRb, p130 and p107. These proteins repress the E2F family of transcription factors, and the E2F-pocket protein complexes act as gene suppressors. Phosphorylation of pRb by the cyclin D/cdc4/6 complex results in the release of E2F transcription factors and transcription of E2F-responsive genes. In many systems, the induction of E2F transcriptional activity results in entry into the cell cycle (From Brennan, 2001).

1.12.2 Interactions of EBV proteins with the cell cycle

Cyclin D2 is probably the first cell cycle protein to be induced following EBV infection of B-cells (Spender *et al*, 1999) and it is accompanied by a loss of the CDKI p27kip1. Phosphorylation of the pocket protein pRb is observed approximately 6 hours later. All these events correspond to those seen during normal B-cell proliferation, thus implying that EBV exploits normal cell pathways to regulate pRb phosphorylation during the cell cycle (Kempkes *et al*, 1995a; Cannell *et al*, 1996).

1.12.2.1 pRb

Retinoblastoma protein (pRb) is a central regulator of cellular proliferation, controlling entry into G_1/S in the cell cycle, mainly through its interaction with the cellular transcription factor E2F, which activates genes important in DNA synthesis (Leiderman *et al*, 2007). EBV has been shown to regulate pRb-modulated pathways and to drive cells through the G_1/S restriction point (Cannell *et al*, 1996) (Figure 1.12.1). Through the up-regulation of cyclins involved in the inactivation of pRb, such as cyclin D2 and cyclin A, or by directly inactivating pRb itself, EBV liberates the E2F transcription factors to drive potentially infected cells through the cell cycle. In this regard, it has been shown that EBNA2 and EBNA-LP are sufficient to activate the expression of cyclin D2 during immortalisation of resting human B lymphocytes (Sinclair *et al*, 1994) and LMP1 is also thought to regulate D2 (Arvanitakis *et al*, 1995). Moreover, the *c-myc* oncogene, a direct target of EBNA2 (Kaiser *et al*, 1999; Spender *et al*, 2001) has also been shown to *trans*-activate the D2 promoter (Bouchard *et al*, 1999). EBV may also repress cyclin dependent kinase inhibitors in order to facilitate the inactivation of pRB. The induction of cyclin D2 is paralleled by the loss of the CDKI p27kip1 in response to EBV (Slingerland and Pagano, 2000; Spender *et al*, 2001) and EBNA3C has been shown to play a role in the regulation of p27kip1 (Parker *et al*, 2000). Since cyclin D2 and p27kip1 together regulate the cyclin dependent kinases, cdk4 and cdk6 activity, the link between EBNA3C and p27kip1 suggests that EBNA3C along with EBNA2, EBNA-LP and LMP1 is implicated in the progression through the G_1 phase of the cell cycle (Brennan, 2001).

Cyclin A, an activator of S phase progression, has been shown to interact with the carboxy terminus of EBNA3C *in vitro*. EBNA3C stimulated cyclin A-dependent kinase activity and rescued p27-mediated inhibition of cyclin A/Cdk2 kinase activity by decreasing the molecular association between cyclin A and p27 in cells (Knight and Robertson, 2004). In addition, EBNA3C can target the SCF^{Skp2} complex, thereby regulating the activity and stability of cyclin A/cdk2 and pRb complexes (Knight and Robertson, 2004; Knight *et al*, 2004; Knight *et al*, 2005a; Knight *et al*, 2005b).

1.12.2.2 p53

The p53 pathway is targeted for inactivation in most human cancers either directly or indirectly, highlighting its critical function as a tumour suppressor gene. p53 is normally activated by cellular stress and mediates a growth-suppressive response that involves cell cycle arrest and apoptosis (Kuribayashi and El-Deiry, 2008). Elevated incidences of p53 mutation have been identified in BL tumour biopsies and BL cell lines (Farrell *et al*, 1991). In addition BL cells with p53 mutations have been shown to be relatively resistant to DNA-damaging drugs such as cisplatin compared to those cell lines harboring wild-type p53 (Allday *et al*, 1995). EBV LMP1 can protect against wild-type p53 mediated apoptosis (Okan *et al*, 1995; Fries *et al*, 1996). Moreover, LMP1 regulates p53 both at transcriptional and translational level (Li *et al*, 2007; Li *et al*, 2008). In addition, BZLF1 has been shown to interact with p53 and inhibit its *trans*-activating function in lymphoid cells (Cayrol and Flemington, 1996) and EBNA-LP can also interact with p53 in vitro, although the functional significance of this interaction has not yet been determined (Szekely *et al*, 1993).

1.12.2.3 c-Myc

c-Myc, a helix-loop-helix leucine zipper transcription factor, is known to be involved in numerous cellular functions such as cell proliferation, apoptosis, metabolism, adhesion, and differentiation in a variety of different cell types. Myc promotes proliferation and growth through both the induction of genes involved in cell cycle control (including *CDK4, CDC25A, cyclin D1, D2, A*, and *E)*, and through the

suppression of growth-arresting genes (such as *GADD45,p15INK4b, p21CIP1, p27KIP1, GAS1)* (Rui and Goodnow, 2006). The C-terminal basic helix–loop–helix zipper domain facilitates binding to DNA sequences with the core consensus sequence 5'-CACGTG'3' known as an "E-box", and requires the association of its hetero-dimeric partner, Max (Brady *et al*, 2007). Myc also possesses an N-terminal *trans*-activation domain through which it drives the expression of a multitude of target genes (Brady *et al*, 2007). To date, there are approaching 1,700 genes which have been identified as Myc-responsive genes (www.myccancergene.org). However, only a minority of the Myc-responsive genes have been implicated as direct target genes (Zeller *et al*, 2006). c-Myc expression is required to drive cells through the G_1 into the S phase of the cell cycle (Figure 1.12.1). A model explaining the role of c-Myc in the apparently diverse roles of apoptosis and proliferation, predicts that c-Myc expression preferentially results in proliferation under favorable growth conditions with this activity being dependent on its apoptosis-promoting activity being blocked (Evan *et al*, 1994; Jamerson *et al*, 2000).

Myc over-expression has been observed in tumour cells from 91% of BL cases. Indeed, chromosomal translocations of *c-myc* into the *Ig* loci is observed in practically all BLs, and the most frequent translocation, the *c-myc/IgH* t(8; 14), accounts for approximately 80% of these. The remaining 20% of cases are split between the translocations with the *IgK* and *IgL* (t(2;8) and t(8;22) respectively). The c-*myc* gene becomes transcriptionally activated as a consequence of the chromosomal translocation and is sustained by up-stream *c-myc* regulators such as NF-κB or Bcl-6 (Rui and Goodnow, 2006). Thus under certain circumstances, Myc deregulation is regarded as priming normal proliferating cells for apoptosis.

It is clear that c-Myc expression is deregulated in EBV-immortalised (non-tumour-derived) B-cells (Cherney *et al*, 1994). Studies using an LCL in which the function of EBNA2 is dependent on the presence of estrogen have revealed that EBNA2 is the EBV protein predominantly responsible for driving *c-Myc* in LCLs (Kempkes *et al*, 1995a) and that c-Myc is a direct target gene of EBNA2 in this context (Kaiser *et al*, 1999; Moore *et al*, 2001). *Myc* is also induced to moderate levels by LMP1 (Schlee *et al*, 2004). In addition EBNA3C has been shown to stabilise *c-Myc* expression, and

this recruitment of both *c-Myc* together with its cofactor Skp2 to *c-Myc*-dependent promoters can enhance *c-Myc*-dependent transcription (Bajaj *et al*, 2008).

1.12.3 Cytokines activated during EBV-mediated immortalisation

Following B lymphocyte activation, EBV also induces the synthesis of several cytokines including TNF, IL-1, IL-6 and IL-10. IL-6 and IL-10 both play a significant role in the growth of EBV-transformed cells (Beatty *et al*, 1997; Kitagawa *et al*, 2000; Haddad *et al*, 2001). LMP1 has been implicated in the regulation of both these cytokines via NF-κB and the p38 stress activated protein kinase (Nakagomi *et al*, 1994; Eliopoulos *et al*, 1997; Eliopoulos *et al*, 1999b; Vockerodt *et al*, 2001). Further studies indicate that IL-10 may also be regulated by the EBERs (Kitagawa *et al*, 2000). The receptor molecules that IL-6 and IL-10 use to mediate their activities both activate the JAK family of tyrosine kinases, and downstream signaling cascades including STAT proteins and the PI3K pathway (Brennan, 2001). Significantly, IL-10 has been shown to act as a co-factor for B-cell growth (Moore *et al*., 2001), and it has been demonstrated that PI3K is important for IL-10-mediated cell growth of both primary monocytes and a murine mast cell line (Crawley *et al*, 1996). Interestingly, EBV vIL-10 enhances the growth transformation of a B-cell infected with EBV (Stuart *et al*, 1995) and both human and EBV-encoded IL-10 can induce LMP1 in the absence of EBNA2 in the Daudi, P3HR1, and other BL cell lines. Furthermore, exposure of the KMH2-EBV cells to CD40-ligand and IL-4 induced LMP1 expression, in the absence of EBNA2 and IL-10 could induce the expression of LMP1 in tonsillar B-cells infected with the non-transforming, EBNA2-deficient EBV strain P3HR1 and enhance LMP1 expression in two EBV-positive NK lymphoma lines suggesting that IL-10 might contribute to the establishment of type II EBV latency (Kis *et al*, 2006).

1.12.4 Signaling Molecules involved in EBV-mediated immortalisation

Constitutively active signaling pathways are a common feature of many malignant cells. Thus, it is important to characterise the potential role of signaling molecules that are activated by EBV-induced cytokines and by the EBV genes themselves. The pathways activated by IL-6 and IL-10 (JAK family of tyrosine kinases, PI3K and STATs) have previously been implicated in lymphocyte growth and transformation (Brennan *et al*, 1997; Bowman *et al*, 2000; Imada and Leonard, 2000; Ihle, 2001). The STAT family of transcription factors are one substrate of the JAK family of tyrosine kinases. STATs have been shown to be active in EBV-immortalised cells (Weber-Nordt *et al*, 1996) and are thought to play a role in oncogenesis (Bowman *et al*, 2000). It has also been reported that LMP1 can activate JAK3 and STAT1 (Gires *et al*, 1999) and a STAT reporter that is known to bind STAT1, STAT3 and STAT5 (Brennan, 2001).

PI3K, a lipid kinase that is involved in lymphocyte survival (Pogue *et al*, 2000; Carey and Scott, 2001) and proliferation (Brennan *et al*, 1997; Craddock *et al*, 1999), can be activated by oncogenic Ras (Vanhaesebroeck and Alessi, 2000). Reports indicate that pathways downstream of the small G protein Ras are active in EBV-immortalised cells (Roberts and Cooper, 1998) and significantly inhibition of PI3K using a chemical inhibitor, inhibits the proliferation of EBV-immortalised cells (Brennan, 2001). Interestingly, LMP2 was found to activate a PI3K-mediated pathway, Akt (Scholle *et al*, 2000; Swart *et al*, 2000). Furthermore, PI3K has also been shown to affect D-type cyclin expression in EBV-immortalised B-cells (Brennan, 2001), suggesting a mechanism for its role in EBV-induced lymphocyte growth.

1.12.5 EBV and the Six Hallmarks of Cancer

The basis for the development of cancerous cells has been explained by the philosophy that cancer cells harbour defects in regulatory pathways that control normal cellular proliferation and homeostasis. It has been suggested that the vast majority of cancer cell genotypes are due to the manifestation of six essential alterations in cell physiology that together dictate malignant growth. These are: (1) self-sufficiency in growth signals, (2) insensitivity to growth-inhibitory signals, (3)

evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis (as reviewed in Hanahan and Weinberg, 2000). These six capabilities are thought to be a common factor in all types of human tumours (Hanahan and Weinberg, 2000). Each of these physiologic changes is represented during tumour development attributed to EBV infection. EBV-infected cells generate many of their own growth signals, including among others, the virally encoded protein LMP1 (Section 1.10.2.1) which is required to drive cells into S phase and through the cell cycle, thereby reducing dependence on stimulation from their normal tissue microenvironment (1). In addition EBV encodes many viral proteins, which target crucial cell cycle regulatory proteins and has been shown to regulate pRb-modulated pathways (through which all anti-proliferative signals are funnelled) driving cells through the G_1/S restriction point, (see Section 1.12.2.1) (2). EBV is known to evade apoptosis through the activation of NF-κB, Bcl-2 family members, A20 and Akt activity (Hayward, 2004) (3) and is known to immortalise B-cells in culture to generate continually proliferating B lymphoblastoid cell lines (Hayward, 2004) (4). It has also been shown that lytically infected cells may contribute to the growth of EBV-associated malignancies by enhancing angiogenesis (Hong *et al*, 2005) (5). Finally, the down-regulation of the adhesion protein E-cadherin in EBV-infected NPC has recently been identified (Krishna *et al*, 2005). E-cadherin acts as an invasion/metastasis-suppressor gene and loss of E-cadherin function has been shown to potentiate tumour cell invasion (6).

1.12.6 Apoptosis

Apoptosis is a genetically controlled pre-programmed cellular suicide, in which individual cells are destroyed while the integrity of the surrounding tissue is preserved. While this targeted cell destruction is essential in many physiological contexts including embryogenesis, immune cell maturation and response, tissue homeostastis and in the cellular response to injury, it also functions as an emergency response to aberrant growth induced by the activation of oncogenes and viral infection and its deregulation is implicated in many pathological states. The process involves dramatic morphological changes including cell shrinkage, nuclear re-organisation, plasma membrane blebbing and eventual fragmentation of the cell into membrane bound apoptotic bodies (Saraste and Pulkki, 2000; Olson and Kornbluth, 2001). The

induction of early cell death in response to viral infection would limit virus production and consequently diminish or eliminate spread of virus progeny in a population (Shen and Shenk, 1995). Thus, in order to maximise their replicative capacity many viruses deregulate the normal cell cycle and secure selective advantage and biological success by developing mechanisms which suppress either the triggers of apoptosis or indeed the programme itself. EBV is one such virus (Shen and Shenk, 1995). By preventing infected lymphocytes from undergoing apoptosis, EBV ensures survival/persistence in the host system.

Apoptosis is orchestrated by a family of cysteine proteases known as the caspases. These molecules are activated upon receipt of divergent pro-apoptotic stimuli and play an essential role in the implementation of apoptosis co-ordinating the efficient dismantling and engulfment of targeted cells (Cryns and Yuan, 1998; Saraste and Pulkki, 2000). Caspases are synthesised as relatively inactive precursors that require proteolytic processing in order to achieve activation (Thornberry and Lazebnik, 1998; Saraste and Pulkki, 2000). The family is generally divided into two distinct groups; initiator caspases at the apex of the cascade, which are activated first by the action of caspase adaptor molecules, such as Fas-associated protein with death domain (FADD) or apoptotic protease-activating factor-1 (Apaf-1). These molecules instigate a caspase cascade, activating the effector caspases further downstream, whose function is principally to dismantle cellular structures. There are three main apoptotic cascades activated by specific initiator caspases: the death receptor pathway, the mitochondrial pathway, and the ER pathway. These are activated by caspase-8, caspase-9, and caspase-12, respectively (Gupta *et al*, 2005).

Two separate pathways of apoptotic cell death can be distinguished by the involvement of the Bcl-2 family proteins and by which caspases are required for their execution (Figure 1.12.2) (Youle and Strasser, 2008). The intrinsic pathway, (also called the Bcl-2-regulated or mitochondrial pathway), is activated by various developmental triggers or cytotoxic insults, such as viral infection, DNA damage and growth-factor deprivation, and is regulated by the Bcl-2 family of proteins. This pathway predominantly leads to the activation of caspase-9 (Hakem *et al*, 1998) but in certain cell types is known to take place in the absence of caspase-9 or Apaf-1 (Marsden *et al*, 2002; Youle and Strasser, 2008). The extrinsic (or death-receptor

pathway) is triggered by ligation of the death receptor molecules such as Fas or tumour necrosis factor (TNF) receptor-1 (TNFR1) (members of the TNF receptor family) which harbour an intracellular death domain with the ability to recruit and activate caspase-8 through FADD at the cell surface (Youle and Strasser, 2008). This recruitment causes subsequent activation of downstream effector caspases, such as caspase-3, -6 or -7, independently of any involvement of the Bcl-2 family (described in Figure 1.12.2). Interestingly, it has been suggested that the two pathways may also be interconnected and that the molecules in one pathway have the ability to influence those of the other. In this regard it has been established that the extrinsic pathway can intersect the intrinsic pathway through caspase-8 cleavage-mediated activation of the pro-apoptotic BH3-only protein Bid (Yin *et al*, 1999; Kaufmann *et al*, 2007) in which the C-terminal truncated form of Bid (tBid) translocates to mitochondria from where it promotes further caspase activation through the intrinsic pathway (Caspase 9) (Yin *et al*, 1999).

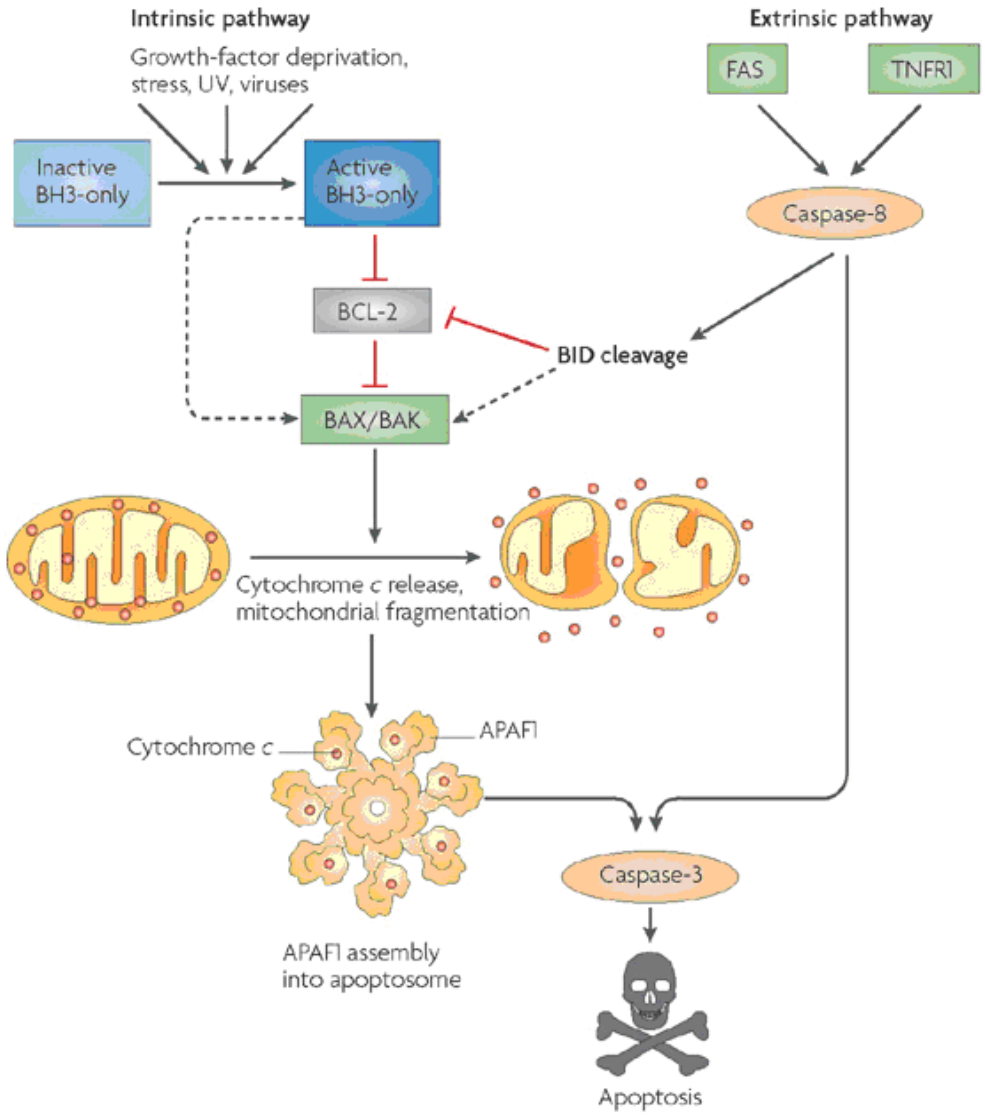


Figure 1.12.2: Schematic depicting intrinsic and extrinsic pathways of apoptosis

The intrinsic apoptotic pathway (left) begins with BH3-only protein induction or post-translational activation, resulting in the inactivation of Bcl-2 family members. This relieves inhibition of Bax and Bak activation, thus promoting apoptosis. Some BH3-only proteins (Bim and Puma), activate Bax/Bak (dotted line). Activated, Bax and Bak promote cytochrome *c* release ultimately leading to the activation of the caspases which in turn orchestrate the destruction of the cell. The extrinsic pathway (right) can bypass the mitochondrial step and activate caspase-8 directly. The Bcl-2 family regulates the intrinsic pathway and can also modulate the extrinsic pathway when cleavage of Bid communicates between the two pathways. A detailed discussion of Bcl-2 family can be found in Section 1.13 (From Youle and Strasser, 2008).

The extrinsic and intrinsic pathways both converge upon the execution phase, which is considered the final chapter of apoptosis, beginning with the activation of the effector caspases. Caspase-3, caspase-6, and caspase-7 function as effector caspases activating cytoplasmic endonucleases and proteases, which degrade nuclear material and cytoskeletal proteins (Elmore, 2007). These effector caspases cleave various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, ultimately causing the morphological and biochemical alterations seen in apoptotic cells (Slee *et al*, 2001). Caspase-3 is thought to be the most important of the effector caspases, and is activated in response to any of the initiator caspases. Caspase-3 specifically activates the endonuclease caspase-activated DNase (CAD). In healthy proliferating cells CAD is complexed with its inhibitor, ICAD (inhibitor of caspase-activated DNase). In apoptotic cells, activated caspase-3 cleaves ICAD to release CAD (Sakahira *et al*, 1998). CAD then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganisation and disintegration of the cell into apoptotic bodies and facilitates disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (Kothakota *et al*, 1997).

Phagocytic absorption of apoptotic cells is the final phase of apoptosis. In *vivo*, apoptotic bodies are promptly cleared via phagocytosis, by either phagocytotic cells or by cells in the direct vicinity of the apoptotic bodies. Phospholipid asymmetry and externalisation of phosphatidylserine (PS) on the surface of apoptotic cells and their fragments is a key characteristic of this stage. A PS receptor is involved in phagocytosis of apoptotic cells. Usually, PS is maintained at the inner layer of the plasma membrane by the action of an ATP dependent PS flippase enzyme. This flippase is inactivated through the action of the caspases, and a scramblase is activated, which ultimately leads to the redistribution of PS to the outer leaflet of the plasma membrane. PS externalisation is widely used to analyse apoptosis in FACS analysis due to PS binding to annexin V (Lawen A, 2003). The mechanism of PS translocation to the outer leaflet of the cell during apoptosis is not completely understood, and research indicates that Fas, caspase-8, and caspase-3 are involved in the regulation of PS externalisation on oxidatively stressed erythrocytes. However, it should be noted that, caspase-independent PS exposure occurs during apoptosis of

primary T lymphocytes (Ferraro-Peyret *et al*, 2002; Mandal *et al*, 2005). The appearance of PS on the outer leaflet of apoptotic cells then assists non-inflammatory phagocytic recognition, facilitating their early uptake and subsequent disposal (Fadok *et al*, 2001).

1.13 Bcl-2 Family proteins

Key players in the determination of cell survival and death are the members of the Bcl-2 family of proteins. The Bcl-2 family, both pro- and anti-apoptotic, are responsible for controlling the intrinsic pathway of apoptosis in mammals (Youle and Strasser, 2008). The founder of this family the B-cell lymphoma-2 (*bcl-2*) gene was discovered at the t(14;18) chromosome translocation breakpoint in B-cell follicular lymphomas, where its transcription becomes excessively driven by the immuno-globulin heavy chain gene promoter and enhancer on chromosome 14 (Bakhshi *et al*, 1985; Tsujimoto *et al*, 1985; Cleary *et al*, 1986). One key early discovery that introduced a new paradigm for carcinogenesis was that over-expression of *bcl-2* does not promote cell proliferation as most previously discovered oncogenes do; rather, over-expression of *bcl-2* inhibits cell death (Vaux *et al*, 1988). The Bcl-2 family of regulators function upstream of the caspase cascade (Igaki and Miura, 2004) and display a range of bioactivities, from inhibition to promotion of apoptosis (Youle and Strasser, 2008).

In mammals, there are at least 23 Bcl-2 family members, including Bcl-2 itself and proteins that have either three-dimensional (3D) structural similarity or a predicted secondary structure that is similar to Bcl-2 (Figure 1.13.1) (Youle and Strasser, 2008). Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4 which correspond to α-helical segments (Adams and Cory, 1998; Gross *et al*, 1999). A characteristic of the Bcl-2 family proteins is their ability to form homo- and hetero-dimers, as well as their ability to become integral membrane proteins (Gross *et al*, 1999).

The anti-apoptotic members of this family (*Bcl-2, Bcl-$_{XL}$, Bcl-w, Mcl-1, A1, Boo/Diva, Bcl-B, and C. elegans CED-9*) are known to contain at least three of the four Bcl-2 homology domains. The pro-apoptotic members can be further characterised into to

sub-categories being either "Bax-like" in their structure (*Bax, Bak, Bcl-$_{XS}$, Bok/Mtd and Bcl-$_{GL}$*) i.e. containing two or three BH domains, or alternatively, the so-called "BH3-only" proteins which share homology with each other and the remainder of the Bcl-2 protein family only through the short BH3 motif (*Bad, Bik, Blk, Bid, Hrk, Bim/Bod, Bmf, Noxa, Puma and C. elegans Egl-1)* (Bouillet and Strasser, 2002). Overall the structures of the BH3-only proteins seem to be unrelated and appear to lack a close evolutionary relationship to the core members of the Bcl-2 family (Aouacheria *et al*, 2005). However, all BH3-only proteins interact with and regulate the core Bcl-2 family proteins to promote apoptosis. The BH3 domains of the Bcl-2 family members are crucial to their function. Indeed, just the 51 amino acids that code the domain in *bak* are enough to have a pro-apoptotic effect, while mutations of the BH3 regions in *bax, bak* and *bik* were found to render the proteins ineffective (Elangovan and Chinnadurai, 1997; Tong *et al*, 2001).

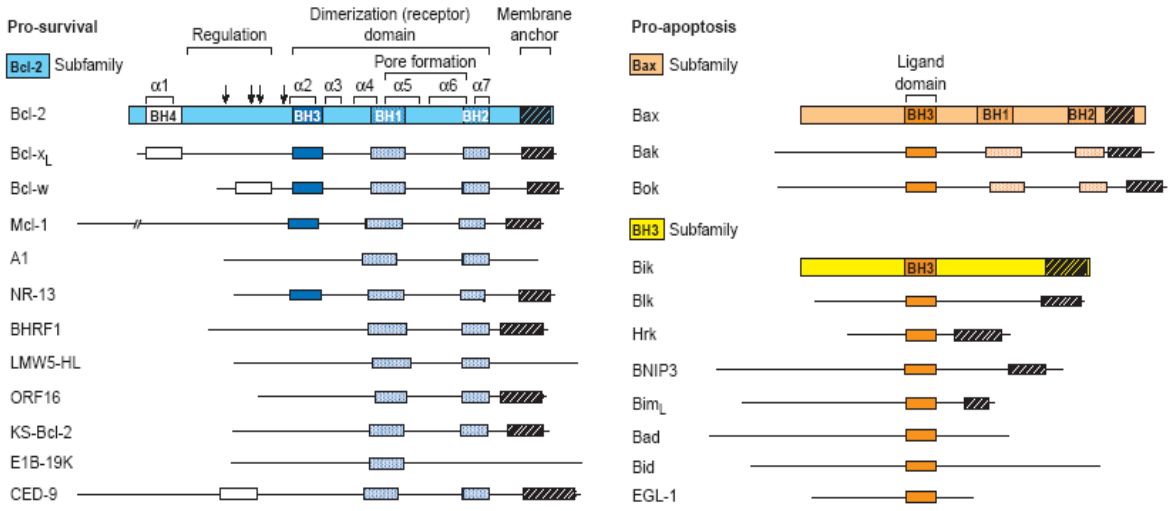


Figure 1.13.1: X-ray crystallography suggests that both pro-apoptotic and anti-apoptotic Bcl-2 family members share a common structure.

Bcl-2 family members possess up to four conserved Bcl-2 homology (BH) domains designated BH1, BH2, BH3 and BH4 which correspond to α-helical segments. Each of the BH domains is indicated above. α1-7 indicates helices in Bcl-$_{XL}$, while the arrows represent phosphorylation sites in Bcl-2. BHRF1 has been shown to harbour a BH3 domain, which is unexposed (From Adams and Cory, 1998).

The pro-apoptotic family members Bax and Bak are critical in the permeabilisation of the outer mitochondrial membrane (OMM) and the subsequent release of apoptogenic molecules such as cytochrome *c* and SMAC/DIABLO into the cytosol, which ultimately leads to caspase activation. The anti-apoptotic family members, such as Bcl-2 and Bcl-$_{XL}$, are known to inhibit the activity of Bax and Bak. A growing body of evidence indicates that BH3-only proteins de-repress Bax and Bak by directly binding and inhibiting Bcl-2 and other anti-apoptotic family members (Willis *et al*, 2007) or alternatively that they may directly activate Bax and Bak (Hacker and Weber, 2007) (Figure 1.12.2).

It has been established that both Bax and Bak promote caspase activation by their effects on mitochondria. These two pro-apoptotic proteins induce the release of proteins from the space between the inner and outer mitochondrial membranes (Newmeyer and Ferguson-Miller, 2003). This process of mitochondrial outer membrane permeabilisation (MOMP) leads to the release of cytochrome *c* and other soluble proteins. While it is generally believed that Bax and Bak form integral membrane pores, the biochemical nature of such pores and how anti-apoptotic Bcl-2 family proteins might regulate them remains to be seen (Chipuk *et al*, 2006). During cytochrome *c* release into the cytosol, Bax and Bak induce mitochondria to fragment into more numerous and smaller units (Martinou and Youle, 2006).

Once the OMM has been permeabilised, soluble proteins diffuse from the intermembrane space into the cytosol, where they promote caspase activation. The best studied of these proteins is cytochrome *c*, which binds to Apaf-1 and leads to the assembly of the apoptosome, which can bind pro-caspase-9 or and thereby induce its activation through a conformational change in the molecule (Wang, 2001; Shi, 2006). Cytochrome *c*–Apaf-1-dependent activation of caspase-9 is absolutely required for neuronal and fibroblast cell-death processes (Hao *et al*, 2005). However, lymphocytes may use alternative Apaf-1-, caspase-9- and cytochrome *c*-independent, but pro-apoptotic Bcl-2-family-member-dependent, pathways for caspase activation (Marsden *et al*, 2002; Hao *et al*, 2005). One such pathway which is Apaf-1-independent, involves the relief of caspase inhibition by inhibitor of apoptosis proteins (IAPs), such as XIAP, which bind and neutralise certain caspases (such as caspase-9 and caspase-3). This inhibitory action of IAPs can be antagonised by the binding of

SMAC/DIABLO, which is released from mitochondria after the activation of Bax and/or Bak (Youle and Strasser, 2008).

Members of the Bcl-2 family are expressed to varying degrees in different cell types. In particular B-cells are known to express a battery of both apoptotic and anti-apoptotic Bcl-2 family members, including Bcl-2, Bcl-xl, Bfl-1, Mcl-1, Bcl-w, Bim, Bax, Hrk, Bid, Noxa, Bak and Puma (Garrison *et al*, 2008) and Bik (Arena *et al*, 2003).

1.14 BH3-only proteins

BH3-only proteins are essential for cell death initiation (Labi *et al*, 2006). A growing body of evidence indicates that different apoptotic stimuli can activate distinct but sometimes overlapping sets of BH3-only proteins (Strasser, 2005). The pro-apoptotic activity of BH3-only proteins is tightly controlled by diverse transcriptional and post-translational mechanisms to prevent inappropriate cell death (Huang and Strasser, 2000). For example, Puma and Noxa are transcriptionally induced in response to DNA damage by p53 whereas other BH3-only proteins, such as Bid or Bad, are thought to be mainly regulated at the post-translational level (Cory and Adams, 2002).

While the molecular basis of BH3-only protein activity is still under investigation, it is clear that BH3-only proteins have the ability to bind to a hydrophobic groove on the surface of Bcl-2-like molecules, formed by their BH1, BH2 and BH3 domains thereby antagonising their pro-survival function (Willis and Adams, 2005). This physical interaction is dependent on a functional BH3-domain within the BH3-only protein. It has been suggested that BH3 only proteins can function via at least three separate modes of action (Letai *et al*, 2002; Kuwana *et al*, 2005; Strasser, 2005) and since the signaling pathways that mediate apoptosis seem to proceed through a succession of interconnected amplification loops, it is conceivable that more than one of these processes takes place (Strasser, 2005).

In one model of BH3-only protein function, this interaction is thought to block the ability of Bcl-2-like pro-survival proteins to regulate the pro-apoptotic Bax/Bak, preventing them from undergoing oligomerisation and causing loss of mitochondrial outer membrane integrity and cell death (Strasser, 2005; Willis *et al*, 2007) (Figure

1.14.1A). It is also been suggested that the BH3-only proteins and Bax/Bak-like proteins function in parallel in a mutually dependent manner (Strasser, 2005) (Figure 1.14.1B). In an alternative model, the BH3-only proteins activate Bax/Bak directly. In support of this model tBid and Bim have this ability. Bid and Bim directly activate Bax, causing its insertion into the mitochondrial membrane where it becomes active. However, Bim and tBid can be bound and neutralised by Bcl-2-like proteins, by sequestration, and the role of other BH3-only proteins in this model is to occupy Bcl-2-like proteins and stop them from sequestering Bim/tBid. Accordingly, these BH3-only proteins (e.g. Bad and Bik) are called sensitisers (Strasser, 2005; Hacker and Weber, 2007) (Figure 1.14.1C).

Additionally a further possibility is that, to keep cells from dying, Bcl-2 (and/or its pro-survival homologues) might bind and inhibit a protein, perhaps a caspase activator (such as Apaf-1), and that apoptotic stimuli might trigger both BH3-only proteins and Bax/Bak-like proteins, which together would disrupt this binding (Strasser, 2005) (as depicted in Figure 1.15.5)

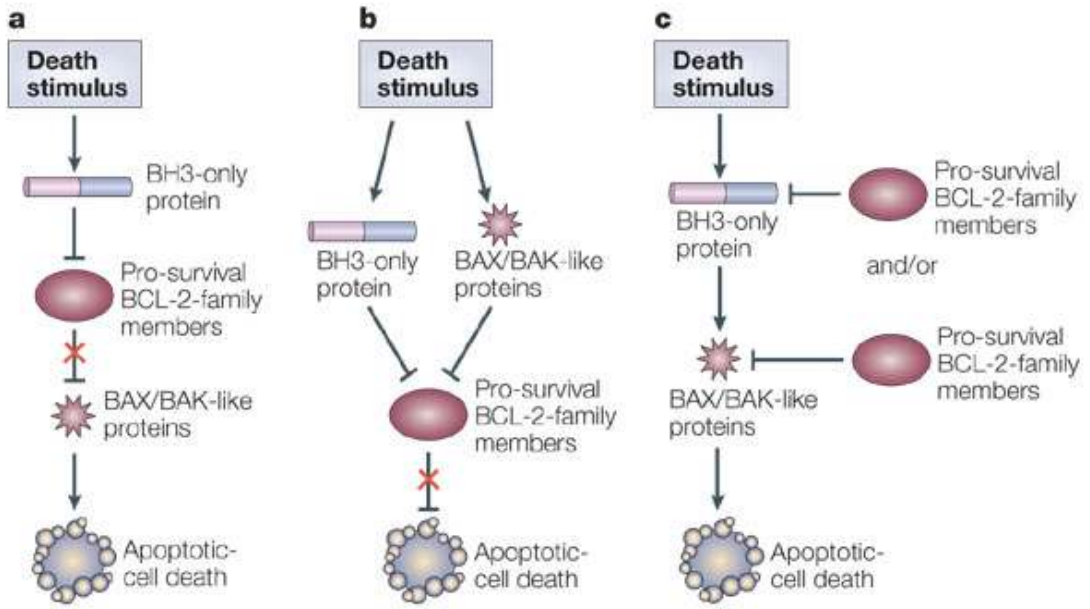


Figure 1.14.1: Models for interaction between BH3-only proteins, Bax/Bak-like proteins and pro-survival Bcl-2-family members in signaling in apoptosis.

Three possible models are illustrated above. (A) BH3-only proteins might inactivate Bcl-2-like pro-survival proteins, which keep cells alive by inactivating Bax/Bak-like proteins. (B) Death stimuli might simultaneously activate BH3-only proteins and Bax/Bak -like proteins, which both block the pro-survival function of Bcl-2 and its homologues. (C) BH3-only proteins might activate the pro-apoptotic activity of Bax/Bak-like proteins directly, and pro-survival Bcl-2-family members might function by binding and inactivating BH3-only proteins and/or Bax/Bak-like proteins. (From Strasser, 2005).

BH3-only proteins have distinct, only partially overlapping, binding preferences for their pro-survival Bcl-2-like relatives (Chen *et al*, 2005), indicating that certain BH3-only proteins can only antagonise a very distinct subset of Bcl-2-like molecules *in vivo* (e.g. Noxa can only block Mcl-1 and A1), while others, such as Bim and Puma, are able to bind with similar affinities to all known Bcl-2 pro-survival proteins and, therefore, may have broader physiological functions than other BH3-only proteins (Labi *et al*, 2006).

Since these proteins are essential initiators of cell death pathways, understanding their regulation may provide novel therapeutic targets to prevent pathological cell death. Inhibitors of BH3-only proteins may alleviate degenerative disorders, whereas small molecule mimetics may be used to kill cancer cells or self-antigen-specific lymphocytes in autoimmune disease (Huang and Strasser, 2000).

1.15 Bik

The regulation of *bcl-2* family members by EBV is a significant research interest the laboratory. Bik, in particular, was a major interest in the present study and will now therefore be discussed in detail.

The human Bcl-2 interacting killer (*bik/nbk)* gene formerly known as BP4 (Han *et al*, 1996b), and BIP1 (Chittenden *et al*, 1995), encodes the 160 amino acid pro-apoptotic Bik protein. Bik was the naissance member of the BH3-only proteins sharing a distinct sequence motif with the Bcl-2 family and a characteristic C-terminal *trans*-membrane segment (Boyd *et al*, 1995; Han *et al*, 1996b) which determines its sub-cellular localisation (Elangovan and Chinnadurai, 1997). Originally identified as a binding partner and antagonist of anti-apoptotic Bcl-2 and its adenovirus homolog, E1B 19K, Bik also displayed affinity for Bcl-$_{XL}$, Bcl-$_{XS}$ and the EBV BHRF1 (Boyd *et al*, 1995; Han *et al*, 1996b; Han *et al*, 1996a). Subsequent sequence analysis indicated that Bik shares only the short BH3 signature domain with other members of the Bcl-2 family and had no homology for the other Bcl-2 signature domains BH1, BH2 and BH4 (Boyd *et al*, 1995). Bik is localised to the endoplasmic reticulum from where it elicits pro-apoptotic signals and, given sufficient time, these signals lead to cell death (Germain *et al*, 2002; Mathai *et al*, 2002; Zhao *et al*, 2008).

1.15.1 Expression

The *bik* transcript has a tissue-specific expression profile (Boyd *et al*, 1995; Han *et al*, 1996a) with strong expression in the kidney (Daniel *et al*, 1999). The gene is also expressed in many tumour lines and in B-cells following antigen receptor stimulation (Verma *et al*, 2000; Jiang and Clark, 2001).

1.15.2 Structure

Bik is localised on chromosome 22q13 and consists of five exons and four introns, spanning a region of 19kb. The translational start site and BH3 domain are located in exon 2 and 3, respectively (Figure 1.15.1). The alpha-helical BH3 region is required

for both death promoting activities and dimerisation with anti-apoptotic proteins (Boyd *et al*, 1995; Tong *et al*, 2001; Letai *et al*, 2002). The C-terminal *trans*-membrane hydrophobic domain and the stop codon are contained within exon 5. The transcriptional initiation site was identified as a G residue (marked as +1, Figure 1.15.1) located approximately 13kb from the translational start site. The gene has a long un-translated sequence in exon 1. No canonical TATA or CCAAT boxes were identified near the transcriptional initiation site (Verma *et al*, 2000).

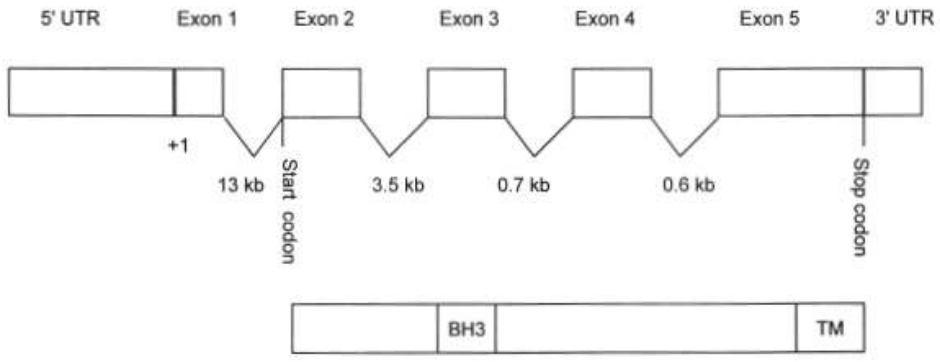


Figure 1.15.1: Arrangement and sizes of exons and introns in the *bik* gene.

Genomic organisation of the *Bik* gene indicating arrangement and sizes of exons and introns (From Verma *et al*, 2000).

1.15.3 Function

Bik is one of the pro-apoptotic members of Bcl-2 family with a death-promoting activity (Boyd *et al*, 1995; Tong *et al*, 2001). Early reports indicated that Bik exhibits death-promoting activities similar to the pro-apoptotic Bax and Bak in that it binds to pro-survival proteins and kills when over-expressed (Boyd *et al*, 1995). Indeed Bik protein is induced in response to a variety of cell stress stimuli, including genotoxic agents (Paquet *et al*, 2004; Mathai *et al*, 2005; Real *et al*, 2006), oncogenic stress (Mathai *et al*, 2002) and hypoxia (Koong *et al*, 2000) and has been shown to induce mitochondrial permeability transition, release of cytochrome *c*, caspase-12, -9, -7 and -3 processing, poly(ADP-ribose) polymerase (PARP) cleavage, and DNA

fragmentation; all of which are indicative of cell death by apoptosis (Tong *et al*, 2001; Germain *et al*, 2002; Zhao *et al*, 2007).

Bik has been implicated in several cancers including human colorectal, prostate, lung and breast cancers, leukaemia, malignant glioma and renal cell carcinoma (Castells *et al*, 1999; Castells *et al*, 2000; Marshansky *et al*, 2001; Tong *et al*, 2001; Naumann *et al*, 2003; Hur *et al*, 2004; Germain *et al*, 2005; Sturm *et al*, 2006; Fu *et al*, 2007) and also displays abnormal expression in acute myeloid leukaemia (AML) compared to normal hematopoietic cells indicating that Bik expression may contribute to the biology of AML (Stirewalt *et al*, 2008).

There is increasing evidence to suggest that repression of *bik* or inactivation of its product may play a role in tumour development. Consistent with this premise, is the evidence that *bik* mutations are a frequent feature of human lymphomas (Arena *et al*, 2003), and the chromatin locus 22q13.3, which contains Bik coding sequence, exhibits deletions in human breast and colorectal cancers (Castells *et al*, 2000). Moreover, loss of *bik*, due to genomic deletion and transcriptional silencing by hypermethylation, is associated with renal cell carcinoma (RCC), suggesting a putative role in the acquisition of apoptosis resistance in tumour cells and significantly, restoration of Bik expression led to apoptotic death in RCC cells but not in non-malignant renal epithelial cells (Sturm *et al*, 2006; Zantl *et al*, 2007). In addition, *bik* down-regulation may contribute to Busulfan (Bu) resistance in hematopoietic stem cell transplantation (HSCT) of patients with chronic or acute myelogenous leukemia (CML or AML) (Valdez *et al*, 2008). Exogenous Bik can sensitise tumour cells to apoptosis (Daniel *et al*, 1999; Panaretakis *et al*, 2002; Oppermann *et al*, 2005) making *bik* a potential therapeutic gene in cancer treatment (Zou *et al*, 2002).

1.15.3.1 A role for Bik in B-cells

Several lines of evidence suggest that the balance between pro-apoptotic Bik and the anti-apoptotic Bcl-$_{XL}$ protein plays a key role in determining B-cell fate and that apoptosis activation signals the up-regulation of Bik protein in order to antagonize the function of Bcl-$_{XL}$. Consistent with this hypothesis, cell surface IgM ligation in human

B104 B-cell lymphoma cells induces Bik accumulation and increases the amount of Bik associated with Bcl-$_{XL}$ ultimately leading to apoptosis through the activation of caspases (Jiang and Clark, 2001). Furthermore, the cytokine transforming growth factor-β (TGF-β)-mediated apoptosis in the Ramos B-lymphoma cell line involves the activation of caspases and is accompanied by Bik up-regulation and a simultaneous loss of Bcl-$_{XL}$ expression (Saltzman *et al*, 1998). The involvement of Bcl-$_{XL}$ in regulating immature B-cell apoptosis is well established (Motoyama *et al*, 1995; Fang *et al*, 1998) and indeed the ability of Bcl-$_{XL}$ to rescue Bik induced apoptosis is also recognised (Boyd *et al*, 1995). Findings have shown that Bik binds to and can antagonise anti-apoptotic Bcl-$_{XL}$ under physiological conditions (Jiang and Clark, 2001; Shimazu *et al*, 2007). Thus it may be the relative proportion of Bcl-$_{XL}$ not associated with Bik that determines whether B-cells survive.

Bik is also weakly down-regulated following CD27 ligation, whereas CD40 ligation (both belong to the TNF/TNFR family) resulted in a similarly weak increase in Bik expression in B-cell receptor (BCR)-mediated apoptosis of Ramos cells. Thus, it is possible that CD27 signals regulate BCR-mediated apoptosis at the mitochondrial level in a manner different from CD40 signals through the alteration of the balance among Bcl-2 family members to regulate mitochondrial permeability. In addition, since CD27 and CD40 inhibit p53 activation it is conceivable that Bik might be involved in the p53-dependent apoptosis pathway in B-cells (Hase *et al*, 2002). The possible involvement of other pro-apoptotic Bcl-2 members such as Bax or Bak in B-cell apoptosis can also not be ruled out.

1.15.4 Mechanism of *bik*-induced apoptosis

It has been well established that Bcl-2 family proteins function at the mitochondria to prevent or promote the release of apoptogenic factors such as cytochrome *c*, AIF and SMAC/Diablo. In addition to their mitochondrial localisation, some anti-apoptotic members of the Bcl-2 family have also been found to be present at the endoplasmic reticulum (ER) and perinulcear membrane regions, suggesting a role of the Bcl-2 family proteins at sites other than mitochondria (Zong *et al*, 2003; Zhao *et al*, 2007). The evidence suggests that Bik may act from the ER, coupling upstream death signals

to events at the surface of the mitochondria via activation of the death effectors Bax and Bak, resulting in cell death (Wei *et al*, 2001; Zong *et al*, 2001).

The ER is well characterised as a calcium (Ca^{2+}) store that sequesters excess cytosolic Ca^{2+} and a reservoir for Ca^{2+} signaling to maintain intracellular calcuim homeostasis (Sambrook, 1990). A growing body of evidence suggests that changes in intracellular Ca^{2+} homeostasis plays a significant role in the modulation of apoptosis (Pinton *et al*, 2001). Indeed, evidence indicates that Bcl-2 family proteins might regulate apoptosis via a mechanism that affects the Ca^{2+} stores within the ER (Foyouzi-Youssefi *et al*, 2000), or alternatively via a mechanism that controls the apoptotic cross talk between the ER and the mitochondria (Hacki *et al*, 2000). Members of the Bcl-2 family appear to differentially regulate intracellular Ca^{2+} level. Translocation of Bax, an apoptotic signaling protein, from the cytosol to the mitochondrial membrane is another step in this apoptosis signaling pathway (Smaili *et al*, 2003).

Bik is exclusively localised to ER rather than mitochondria through its *trans*-membrane domain (Germain *et al*, 2002; Zhao *et al*, 2008). Bik can induce changes in the content of cytosolic as well as ER Ca^{2+} stores (Mathai *et al*, 2005; Zhao *et al*, 2008) and this process is thought to be mediated through a Bax/Bak regulated mechanism (Mathai *et al*, 2005). Recent findings have further revealed that the depletion of ER Ca^{2+} stores rather than the elevation of intracellular Ca^{2+} or the extra-cellular Ca^{2+} influx play an important role in Bik-induced apoptosis (Zhao *et al*, 2008). Furthermore, it has previously been demonstrated that ER Bik initiates dynamin-related protein-1 (DRP1)-regulated remodelling of mitochondrial cristae during apoptosis in a manner dependent on the transmission of Ca^{2+} to the mitochondria (Germain *et al*, 2005) further supporting a role for Bik in the regulation of the ER Ca^{2+} stores.

Exactly how the over-expression of Bik affects the intracellular Ca^{2+} homeostasis is presently unknown. Bik may oligomerise with the other Bcl-2 member proteins and create a non-selective or selective ion pore across the ER membrane causing leakage of Ca^{2+} from the ER Ca^{2+} stores (Zhao *et al*, 2008). Alternatively, Bik protein may interact directly or indirectly with the ER Ca^{2+} pump, or other Ca^{2+} channels.

Upon induction of apoptosis Bax molecules translocate from the cytosol to mitochondrial membranes where it is thought to oligomerise into an active *trans*-membrane pore (Hsu *et al*, 1997; Wolter *et al*, 1997; Desagher *et al*, 1999). Bik is reported to function through the initiation of cell death signaling, whereas Bax/Bak-like proteins function further downstream (Zong *et al*, 2001; Lindsten and Thompson, 2006). Further reiterating this point is the finding that Bik induces exposure of an N-terminal Bax epitope mediating its insertion into the outer mitochondrial membrane, release of cytochrome *c*, and cell death in H1299 cells (Gillissen *et al*, 2003). Since the interaction of Bik with Bax is associated with a change in conformation of Bax leading to cytochrome c release (Gillissen *et al*, 2003), it would appear then, that Bik functions as a positive death effector, engaging downstream components of the cell death pathway. Furthermore, Bik can co-operate with Noxa, another BH3 protein, to release cytochrome *c* from the mitochondria and this pathways involves conformational changes in Bax leading to its activation and ultimately caspase activation (Germain *et al*, 2005)

Thus it appears that Bik initiates a pathway from its location at the ER that stimulates the release of Ca^{2+} from the ER via activation of the death effectors Bax and Bak, which leads to cytochrome *c* release and signaling of apoptosis from mitochondria (Germain *et al*, 2002; Germain *et al*, 2005). The collapse of membrane potential along with the release of cytochrome *c* from mitochondria is followed by the activation of caspases (Smaili *et al*, 2003). Accordingly Bik, is known to elicit pro-apoptotic signals which lead to cell death by pathways which involve the activation of caspases (Germain *et al*, 2002; Gillissen *et al*, 2003; Mathai *et al*, 2005; Zhao *et al*, 2007) which can be inhibited by the broad range caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD-fmk) (Mathai *et al*, 2002; Naumann *et al*, 2003; Hur *et al*, 2004). In one study Bik induced the depolarisation of mitochondrial membrane potential (MMP) through a caspase-12-dependent pathway, leading to the activation of initiator caspase-9 and ultimate cell death in 293 and Hep3B cells suggesting that caspase-12 may directly or indirectly influence the intrinsic apoptosis signal transduction pathways triggered by Bik at the ER (Zhao *et al*, 2007). Bik has also been shown to induce the catalytic activation of caspase-9, caspase-7 and caspase-3 processing in chemoresistant human tumour prostate and

colon cell lines (Tong *et al*, 2001). A model for caspase-dependent Bik-induced apoptosis can be seen in Figure 1.15.2.

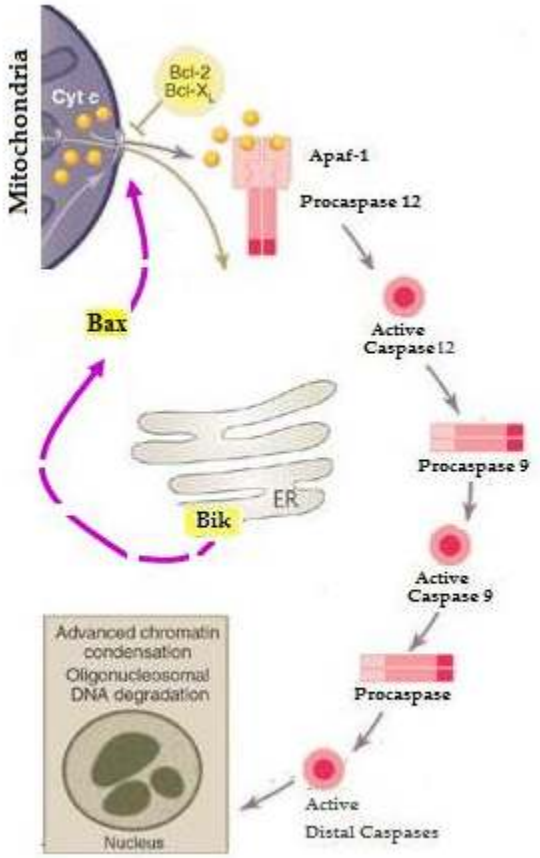


Figure 1.15.2: The caspase-dependent *bik*-induced apoptotic pathway.

Upon apoptotic stimulation Bik induces the depletion of ER Ca^{2+} stores, exposure of an N-terminal Bax epitope mediating its insertion into the outer mitochondrial membrane, release of cytochrome *c*, and activation of a caspase-12-dependent pathway, leading to the activation of initiator caspase-9 and ultimately cell death. Adapted from Hunot and Flavell, 2001.

Further evidence for the involvement of caspases in Bik-induced apoptosis is presented in a model in which Bik triggers apoptosis through the activation of caspases distal to the CrmA block (which preferentially inhibits proximal components of the caspase protease cascade, including caspase-8 Muzio *et al*, 1997) but upstream of the IAPs (the inhibitors of apoptosis molecules, that block caspase catalytic activity, Cp-IAP and Op-IAP (Clem and Miller, 1994; Orth and Dixit, 1997). A representation of this model is seen in Figure 1.15.3.

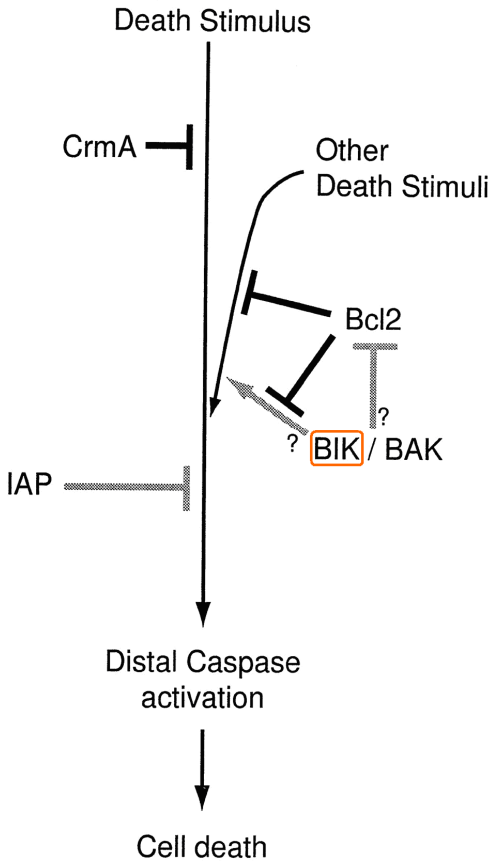


Figure 1.15.3: Relative order of Bik in the death pathway.

At the apex of the cascade is the death signals that are susceptible to CrmA inhibition. Downstream of this block, other Bcl-2-inhibitable death stimuli can enter the death pathway. Bik and Bak/Bax may inhibit Bcl-2/Bcl-$_{XL}$ directly to release an unidentified cytotoxic factor. Alternatively, Bik and Bak/Bax may activate the pathway directly. Regardless, IAPs function downstream of Bik and Bak/Bax. Activation of distal caspases results in cell death (Adapted from Orth and Dixit, 1997).

Interestingly it has also been reported that Bik can induce cell death in a caspase-independent fashion in human melanoma cells (Oppermann *et al*, 2005) and that inhibition of caspases by treatment with zVAD-fmk in the absence of Bcl-2 actually enhances Bik-induced cell death in mouse embryonic fibroblasts (MEFs) (Rashmi *et al*, 2007). Interestingly a pathway in which Bik-mediated cell death induces the release of cytochrome *c* but not mitochondrial membrane potential loss and without modulating voltage-dependent anion channel activity (VDAC), (an important component of the PTP), has also been reported, suggesting that Bik may target molecules other than the PTP or act alone to induce cytochrome *c* release (Shimizu

and Tsujimoto, 2000). Furthermore, findings have suggested that Bik can induce cytochrome *c* release from mitochondria independently of Bax in H1299 cells. This report favoured a direct induction of the mitochondrial apoptotic signaling cascade by *bik* and subsequent activation of caspases (Germain *et al*, 2002). It is conceivable that *bik* could be acting on separate distinct pathways, in response to different stimuli.

A mechanism of Bik-induced apoptosis regulated by Bcl-2 family members, such as Bax or Bcl-2/Bcl-$_{XL}$ cannot be excluded. The pro-apoptotic activity of BH3-only proteins is proposed to be governed by their propensity to interfere with at least a dual layer of protection of cells from the pro-apoptotic activity of Bak (Willis and Adams, 2005). It has previously been shown that binding of Bik inactivates Bcl-$_{XL}$ and releases both Bax and Bak. While the interaction between Bik and Bcl-$_{XL}$ seems to be stronger, Bik is also known to bind to Bcl-2 (Elangovan and Chinnadurai, 1997; Verma *et al*, 2001; Gillissen *et al*, 2003), indicating that Bcl-2 is at least in part inhibited by Bik. Once the capacity of Bcl-2 to bind Bax has been surpassed, the liberated Bax protein becomes activated. Notably, the Bax inhibitors Bcl-2 and Bcl-$_{XL}$ are not stabilised by Bik. In contrast, Bak, released from Bcl-$_{XL}$ upon Bik expression, is still kept in check by Mcl-1, and this is enforced by the up-regulation of the Bak inhibitor Mcl-1 by Bik. Mcl-1-mediated repression of Bak thereby provides a molecular rationale for the strict Bax dependency of Bik-induced cell death. Thus it appears that Mcl-1 determines the Bax dependency of Bik-induced apoptosis (Gillissen *et al*, 2007). In this instance Bik appears to act as de-repressor molecule binding to distinct anti-apoptotic proteins. The consequently de-repressed pro-apoptotic multi-domain proteins can then be counteracted by a second set of anti-apoptotic proteins. This adds a higher degree of specificity and the outcome of triggering this tightly balanced interaction network would be determined by both the specific BH3-only proteins induced by a given death stimulus and the abundance of their anti-apoptotic counterparts forming this second obstacle to apoptosis (Gillissen *et al*, 2007). A diagram depicting a model for the regulation of Bik-induced apoptosis by Bcl-2 family members can be seen in Figure 1.15.4.

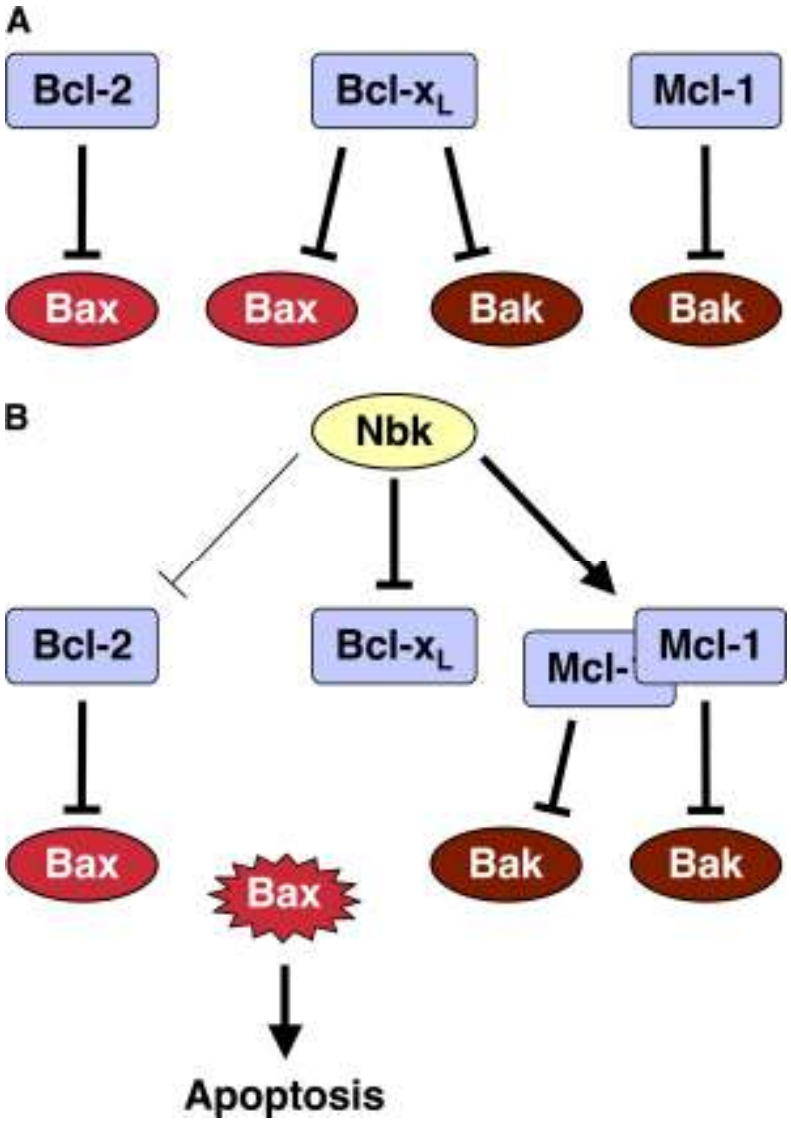


Figure 1.15.4: Model for the regulation of Bik/Nbk-induced apoptosis by Bcl-2 family members.

(A) In healthy cells, Bax is inactivated by Bcl-2 and Bcl-$_{XL}$, whereas Bak is sequestered by Bcl-$_{XL}$ and Mcl-1. (B) Bik/Nbk activates the pro-apoptotic Bax through neutralisation of the anti-apoptotic Bcl-$_{XL}$. In contrast, increased levels of the anti-apoptotic Mcl-1 keep in check Bak that was released from Bcl-$_{XL}$ upon competition with Nbk. From Gillissen *et al*, 2007.

1.15.5 Regulation

Regulation of *bik* on several different levels has been reported. Evidence of roles for transcriptional regulation, methylation, phosphorylation and protein degradation by the proteasome have all been indicated. Additionally, the specificity of the interplay between hetero-dimerising partners is also thought to be associated with *bik* control. Indeed stringent regulation of *bik* gene expression at various steps is probably essential, given the fact that Bik is predicted to possess an already exposed BH3 domain and is thus considered constitutively active (McDonnell *et al*, 1999). It is clear that external and internal stimuli lead to regulation of *bik* at several different levels, including tissue-specific expression (Daniel *et al*, 1999), transcriptional and translational control and intracellular localisation (Germain *et al*, 2002; Mathai *et al*, 2002; Zhao *et al*, 2008). Evidence that *bik* is transcriptionally regulated by a number of transcription factors including $p75^{NTR}$, SFRP1, IgM, E4orf6, E1A, GRP78/BiP, the E2F transcription factors and TGF-β has been demonstrated (Saltzman *et al*, 1998; Jiang and Clark, 2001; Mathai *et al*, 2002; Tabassum *et al*, 2003; Han and Amar, 2004). Furthermore, apoptosis triggered by *bik* can be either p53-dependent or independent depending on the induction stimulus (Han *et al*, 1996a; Bartke *et al*, 2001; Mathai *et al*, 2002).

The regulation of Bik function by phosphorylation has also been verified (Verma *et al*, 2001). Bik is phosphorylated on consensus CKII phosphorylation sites (Thr-33 and/or Ser-35) (Verma *et al*, 2001) an event that appears to result in a gain in function (Verma *et al*, 2001). Mutation of the phosphorylation sites, in which the threonine and serine residues were changed to alanine residues, indicated that phosphorylation of Bik is required for its apoptotic activity, without significantly affecting its ability to hetero-dimerise with Bcl-2 and $Bcl\text{-}_{XL}$ (Verma *et al*, 2001). The precise mechanism by which phosphorylation plays a role in the apoptotic activity of Bik is not known, although it is possible that phosphorylation of Bik may play role in interaction with other potential cellular targets or that the activity of Bik may be activated by phosphorylation in response to different apoptotic stimuli in various human tissues (Verma *et al*, 2001).

The methylation status of *bik* remains uncertain. While treatment with the DNA methylation inhibitor, zebularine induced genomic de-methylation of a multiple myeloma cell line KAS-6/1, and induced the expression of Bik (Pompeia *et al*, 2004), conflicting reports argue that DNMT1 mediated *bik* suppression does not involve a change in the DNA methylation state or histone modification, maintaining that DNMT1 is capable of regulating gene expression by a DNA methylation independent pathway (Milutinovic *et al*, 2004). A closer look at the mechanism of action of zebularine, reveals that this drug acts by forming a complex with DNMT1 in the position normally incorporated by the base targeted for methylation (Zhou *et al*, 2002). Thus, the gene targeted to disrupt methylation may also suppress Bik independently of the DNA methylation state (Pompeia *et al*, 2004). Other studies have indicated a role for DNA methylation in transcriptional silencing of the *bik* gene. Exposure to 5'-aza-2'-deoxycytidine resulted in strong up-regulation of *bik* mRNA in renal cell carcinoma (RCC) cell lines (Sturm *et al*, 2006) and was shown to be induced by the histone deacetylase inhibitor, sodium butyrate in human hepatoma cells (Ogawa *et al*, 2004). Increases in *bik* mRNA expression were observed in both HepG2 and Hep3B cells following treatment with 5-aza-2′-deoxycytidine or histone deacetylase inhibitor sodium butyrate given separately. Bik protein however was undetectable (Wang *et al*, 1998). A combination treatment of both 5-aza-2′-deoxycytidine plus depsipeptide has shown a synergistic effect on *bik* gene induction in a variety of cancer cell lines (Dai *et al*, 2006). It may be the case that control of *bik* expression by epigenetic mechanisms occurs in a cell type-specific manner.

The realisation that Bik may rapidly turnover during apoptosis suggests another level of control by means of Bik degradation (Marshansky *et al*, 2001). It has been postulated that the accumulation of Bik protein may be a direct result of its reduced degradation in the cell (Marshansky *et al*, 2001). Indeed, reports suggest that Bik is one of the mediators of proteasome inhibitor-induced apoptosis and that the proteasome inhibitors Bortezomib (Nikrad *et al*, 2005) and lactacystin (Marshansky *et al*, 2001) can regulate Bik expression, through stabilisation of the protein. This accumulation of Bik is sufficient to induce apoptosis indicating that it maybe the accumulation of Bik rather than the expression per se that determines whether a cell undergoes apoptosis (Jiang and Clark, 2001).

Akin to other BH3-only proteins, Bik binds to pro-survival family members, killing cells when over-expressed (Boyd *et al*, 1995). Not unlike other pro-apoptotic members of the Bcl-2 family the BH3 domain of *bik* has proven critical for pro-apoptotic activities; dimerisation as well as for cytochrome *c* release and apoptosis (Boyd *et al*, 1995; Cosulich *et al*, 1997; Hegde *et al*, 1998; Germain *et al*, 2002). Since BH3-only pro-apoptotic proteins share only the BH3 domain in common (Bouillet and Strasser, 2002), it has been postulated as to whether the BH3 domain is a death effector module eliciting its cell death activity through the inactivation of the anti-apoptotic proteins by hetero-dimerisation, thus disrupting the complex of the death suppressor with a caspase activating molecule such as Apaf-1, which in turn results in the activation of caspases and eventually death of the cell (Boyd *et al*, 1995; Kelekar and Thompson, 1998; Lutz, 2000; Chittenden, 2002) as represented in Figure 1.15.5.

Accordingly then**,** regulatory control could be directly associated with the specificity and nature of the interplay between Bik and its hetero-dimerising partners (Han *et al*, 1996a; Elangovan and Chinnadurai, 1997). In this regard, the death promoting activity of Bik can be suppressed by co-expression of Bcl-2, Bcl-$_{XL}$ and BHRF1, and E1B-19k (Boyd *et al*, 1995; Elangovan and Chinnadurai, 1997). Bik also binds with Bcl-$_{XS}$, a death-promoting protein that lacks the BH1 and BH2 domains, possibly indicative of a co-operation effect between the two proteins (Boyd *et al*, 1995). It has long been recognised that the relative levels of available Bcl-2 family dimerisation partners in the cell may shift the balance of cell fate either in favour of viability or of cell death, following exposure to an appropriate stress (Han *et al*, 1996a). The assumption that certain BH3-only ligands possess a degree of specificity for their pro-survival receptors would render apoptosis of the Bcl-2 family a sophisticated affair, with Bik functioning as a naturally dominant negative antagonist whose role in the cell is to sequester and inactivate anti-apoptotic proteins.

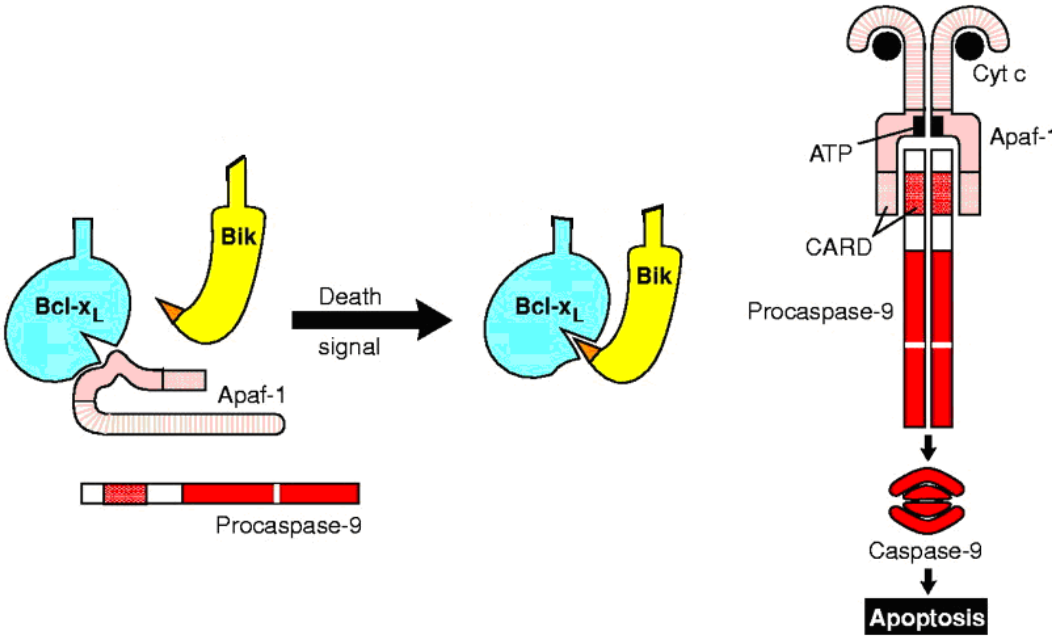


Figure 1.15.5: Model for Apaf-1 regulation by the Bcl-2 family.

A pro-survival member Bcl-2 family member such as Bcl-x_L may bind Apaf-1 and prevent it from activating procaspase-9 (or another initiating pro-caspase). A death signal may, for example, provoke interaction of a BH3 family member such as Bik, or perhaps a Bax family member with Bcl-$_{XL}$, preventing it from neutralising Apaf-1. In the presence of cytochrome c released from mitochondria and ATP, Apaf-1 can then bind to procaspase-9 and promote its dimerisation and activation by autocatalysis. Caspase-9 subsequently activates effector caspases (Adapted from Adams and Cory, 1998).

Association of Bik with anti-apoptotic proteins indeed hinders Bik function (Boyd *et al*, 1995). However, mutational analysis of the BH3 domain in *bik*, suggests that hetero-dimerisation via the BH3 domain with survival proteins alone is insufficient to explain their cell death inducing activity (Elangovan and Chinnadurai, 1997). It has also been hypothesised that sequences in the C-terminal region of Bik might also be required for the pro-apoptotic activity of Bik, in addition to the activity of the BH3 domain. The precise biochemical activity of the C-terminal region of Bik is not known but it is conceivable that the C-terminal sequences may function in concert with the BH3 domain to enforce apoptosis. Another, simpler rationalisation may be that the C-terminal region plays a role in Bik protein folding is also possible (Elangovan and Chinnadurai, 1997). Interestingly, this region is known to contain a sequence motif

similar to the substrate binding motif of the caspase family, and it was postulated that this motif could modulate the substrate-caspase complexes. Indeed a very recent study demonstrated that the *trans*-membrane domain not only decided the sub-cellular localisation of Bik, but also had an important role in Bik-induced apoptosis in Hep3B cells (Zhao *et al*, 2008).

Thus the regulation of Bik appears complex. It may be that distinct regulatory mechanisms involving phosphorylation, turnover and altered mRNA levels, may be engaged in response to an array of different signaling molecules, such as survival factors, growth factors and death inducers, including viral infection (Lutz, 2000) and perhaps dependent on the cellular context (Mathai *et al*, 2002) providing multiple means to regulate this pro-apoptotic protein.

1.15.6 EBV regulation of the Cellular Apoptotic Programme

A central component of the overall EBV strategy and its role in the development of related malignant disease is the ability of the viral proteins to suppress the cellular apoptotic program. In this regard, EBV has developed mechanisms that suppress the apoptotic programme of the host in order to delay cell death during the lytic stage of infection, until virion numbers have been sufficiently amplified to transmit infection to other individuals via saliva, and also to ensure persistence in the host memory B-cell compartment. EBV genes have been shown to manipulate the cell survival machinery of the infected cell by directly regulating the apoptotic death machinery, specifically by inducing the expression of several anti-apoptotic proteins such as Bcl-2, Bfl-1, Mcl-1, A20, c-IAP-2 and also through the down-regulation of expression of pro-apoptotic proteins such as Bax and Bim (Laherty *et al*, 1992; Fries *et al*, 1996; Wang *et al*, 1996; D'Souza *et al*, 2000; Clybouw *et al*, 2005; Grimm *et al*, 2005; Pegman *et al*, 2006; Anderton *et al*, 2007).

Group I BL cell lines which display type I latency, only expressing EBNA1, EBERs and BARTs and many EBV-negative BL lines can readily be triggered into apoptosis,

whereas Group III BL cell lines, which express the complete set of EBV latent proteins, are relatively resistant to a variety apoptotic triggers, including growth factor withdrawal, Ca^{2+} ionophore treatment and over-expression of the p53 tumour suppressor gene (Henderson *et al*, 1991; Okan *et al*, 1995). EBV-negative BL cells converted to the type III latency state by infection with the B95-8 strain of EBV also display elevated thresholds of resistance to apoptotic stimuli (Gregory *et al*, 1991), thus implicating EBV latent genes in cell survival.

1.15.6.1 EBV up-regulates *bcl-2*

Type I BLs express little or no Bcl-2, group III BL cells lines display high levels of the Bcl-2 protein (Henderson *et al*, 1991). Bcl-2 is a prototype anti-apoptotic protein that interacts with the mitochondrial membrane and inhibits the action of caspases (Adams and Cory, 1998). Transfection of individual EBV latent genes into EBV-negative BL cell lines has shown that up-regulation of Bcl-2 expression correlates with the expression of three EBV proteins; LMP1, (Henderson *et al*, 1991; Rowe *et al*, 1994) EBNA2, (Finke *et al*, 1992) and EBNA3B (Silins and Sculley, 1995). Significantly, over-expression of Bcl-2 in group I BL cell lines following gene transfer results in reduced apoptosis in response to a number of stimuli compared with group III cell lines, and the inhibition of cell death was correlated to the amount of Bcl-2 expressed (Henderson *et al*, 1991; Milner *et al*, 1992). Indeed, transfections with EBV LMP1 also resulted in enhanced survival of these cells and the ability of LMP1 to confer resistance to apoptosis to the type I BLs resulted from its ability to induce endogenous Bcl-2 expression (Gregory *et al*, 1991; Henderson *et al*, 1991). While LMP1 and EBNA2 appear to up-regulate Bcl-2 expression *in vitro*, the recirculating B-cells of peripheral blood which are a target of EBV, already express relatively high levels of Bcl-2 and EBV infection and so expression of LMP1 or EBNA2 or the combined expression of both, does not significantly impact Bcl-2 expression. Thus it may be that the role of LMP1/EBNA2 *in vivo* is to maintain the high constitutive level of Bcl-2 rather than to induce it (Martin *et al*, 1993).

1.15.6.2 EBV up-regulates *A20*

A20 is an inducible zinc finger protein that confers resistance to TNFα cytoxicity (Laherty *et al*, 1992) and is induced in response to a wide variety of stimuli, including cytokines such as TNF, IL-1, and CD40, as well as bacterial and viral products such as lipopolysaccharide (LPS), and human T-cell leukemia virus type I Tax in many cell types, including fibroblasts, lymphocytes and endothelial cells (Beyaert *et al*, 2000). A20 is constitutively expressed in EBV-immortalised B-cells and transfection experiments have demonstrated that LMP1 induces the expression of A20 in both lymphocytes and epithelial cells (Laherty *et al*, 1992; Fries *et al*, 1996; Spender *et al*, 1999). Studies of the A20 promoter have shown that LMP1 transcriptionally activates the A20 gene through *cis*-acting NF-κB binding sites with a role for LMP1-inducible binding of an NF-κB-like factor to a consensus binding sites within the A20 promoter. A20 has also been identified as functioning in a negative feedback loop by inhibiting the activation of NF-κB from both CTAR1 and CTAR2 of the LMP1 protein. This effect is mediated by the binding of A20 to TRAF2 (Eliopoulos *et al*, 1999a).

1.15.6.3 EBV up-regulates *mcl-1*

Maximal Bcl-2 up-regulation by LMP1 requires 48 to 72 hours (Rowe *et al*, 1994). Up-regulation of the Bcl-2 homologue Mcl-1 by LMP1 precedes the induction of Bcl-2 and is transient; and levels of Mcl-1 decrease when Bcl-2 levels begin to increase. Mcl-1 is an anti-apoptotic protein that has been shown to contribute to the prolonged existence of chronic lymphotrophic leukaemia B-cells and its higher expression is correlated with resistance to chemotherapy treatment (Moshynska *et al*, 2004). It is thought that Mcl-1 functions as a rapidly inducible, short-term effector of cell viability (Chao *et al*, 1998). LMP1 also blocked the decline in Mcl-1 levels in response to apoptotic stimulation triggered by elevated cyclic AMP. This effect of LMP1 was associated with a delayed cell death in the EBV-negative BL cell line BL41. The maintenance of Mcl-1 expression by LMP1 is likely to be a crucial immediate-early response that enables cells to survive until Bcl-2 can be up-regulated (Wang *et al*, 1996).

1.15.6.4 EBV up-regulates *c-IAP2*

c-IAP2 is a member of the cellular inhibitor of apoptosis protein family (c-IAP), and was originally identified as a molecule recruited to the TNF receptor complex receptor via TRAF1 and TRAF2 (Rothe *et al*, 1995). Its expression is preferentially up-regulated by TNF and other stimuli activating NF-κB including IL-1, LPS, and CD30 stimulation (Craxton *et al*, 1998; Stehlik *et al*, 1998). Transcriptional activation by TNF or IL-1 is mediated co-operatively by two NF-κB binding sites bound by the NF-κB p50/p65 hetero-dimer. In addition, over-expression of CD40 or LMP1 resulted in the *c-IAP2* promoter-driven reporter gene activation (Hong *et al*, 2000). It has been postulated that the LMP1-inducible c-IAP2 protein functions as a pro-survival factor during EBV-infection, acting independently or in concert with TRAF1 or other LMP1-inducible anti-apoptotic proteins such as A20 and Bcl-2 (Hong *et al*, 2000).

1.15.6.5 EBV up-regulates *bfl-1*

Studies in our laboratory have revealed the up-regulation of another anti-apoptotic gene, *bfl-1*/*A1* during EBV infection. The pro-survival Bcl-2-family member Bfl-1/A1 is a transcriptional target of NF-κB that is over-expressed in many human tumours and is a means by which NF-κB is known to inhibit apoptosis (Karsan *et al*, 1996; Grumont *et al*, 1999; Somogyi *et al*, 2001). Two EBV proteins LMP1 and EBNA2 have been shown to up-regulate expression of this cellular gene in the laboratory (D'Souza *et al*, 2000; D'Souza *et al*, 2004; Pegman *et al*, 2006). In this regard, expression of LMP1 in EBV-negative BL cell lines coincides with a dramatic increase in *bfl-1* mRNA (D'Souza *et al*, 2000) and *bfl-1* expression was found to protect EBV-positive cells against serum depletion-induced apoptosis. Moreover, expression of LMP1 in EBV-negative cell lines was found to *trans*-activate the *bfl-1* promoter through interactions with components of the tumour necrosis factor receptor (TNFR)/CD40 signaling pathway. This process is NF-κB dependent, and involves the recruitment of TNFR-associated factor 2, and is mediated to a greater extent by CTAR2 relative to the CTAR1 domain of LMP1 (D'Souza *et al*, 2004). EBNA2 also up-regulates *bfl-1* expression. EBNA2 *trans*-activation of *bfl-1* requires CBF1, and there is an essential role for a core consensus CBF1-binding site on the *bfl-1* promoter. Interestingly *bfl-1* expression is induced and maintained at high levels by

the EBV growth program in an LCL, and withdrawal of either EBNA2 or LMP1 does not lead to a reduction in *bfl-1* mRNA levels in this context, whereas the simultaneous loss of both EBV proteins results in a major decrease in *bfl-1* expression (Pegman *et al*, 2006).

1.15.6.6 EBV down-regulates *bax*.

Bax is a member of the Bcl-2 family that, together with Bak, is required for permeabilisation of the OMM (Wei *et al*, 2001). EBV LMP1 inhibits *bax* promoter activity through activation of NF-κB signaling via CTAR-1 and CTAR-2. The *bax* promoter harbours 3 κB consensus sites (Bentires-Alj *et al*, 2001) of which LMP1 is known to activate 2, namely κB2 and κB3. LMP1 induced binding of the NF-κB hetero-dimer p65/p50 to the κB2 site and of the p50/p50 homo-dimer to the κB3 site. Promoter mutation analysis revealed that the κB2 site is necessary for inhibition of *bax* promoter activity and the κB3 site, for its activation. However, the activation of the *bax* promoter by LMP1 was observed only in the presence of specific inhibitors of p65/p50. In all other cases, LMP1 inhibited *bax* promoter activity. Moreover, in cells with impaired Bax function or expression the anti-apoptotic activity of LMP1 is significantly reduced indicating that Bax is an important target of the anti-apoptotic activity of LMP1 (Grimm *et al*, 2005).

1.15.6.7 EBV down-regulates *bim*.

Bim is a BH3-only protein whose over-expression in cells leads to cytochrome *c* release through the activation of Bax (Hacker and Weber, 2007). All mature B-cells express significant amounts of BH3-only Bim (O'Reilly *et al*, 2000; Strasser, 2005). Infection of BL cells with EBV results in a significant down-regulation in the expression of all three isoforms of the *bim* gene (Clybouw *et al*, 2005; Leao *et al*, 2007). Moreover, this down-regulation appears not to require expression of EBNA2 or the LMP proteins (Leao *et al*, 2007) and is in fact dependent on the co-operation of EBNA3A and EBNA3C (Anderton *et al*, 2007). It has been reported that EBV infection may lead to post-translational modulation of Bim expression, involving the phosphorylation of BimEL by the EBV-activated kinase ERK1/2, followed by its

degradation through the proteasome pathway (Clybouw *et al*, 2005). However, a pathway in which EBNA3A and EBNA3C co-operate to regulate Bim expression at the level of transcription has also been described (Anderton *et al*, 2007).

1.15.7 EBV lytic proteins encode anti-apoptotic functions.

During the lytic cycle of virus replication, repressors of apoptosis can provide a selective advantage by protecting the cell from death. EBV encodes its own gene products which can delay apoptosis in order to maximise viral production and spread of virus progeny to other individuals. These genes include BHRF1, BZLF1 and BALF1 (Henderson *et al*, 1993; Tarodi *et al*, 1994; Dawson *et al*, 1995; Marshall *et al*, 1999). As indicated earlier, EBV also encodes a viral homologue of IL-10 (Stuart *et al*, 1995)

BHRF1 promotes the survival and proliferation of lytically infected cells (Dawson *et al*, 1998) and the highly conserved nature of BHRF1 amoung different EBV isolates at both the sequence and functional level supports the proposed vital role of BHRF1 in delaying cell death, thereby maximising the production of progeny virus and facilitating the establishment of virus persistence (Khanim *et al*, 1997). BZLF1 has been shown to interact with p53 and inhibit its *trans*-activating function in lymphoid cells, thereby providing a mechanism for preventing p53-mediated apoptosis (Cayrol and Flemington, 1996). The BALF1 ORF in EBV possesses significant sequence similarity to other anti-apoptotic viral Bcl-2 homologs and the cellular Bcl-2 and Bcl-$_{XL}$. A recombinant GFP-BALF1 fusion protein co-localises with mitochondrial dyes, suppresses apoptosis, and hetero-dimerises with Bax and Bak (Marshall *et al*, 1999).

While the growth stimulatory and anti-apoptotic genes induced by the initial EBV infection almost certainly prolong survival in the lytic stage of the lifecycle, it is also likely that they may augment the likelihood of the virus-infected cell progressing into the memory B-cell pool. Taken together, these interactions account, at least in part, for the ability of EBV to induce cell proliferation and survival, which is essential for both EBV-induced immortalisation of B-cells and establishment of life-long persistence in the memory B-cell compartment.

1.16 Objectives of the study

The modulation of anti- and pro-apoptotic proteins by EBV plays a central role in the overall virus strategy, and in the development of virus-associated malignancies. Previous work in our laboratory has demonstrated that the cellular *bfl-1* gene plays an important role in regulating the survival of EBV-infected B-cells (D'Souza *et al*, 2000; D'Souza *et al*, 2004; Pegman *et al*, 2006). In a related study in our laboratory, it was observed, that *bfl-1* is expressed in H/RS cells *in vivo* and that its expression is regulated by NF-κB. Bfl-1 protected H/RS cells from apoptosis induced by NF-κB inhibitors and knockdown of *bfl-1* expression significantly increased the chemosensitivity of cultured H/RS cells (Loughran, S.T. manuscript in preparation).

Preliminary results in the laboratory had indicated that repressed levels of *bik* mRNA appeared to be a feature of the BL cell line expressing a group III phenotype but not the BL group I phenotype, indicating that the pro-apoptotic *bik* gene could potentially be an important target for down-regulation by EBV latent genes in the mediation of an increased anti-apoptotic threshold of EBV infected cells.

The down-regulation of *bik* by EBV in B-cells would have implications for the biology of EBV, as EBV-mediated down-regulation of this gene may contribute to the survival of EBV-infected B-cells, since cells similar to LCLs are present in the circulation and during primary infection by the virus (Qu and Rowe, 1992; Tierney *et al*, 1994). Additionally, the suppression of *bik* may contribute to the development of EBV-associated B-cell malignancies, such as post-transplant lymphoproliferative disorders and BL tumours. Mechanistic studies into the contribution of *bik* to cell survival will provide important information about both normal B-cell development and potential routes to B-cell malignancy.

The main focus of the current study was thus to

- Establish a role for EBV in the regulation of the cellular *bik* gene.
- Elucidate the mechanisms by which this pro-apoptotic gene is regulated and in particular to determine if EBNA2 is involved in its regulation.

- Determine the significance of the loss of Bik expression to the survival of EBV-infected cells in culture.

In summary, this thesis presents the novel finding that EBV down-regulates the cellular *bik* gene in B-cells proliferating due to the EBV growth programme, with a key role for EBNA2 and possibly c-Myc in this regulation. Significantly, restoration of Bik expression in LCLs leads to apoptotic cell death. The down-regulation of *bik* by EBNA2 highlights the protective effect of EBNA2 in EBV-infected cells and which may complement the LMP1-induced survival signals mediated through NF-κB.

CHAPTER 2

Materials & Methods

2.1 Materials

2.1.1 Biological Materials

2.1.1.1 Cell lines

Table 2.1-1: Cell lines used in the study

Cell Lines	EBV Status	Cell Classification	Description
AG876	+	Type III BL	Type III BL cell line expressing all of the EBV latent genes.
Akata-4E3	-	Type I BL	During cultivation of the Epstein-Barr virus (EBV)-positive Burkitt's lymphoma (BL) line Akata, EBV DNA was lost from some of the cells and EBV-negative clones were isolated (Shimizu *et al*, 1994).
BJAB	-	Burkitt-like lymphoma cell line	BJAB is a B-cell derived from a patient with EBV-negative African Burkitt's lymphoma. The cells do not contain detectable amounts of EBV DNA, nor do they express the EBV-determined nuclear antigen EBNA. However the cells have the characteristics of B-type lymphocytes and carry receptors for EBV (Menezes *et al*, 1975).
BJAB-ER/EBNA2(K3) (BJABK3)	-	Stable transfectant	EBV negative BL-like cell line stably transfected with an EBNA2 expression plasmid in which EBNA2 activation domain is fused to the estrogen binding domain, rendering the activation of EBNA2 dependent on the presence of estrogen (Kempkes *et al*, 1996)
BL41	+	Type I	These cells are a matched set. BL41 is an early passage BL cell line expressing EBNA1 as the only viral protein, BL41-B95.8 is the cell line stably transformed with the EBV strain B95.8 expressing all the EBV latent genes (Calender *et al*, 1987). IARC 171 is a spontaneously transformed Lymphoblastoid Cell Line derived from the same patient.
BL41-B95.8	+	Type III	
IARC 171	+	LCL	
BL41-P3HR1	+	Type I	BL41-P3HR1 is the BL41 cell line stably transformed with the EBV strain P3HR1 expressing all the EBV latent genes except EBNA2 and EBNA-LP (Calender *et al*, 1987).
BL41-ER/EBNA2 (K3) (BL41K3)	-	BL (stable transfectant)	EBV negative BL cell line stably transfected with an EBNA2 expression plasmid in which the EBNA2 activation domain is fused to the estrogen binding domain, rendering the activation of EBNA2 dependent on the presence of estrogen (Kempkes *et al*, 1996).

BL41P3HR1-ER/EBNA2 (9A) (BL419A)	+	EBV Positive BL (Stable transfectant)	EBV negative BL infected with the P3HR1 strain of EBV and subsequently transfected with an EBNA2 fusion protein where EBNA2 activation is dependent on the presence of estrogen as above (Kempkes *et al*, 1996)
DAUDI	+	EBV positive BL Type III	DAUDI, described by (Klein *et al*, 1968), is deleted for EBNA-2 and does not express LMP1. This cell line expresses only EBNA1 and LMP2A. The EBV genome in Daudi cells has a deletion similar to that observed in a non-transforming strain (P3HR-1) of the virus (Jones *et al*, 1984).
DG75	-	EBV negative BL	Lymphoid B-cell line derived from an Israeli Burkitt-like lymphoma case (Ben-Bassat *et al*, 1977).
DG75-tTA-EBNA2	-	Stable transfectant	Tetracycline regulated system whereby the expression of EBNA2 can be induced by the removal of tetracycline from the growth media (Floettmann *et al*, 1996).
DG75-tTA-LMP1	-	Stable transfectant	Tetracycline regulated system whereby the expression of LMP1 can be induced by the removal of tetracycline from the growth media (Floettmann *et al*, 1996).
EREB 2.5	+	LCL	An LCL established by co-infecting B-cells with a 28kb mini-EBV plasmid that only expresses estrogen-responsive EBNA2, together with the EBV P3HR1 strain in which it's own EBNA2 gene is deleted (Kempkes *et al*, 1995a).
HEK-293	-		Human embryonic kidney cell line (Graham *et al*, 1977).
IARC 290B	-	LCL	IARC 290B is a spontaneously transformed LCL.
IB4	-	LCL	An LCL established by infection of umbilical cord blood lymphocytes with the EBV strain B95.8 (King *et al*, 1980)
KEM BL	+	Type I BL	Early passage BL cell lines expressing EBNA1 as the only viral gene.
L428	-	EBV negative HL	Established from the pleural effusion of a 37-year-old woman with HL (stage IVB, nodular sclerosis, refractory, terminal) in 1978 (Schaadt *et al*, 1979).
LCL 327	+	LCL	An EBV immortalised B-cell line (Allday *et al*, 1995).
Mutu I	+	Type I	Early passage BL cell expressing EBNA1 as the only viral gene (Gregory *et al*, 1990).

Mutu III	+	Type III	Stable clone of early passage BL cell line Mutu I which has upon serial passage drifted to express the full compliment of EBV latent genes (Gregory *et al*, 1990).
OKU BL	+	Type I	These cells are a matched pair. OKU BL is a group I BL cell line expressing only EBNA1.
OKU LCL	+	LCL	OKU LCL was established upon infection of peripheral lymphocytes from the same patient from whom OKU BL was derived with the EBV strain B95.8.
P493-6	+	LCL	An EREB 2-5-derived cell line, P493-6, in which c-Myc is expressed under the control of a tetracycline-regulated promoter (Pajic *et al*, 2000).
RAEL BL	+	Type I BL	Early passage BL cell lines expressing EBNA1 as the only viral gene.
X50-7	-	LCL	X50-7 is a spontaneously transformed LCL.

All BL cell lines and LCLs were obtained from Professor Martin Rowe, University of Wales, Cardiff, Wales. The estrogen-responsive cell line EREB 2-5 was a gift from Dr. Ursula Zimber-Strobl, GSF-National Research Centre for Environment and Health, Munich, Germany. The EREB 2-5-derived cell line, P493-6 was a gift from Prof. Dr. med. Georg W. Bornkamm, Institute of Clinical Molecular Biology and Tumor Genetics Munich, Germany. The estrogen responsive cell lines BL41K3, BL419A and BJABK3 were gifts from Dr. Bettina Kempkes GSF-National Research Centre for Environment and Health, Munich, Germany. Akata-4E3 was received from Professor Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.

2.1.1.2 Antibodies used in the study

Table 2.1-2: Antibodies used in the study

Antibody	Name/Code	Description	Supplier
Goat Anti-BIK	NBK (N-19): sc-1710	An affinity purified Goat Polyclonal IgG raised against a peptide mapping at the N-terminus of Bik/NBK of human origin	Santa Cruz
Mouse Anti-Bik	Mouse Anti-Human Bik Clone C33-1 (557040)	Monoclonal IgG raised against amino acids 40-114.	BD Biosciences
Rabbit Anti-Bax-Horseradish Peroxidase (HRP)	Bax (N-20): sc-493	Affinity purified rabbit polyclonal antibody raised against a peptide mapping at the N-terminus of Bax of human origin.	Santa Cruz
Rabbit-Anti *c*-Myc	*c*-Myc (N-262) :sc-764	Rabbit Polyclonal IgG raised against amino acids 1-262 mapping at the N-terminus of c-Myc of human origin.	Santa Cruz
Mouse Anti- β - Actin Clone AC-15	Anti- β – Actin A1978	Monoclonal IgG Raised against β-cytoplasmic actin N-terminal peptide.	Sigma
Donkey Anti-Goat IgG-HRP	Anti-Goat IgG-HRP V8051	Secondary antibody developed in donkeys against goat IgG; it has been affinity-purified and conjugated to HRP.	Promega
Goat Anti-Mouse IgG Alkaline Phosphatase (AP) Conjugate	Anti-Mouse IgG (AP) Conjugate S3721	Affinity-purified goat anti-mouse antibody	Promega
Mouse anti-EBNA2	PE2	An affinity purified monoclonal antibody that reacts with the latent gene product EBNA2.	Professor Martin Rowe, University of Cardiff, Wales
Mouse anti-LMP1	CS1-4	An affinity purified monoclonal antibody that reacts with EBV-encoded latent gene product LMP1.	Professor Martin Rowe, University of Cardiff, Wales

Rat Anti-HA-HRP	Anti-HA-peroxidase high affinity (12013819001)	HRP-conjugated rat monoclonal antibody that recognises the HA peptide sequence (YPYDVPDYA) derived from the influenza HA protein.	Roche.
Mouse anti-p53	Anti-p53 (OP43)	Monoclonal IgG raised against amino acids 21-25 of human p53	Merck

2.1.1.3 Bacterial strains used in the study

Table 2.1-3: Bacterial strains used in the study

Bacterial Strain	Genotype
***E. coli* JM109**	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ-, Δ(*lac-pro*AB), [F´, *tra*D36, *pro*A + B + , lacl ^{q} ZΔM15]

2.1.1.4 Expression and Reporter Constructs

Table: 2.1-4: Plasmids used in this study

Plasmid	Source	Description
pSG5	Stratagene	The pSG5 Vector is a eukaryotic expression vector, harbouring the SV40 promoter and the ampicillin resistance gene.
pSG5-EBNA2	Lindsey Spender, Ludwig Institute for Cancer Research, Imperial College School of Medicine, London.	pSG5EBNA2 expresses the wild type EBNA2 gene (from the EBV strain B95.8) cloned into pSG5.
pSG5EBNA2ww323sr	Professor Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.	pSG5EBNA2ww323sr is identical to pSG5-EBNA2 except that EBNA2 has had two tryptophan residues mutated to serine and arginine residues at positions 323 and 324 respectively. pSG5EBNA2ww323sr therefore, does not bind RBP-Jκ/CBF1 (Ling *et al*, 1993a).

pGL2-Basic	Promega	The pGL2-Basic Vector is a reporter construct lacking eukaryotic promoter and enhancer sequences, The vector carries the coding region for firefly (*Photinus pyralis*) luciferase.
-1374/+81- *bfl1*-Luc	Dr. Brendan D'Souza, Laboratory for molecular and cellular biology. Dublin City University, Glasnevin, Dublin 9.	–1374/+81 Bfl-1 is a reporter construct for *bfl-1* in which the *bfl-1* promoter from the -1374/+81 Bfl-1 CAT construct was inserted into the pGL2 basic vector which contains the luciferase reporter (D'Souza *et al*, 2000).
-1710/+203-*bik*-Luc -855/+203-*bik*-Luc -191/+203-*bik*-Luc	This study	Reporter construct for *bik* in which the full length 1.9kb *bik* promoter from the -1710/+203 Bik CAT construct was cloned into the promoterless vector pGL2-basic which expresses the luciferase reporter gene. -855/+203-*bik*-Luc and -191/+203-*bik*-Luc are progressive deletion fragments of the *bik* promoter region containing the regions –855 to +203 and -191 to +203 cloned into pGL2-basic.
pGL3-Basic	Promega	The pGL3-Basic is a reporter construct containing the backbone of the pGL2-Basic with increased expression, and contains a modified coding region for firefly luciferase that has been optimised for monitoring transcriptional activity in transfected eukaryotic cells. pGL3-Basic lacks eukaryotic promoter and enhancer sequences.
pGL3-1710/+203-*bik*-Luc pGL3-855/+203-*bik*-Luc pGL3-191/+203-*bik*-Luc	This study	Full length 1.9kb reporter construct for *bik* as above but in the pGL3-basic background. pGL3-855/+203-*bik*-Luc and pGL3-191/+203-*bik*-Luc are progressive deletion fragments in pGL3-basic also as above.
pcDNA3	Invitrogen	The pcDNA3 Vector is a eukaryotic expression vector, harbouring the CMV promoter and the ampicillin and G418 resistance gene.
pcDNA3-Myc-*bik*	Professor Eileen White, Department of Molecular Biology and Biochemistry, and Cancer Institute of New Jersey, Rutgers University, Piscataway, New Jersey 08854	Expresses the Bik protein tagged to the Myc-epiptope in pcDNA3.

pcDNA3-HA-*bik*	Dr. G. Chinnadurai, Saint Louis University School of Medicine, Missouri. U.S.A.	Expresses Bik protein tagged to the influenza virus hemaglutinin (HA) epitope in pcDNA3.
pcDNA3-HA-*bik*-ΔBH3	Dr. G. Chinnadurai, Saint Louis University School of Medicine, Missouri. U.S.A.	pcDNA3-HA-*bik*-ΔBH3 is an expression vector for Bik protein as before but containing a deletion in the BH3 domain which abrogates the death promoting activity of this protein.
pcDNA3-HA-*bfl-1*	Dr. G. Chinnadurai, Saint Louis University School of Medicine, Missouri, U.S.A.	Expresses Bfl-1 protein tagged to the influenza virus HA epitope in pcDNA3 (D'Sa-Eipper *et al*, 1996).
pcDNA3-HA-Myc	Prof. Dr. med. Georg W. Bornkamm**,** Institute of Clinical Molecular Biology and Tumor Genetics (München, Germany)	Expresses the Myc protein tagged to the HA-epiptope in pcDNA3.
pCMV-LacZ	Clontech	pCMV-LacZ contains *E.coli* β-galactosidase gene under the control of the CMV promoter-enhancer.
pGa50-7 pGa981-6	Dr. Bettina Kempkes, Institute of Clinical Molecular Biology and Tumour Genetics, GSF, Munich, Germany.	pGa50-7 is a β-globin minimal promoter construct. pGa981-6 is a reporter construct (Minoguchi *et al*, 1997) which was generated using a 50bp oligonucleotide containing both CBF1 binding sites of the EBV *TP1* promoter, which was then ligated as a hexamer into pGa50-7 (Laux *et al*, 1994b).
p-1710/+203-BikCAT	Dr. G. Chinnadurai, Saint Louis University School of Medicine, Missouri. U.S.A.	p-1710/+203Bik CAT contains the region –1710 to +203 of the human *bik* gene cloned into the pBLCAT-2 ΔTK vector (in which the *tk* promoter has been deleted) (Verma *et al*, 2000), expressing a CAT reporter gene.
pJT125 pRSP 83	Professor Elliot Kieff. Department of microbiology and molecular genetics, Harvard Medical School, Massachussetts. Dr. Paul Ling, Department of Molecular Virology and Microbiology, Baylor College of Medicine. Baylor Plaza, Houston, Texas.	pSG5LP expression plasmid containing two W repeats is also known as pJT125 and the non functional partner for this (pRSP83) also consists of the EBNA-LP isoform with 2 W repeats however site directed mutagenesis changed three serine residues in each of the W2 repeats to alanine residues. The EBNA-LP expression plasmids are cloned into the pSG5 vector (Harada,and Kieff; Peng *et al*., 2000.

pRTS-1	Prof. Dr. med. Georg W. Bornkamm, Institute of Clinical Molecular Biology and Tumor Genetics (München, Germany)	Novel tetracycline-inducible vector carrying all the elements for conditional gene expression including the gene of interest on one EBV-derived episomally replicating plasmid (Bornkamm *et al*, 2005).
pRTS-1-HA-*bik* pRTS-1-HA- *bik*-ΔBH3	This Study	Generated by insertion of HA-*bik* ORF from pcDNA3-HA-*bik* into pRTS-1, generating a tetracycline regulatable vector capable of conditional expression of HA-tagged Bik protein. pRTS-1-HA-*bik*-ΔBH3 was generated by insertion of HA-*bik*-ΔBH3 ORF from pcDNA3-HA-*bik*-ΔBH3 into pRTS-1.
pRTS-1-Myc-*bik*	This Study	Generated by insertion of Myc-*bik* ORF from pcDNA3-Myc-*bik* into pRTS-1, generating a tetracycline regulatable vector capable of conditional expression of Myc-tagged Bik protein.
pRTS-1-HA-*bfl-1*	Dr. Sinead Loughran Laboratory for molecular and cellular biology. Dublin City University, Glasnevin, Dublin 9.	Generated by insertion of HA-*bfl*-1 ORF into pRTS-1, capable of conditional expression of HA-tagged Bfl-1 protein.

2.1.1.5 Oligonucleotides

Table 2.1-5: Oligonucleotides used in this Study

Target	Primer Sequence
BIK ORF F BIK ORF R (Sequencing)	5'-GGT ACC ATG GAG CAA AAG CT-3' 5'-CTC GAG CCT CCA GGG CAG TG-3'
BikPromFor BikPromRev	5'-CGC GGA TCC ACC CAC TCT GCC CTT GA-3' 5'-CGC GGA TCC TGG CAG CGT CTG TAA GC-3'
BikPromFor2 BikPromRev2	5'-CCG CTC GAG CAC CCA CTC TGC CCT TGA-3' 5'-CGG GGT ACC CAC CCA CTC TGC CCT TGA-3'
MycBikForSfiI MycBikRevSfiI	5'-TCT ATG GGC CTC ACT GGC CGG TAC CAT GGA GCA AAA GCT-3' 5'-TCT ATG GGC CTC ACT GGC CCT CGA GCC TCC AGG GCA GTG-3'

HABikForSfiI	5’-TCT ATG GGC CTC ACT GGC CAT GTA CCC ATA CGA TGT T-3’
HABikRevSfiI	5’-TCT ATG GGC CTC ACT GGC CTC ACT TGA GCA GCA GGT-3’
pEBNAseqFor (Sequencing primer)	5’-CAA GCT TTT CGG CCT CAC TGG CC-3’
GAPDH-F	5’-TGC ACC ACC AAC TGC TTA-3’
GAPDH-R	5’-GAT GAT GTT CTG GAG AGC-3’
pGL2-F	5’-GTA TCT TAT GGT ACT GTA ACT G-3’
pGL2-R (sequencing primers)	5’- CTT TAT GTT TTT GGC GTC TTC C-3’
pcDNA3-For	5’-GGC TAA CTA GAG AAC CCA CTG-3’
pcDNA3-Rev (sequencing primers)	5’-GGC AAC TAG AAG GCA CAG TC-3’

All oligonucleotides were synthesised by and obtained from MWG-Biotech, Ebersberg, Germany.

2.1.2 Bioinformatics software

A number of web-based bioinformatics tools listed in Table 2.1-6 were used routinely in this study.

Table 2.1-6: Web-based bioinformatics tools used in this study

Tool	Source	Use
BLAST	http://www.ncbi.nlm.nih.gov/BLAST	Comparison of nucleotide or protein sequences to GenBank sequence database. Statistical significance of matches calculated.
BioSeq File Format converter	http://bioinformatics.org/JaMBW/1/2/index.html	Conversion of nucleotide sequences to other formats including FASTA and GenBank.
CpG Island Searcher	http://cpgislands.usc.edu/cpg.aspx	The CpG island searcher screens for CpG islands which meet the criteria selected in submitted DNA sequences
MatInspector	http://www.genomatix.de/products/MatInspector/MatInspector1.html	Nucleic Acid Sequence Searches, to identify putative transcription factor binding sites.
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/index.php	Identification of restriction enzymes sites along a DNA sequence, and prediction of fragment sizes.

NetPrimerLaunch	http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html	Primer analysis software for prediction of primer properties including, T_m, GC content, probability of primer-dimers/hairpins formation.
TESS	http://dot.imgen.bcm.tmc.edu/	Nucleic Acid Sequence Searches, to identify putative transcription factor binding sites.
Translate tool	http://www.expasy.org/tools/dna.html	Translation of a nucleotide (DNA/RNA) sequence to a protein sequence.

2.1.3 Chemical and Molecular Biology Reagents

Table 2.1-7: Chemical and molecular biology reagents used in this study

Supplier	Product/Code
Amersham	Rainbow Molecular Weight Markers (RPN800)
Amaxa GmBH	Cell Line Nucleofector Kit T (VCA-1002), Kit V (VCA-1003)
Applied Biosystems	AmpliTaq Gold DNA Polymerase (N8080240), Bax Real Time Assay (Hs00180269_m1), Bik Real Time Assay (Hs00154189_m1), Gapdh Real Time Assay (Hs99999905_m1), Taqman 2X Universal PCR Master Mix No AmpErase UNG (58004008-01).
ASP	Presept Disinfectant Tablets (SPR25)
BD Biosciences	Annexin V-PE kit code 559763, FACS flow (342003).
BDH	Bromophenol blue (44385), EDTA (280254D), Glycine (444495D), Hydrochloric acid (28507BF), Isopropanol (296946H), Magnesium sulphate (29117), Methanol (29192BL), Nitrocellulose membrane (436107E), Potassium acetate (295814P), Sodium dihydrogen orthophosphate (30716), Sucrose (102745C), Tris(hydroxymethyl)methylamine (271195Y).
Eppendorf	Perfectprep Gel Cleanup (955152000)
Invitrogen	1 Kb DNA ladder (15615-016),100 bp DNA ladder (15628-019), Foetal calf serum (10270-106), HEPES (15630-056), Glutamax 1640 (61870-010), RPMI 1640 (31870-025), Trypan blue (15250-061), Trypsin (25090-028), Sodium Pyruvate (11360-039), SYBR Gold Nucleic Acid Gel Stain (S11494), Lipofectamine (18324-012).
Kodak	X-ray film (FS388).
Labkem	Nitrocellulose Membrane (436107E)
Labscan	Chloroform (A3505E).
Lennox Chemicals	IMS (B0982)
Merck	Calcium chloride (23821000), Glacial acetic acid (100632511), Magnesium chloride (1058321000), Potassium Hydroxide (50321000), Sodium carbonate (A654792), di-Sodium hydrogen phosphate (1065860500), 5-aza-2'-Deoxycytidine (189825), zVAD-FMK (219007), Anti-p53 (OP43).
National diagnostics	Acrylagel (EC810), Bis-acrylagel (EC820).
Millipore	Amicon Ultrafilter (UFC8 00508)
New England Biolabs	Alkaline Phosphatase (CIP) & 10X dephosphorylation buffer (M0290S). T4 DNA Ligase & 10X ligation buffer (M0202L), Restriction enzymes.
Oxoid	Agar (L13), PBS tablets (BR14), Tryptone (L42), Yeast extract (L21), PBS (Br14A)
Pierce	BCA Protein assay kit (23227), SuperSignal® West Pico Chemiluminescent Substrate (34080).

Promega	dNTPs (U1330), Luciferase Assay System (E1501), Magnesium chloride 25mM (A3511), M-MLV reverse transcriptase & RT buffer (M1701), 5X Reporter lysis buffer (E3971), RNasin (N2111), Wizard® PCR Preps DNA purification system (A7170), Donkey Anti-Goat HRP V8051, Goat Anti-mouse Alkaline phosphatase conjugate S3721.
Roche	Rat Anti-HA-HRP (12013819001), Taq polymerase & 10 X enzyme buffer (1146173), Hygromycin B (843555), Leupeptin (1017128).
Qiagen	Qiagen® Plasmid Purification Kit (12143), Quantitect ™ SYBR® Green PCR Kit (204143).
Santa Cruz Biotechnology	Goat Anti-Bik sc1710, Rabbti Anti-Bax-HRP sc493, Rabbit Anti-c-Myc sc764
Sigma-Aldrich Chemical Co.	3M sodium acetate, pH5.2 (S7899), Agarose (A5093), Ampicillin (A9518), Aprotinin (A4529), Ammonium Persulfate (215589), BCIP-NBT (B1911), Bovine serum albumin (A9647), Chlorofrom:isoamyl alcohol (24:1) (C0549), Colorburst electrophoresis markers (C4105), Coomassie Brilliant Blue R (B0149), Copper (II) Sulphate pentahydrate (31293), DEAE-dextran (D9855), DEPC (D5758), DMSO (D8779), β-estradiol (E8875), Ethidium bromide (E4391), Geneticin (G418) (A1720), Glucose (G7528), Glycerol (G5516), Hydrogen peroxide (H1009), Hydroquinone (H9003), L-glutamine (G7513), Manganese Chloride (M3634), MOPS (M3183), ONPG (N1127), Penicillin/streptomycin (P0781), Phenol:chloroform:isoamyl alcohol (25:24:1) (P3803), PMSF (P7626), Ponceau S (P7170), Potassium chloride (P4504), Puromycin (P8833), RedTaq (D4309), RNase A (R6513), Rubidium chloride (R2252), Lauryl Sulfate (L6026), Sodium Butyrate (B5887), Sodium chloride (S3014), Sodium hydroxide (S5881), α-Thioglycerol (M1753),Tetracycline (T7660), TEMED (T7024), Tri reagent (T9424),3,3',5,5'-Tetramethylbenz idine (TMB) (T0565), Triton® X-100(T8787), Trizma® Base (T1503), Trichostatin A (T8552) Tween 20 (P1379), Water (Molecular Grade) (W4502), Mouse Anti-β-Actin A1978, Sodium Citrate (S1804) MG132 (C2211), Actinomycin D (A1410).

2.2 Methods

2.2.1 DNA Manipulation*

2.2.1.1 DNA Storage

DNA samples were stored in Tris-EDTA (TE) buffer (pH 8.0) at 4˚C. EDTA was used to chelate heavy metal ions that are needed for DNase activity while storage at pH 8.0 minimises deamidation. DNA was also stored in sterile distilled H_2O (dH_2O) at -20˚C.

2.2.1.2 Phenol/chloroform extraction and ethanol precipitation

The standard method to concentrate nucleic acid samples, remove proteins or change the buffers in which a sample was dissolved is a Phenol/chloroform extraction followed by ethanol precipitation. DNA partitions into the aqueous phase, denatured proteins collect at the interface, and lipids partition into the organic phase. Nucleic acids are routinely recovered from aqueous solutions using ethanol precipitation in the presence of sodium acetate.

An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution, mixed by vortexing and centrifuged for 5 mins at 13,000 x g at room temperature. The upper aqueous phase was removed, avoiding any material at the interphase, and placed in a sterile microfuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase (to remove any residual phenol), vortexed as before, and centrifuged for 5 mins at 13,000 x g at room temperature. Again the upper aqueous phase was removed to a fresh tube. One-tenth volume of 3M sodium acetate (pH 5.2) was added to the solution of DNA to aid precipitation, mixed and followed by 2 and a half volumes of 100% (v/v) ethanol. This mixture was vortexed and incubated at -20˚C for a minimum of 20 mins. When dealing with very small quantities of DNA samples were precipitated in ethanol at -20^{o}C overnight. The DNA samples were then centrifuged for 20 mins at 12,000 x g at 4˚C, the supernatant was removed and pellets were washed with 300µl 70% (v/v) ethanol to remove excess salts. The tube was centrifuged for 5 mins at 10,000 x g, the

* Preparations of all solutions used in DNA manipulation are outlined in Appendix I.

supernatant was removed and pellets were air dried for approximately 10 mins. Pellets were re-suspended in an appropriate volume of sterile TE (pH 8.0) or dH_2O and were stored at 4˚C or -20˚C respectively.

2.2.1.3 Restriction digestion of DNA

Restriction enzymes specifically bind and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence, which is known as the recognition site. Restriction digestion of DNA was carried out for identification purposes, plasmid linearisation or to excise fragments from plasmid DNA. Restriction digestion patterns were predicted for DNA sequences using the NEBCutter or the WebCutter 2.0 bioinformatics tool (Table 2.1-6). All restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). Some enzymes required the addition of Bovine Serum Albumin (BSA) to the reaction to stabilise the enzyme, (working concentration 1X). DNA digestion reactions were performed according to manufacturers' instructions (New England Biolabs) and incubated for 3 hours at the optimum enzyme temperature (between 37˚C and 50˚C, usually 37˚C).

2.2.1.4 Dephosphorylation of linearised plasmid DNA

T4 DNA ligase catalyses the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA during ligation reactions. Calf intestinal phosphatase (CIP) is an enzyme that catalyses the removal of 5′ phosphate groups from DNA. Since CIP-treated fragments lack the 5′ phosphoryl termini required by ligases, they cannot self-ligate. Therefore in order to minimise re-circulisation of linearised DNA required for ligation and thus decreased the vector background in cloning strategies, treatment with CIP is required.

Digested DNA (<100 ng/µl) was de-phosporlylated using CIP in a 100µl volume (CIP was added 1 unit/100 pmoles for cohesive termini). The solution was mixed by pipetting, centrifuged briefly and incubated for 1 hour at 37˚C. This was followed by an enzyme denaturation step achieved by heating to 75˚C for 10 mins. The DNA was

then purified by phenol/chloroform extraction and ethanol precipitation, or gel purification. The purified linearised, CIP-treated DNA was stored at 4˚C until required for ligation.

2.2.1.5 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) uses short synthetic primers to amplify a specific region of DNA through multiple rounds of denaturation, annealing and elongation. PCR was used to amplify fragments of DNA for subsequent sub-cloning, for identification purposes and also for screening and sequencing. Primers are designed to allow polymerisation of both strands of the DNA using the thermostable enzyme DNA polymerase.

PCR reactions were set up by the addition of reagents in the following order:

Table 2.2-1: PCR Reaction Components

Component	Volume
dH_2O	37µl
Template DNA (~500ng)	1µl
Forward primer (40pmole)	2.5µl
Reverse primer (40pmole)	2.5µl
10X enzyme buffer (+$MgCl_2$)	5µl
dNTP mix (each at 10mM)	1µl
Taq polymerase (5 U/µl)	1µl

All PCR reactions were carried out in a Hybaid thermocycler under the following conditions:

Table 2.2-2: PCR Reaction Conditions

Stage	Step	Number of cycles
Denaturation	95 ˚C, 5 mins	1 Cycle
Denaturation	95 ˚C, 45 seconds	35 Cycles
Annealing	**X** ˚C, 45 seconds	
Extension	72 ˚C, 1 min	
Extension	72 ˚C, 15 mins	1 Cycle

X = Annealing temperature was calculated by subtracting 5 ˚C from the melting temperature (T_m*) of a primer pair. Annealing temperatures and primers are listed in Table 2.1-5.

2.2.1.6 Purification of PCR products

PCR products were purified using the Promega Wizard® PCR Preps DNA Purification System to remove contaminants including primer-dimers and primers used for amplification according to the manufacturers protocol (Wizard® PCR Preps DNA Purification System, Technical Bulletin, 2005). The purified DNA was stored at 4˚C until required for further use.

2.2.1.7 Ligation of DNA molecules

T4 DNA ligase catalyses the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in double-stranded DNA. This enzyme joins blunt end and cohesive end termini. Cohesive end ligations of equimolar concentrations of vector and insert DNA (final concentration 1µg), used in the cloning reactions were generally carried out overnight at 16˚C (or alternatively for 3 hours at room temperature (20-25°C) in a commercial ligation buffer (containing 5mM ATP)

* T_m = [2*(A/T content) + 4*(G/C content)].

with 10 units of T4 DNA ligase/ml in a total volume of 10µl. After ligation, the samples were heated at 65˚C for 10 mins to inactivate the ligase and stored at -20˚C until required for transformation.

2.2.1.8 Preparation of competent cells

Competence is the ability of a cell to take up extracellular DNA from its environment. *E. coli* cells are more likely to incorporate foreign DNA if the cultures are treated to make them transiently permeable to DNA. In this regard, a modified Rhubidium chloride ($RbCl_2$) method was employed to prepare competent cells. A JM109 *E.coli* strain was streaked from a glycerol stock on to a Luria-Bertani (LB) (Appendix I) agar plate and incubated at 37˚C overnight. An isolated colony was then picked using a sterile inoculating loop and used to inoculate 2.5ml of LB broth which was incubated at 37˚C in a shaking incubator at 200 rpm overnight. This culture was then used to inoculate 250 ml of sterile LB broth supplemented with 20mM $MgSO_4$. The 250 ml culture was incubated in a 1L flask at 37˚C until the O.D. of the culture was between 0.4 and 0.8, at 640 nm (approximately 4-5 hours). The cells were then transferred to two sterile 250ml centrifuge tubes and collected by centrifugation at 4,500 x g, 4˚C for 5 mins. The cells were then gently re-suspended in 0.4 of their original volume in ice cold TFB1 (Appendix I) (100ml for 250ml culture). Cells were kept on ice for all subsequent steps and pipettes, tubes and flasks were chilled. The re-suspended cells were then incubated on ice at 4˚C for 5 mins followed by centrifugation at 4,500 x g at 4˚C for 5 mins. Cells were then gently re-suspended in 1/25 of the original volume of ice-cold TFB2 (Appendix I) (10ml for a 250ml culture). These cells were then dispensed into 100µl aliquots and incubated on ice for 1 hour. Prior to storage the aliquoted cells were snap frozen in a dry ice/isopropanol bath. JM109 competent cells prepared by this method and stored at -80˚C are stable for 1 year.

2.2.1.9 Transformations

A 95µl aliquot of competent cells was placed in a pre-chilled microfuge tube containing 5µl DNA (~100ng/10µl). The contents of the tube were mixed gently by flicking, and incubated on ice for 30 mins, during which time an aliquot of SOC medium (Appendix I) was pre-heated to 37˚C. After 30 mins on ice the cells were heat-shocked in a water bath at 42˚C for 55-65 seconds, and were then removed immediately to ice for a further 2 mins. A 900µl aliquot of preheated SOC was added to the cells which were then incubated at 37˚C in a shaking incubator for 1 hour 30 mins. The cells were then collected by centrifugation for 3 mins at 6,000 x g following which, and 900µl of supernatant was removed and discarded. The cells were re-suspended in the remaining supernatant and plated out, along with the controls, on LB plates containing antibiotic at the appropriate concentration, and incubated overnight at 37˚C. Plasmids carrying the antibiotic resistance gene confer this resistance to the transformed cells and thus only transformed cells will yield colonies. These colonies were subsequently used to prepare broth cultures for DNA mini-preparations.

2.2.1.10 Small scale preparation of plasmid DNA (Miniprep)

A single bacterial colony was used to inoculate 5ml of LB medium containing the appropriate antibiotic and incubated with shaking (200rpm) overnight at 37˚C. An aliquot (1.5ml) of this culture was transferred to a sterile microfuge tube and centrifuged for 30 seconds at room temperature; the remainder was stored at 4˚C. The supernatent was removed from the tube using pipettes, leaving the cells as dry as possible. The cells were then re-suspended in 100µl of Buffer P1 (Appendix I) by vortexing. 200µl of freshly prepared Lysis solution; Buffer P2 (Appendix I) was then added, the tube contents were mixed by inverting the tube rapidly a number of times. 150µl of ice-cold neutralisation Buffer P3 (Appendix I) was added to stop the reaction and the tubes were vortexed gently for 10 seconds.

The lysate was centrifuged for 5 mins at 12,000 x g, the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate. A phenol chloroform extraction and ethanol precipitaion was carried out as described

previously in Section 2.2.1.2. The pellet was air-dried, then resuspended in 20µl of dH_20. 1µl of DNase-free RNase A (20µg/ml) was also added, and the mixture was vortexed, incubated at 37˚C for 1 hour and then stored at 4˚C (Adapted from Maniatis *et al*, 1982).

2.2.1.11 Glycerol Stocks

Glycerol stocks enable long term storage of bacteria since glycerol prevents water in the broth from forming ice crystals that can puncture and kill the bacterial cells. Glycerol stocks of bacterial cultures were prepared by the adding 0.5ml of a 50% (v/v) glycerol solution to 0.5ml of the overnight bacterial culture of interest, mixing and storing at -80˚C for future use.

2.2.1.12 Qiagen® plasmid DNA purification protocol (Midiprep)

Plasmid DNA was isolated and purified using the Qiagen® Plasmid Midi Kit. The process of plasmid isolation involves the lysis of the cells by a treatment with lysozyme followed by SDS which denatures proteins after the lysis of the membranes. The precipitation of the SDS-protein complex with high-molarity salt specifically subtracts the chromosomal DNA from the supernatant, since it remains attached to the mesosome.

A glycerol stock of the bacteria of interest was streaked on an LB agar plate (containing the appropriate antibiotic) and incubated overnight at 37˚C. An isolated colony from this plate was used to inoculate a 5ml starter culture (with the appropriate antibiotic) and incubated in a shaking incubator at 225rpm 37˚C for 8 hours. 100µl of the starter culture was used to inoculate 50ml of LB (containing the appropriate antibiotic) in a 250ml sterile flask and incubated overnight in a shaking incubator at 37˚C. It was important that the O.D. of the culture was between 1 and 1.5 at 600nm. The bacteria culture was transferred to a centrifuge tube and the bacterial cells were harvested by centrifugation at 6,000 x g for 15 mins at 4˚C using a JA-20 rotor in a Beckman centrifuge. The supernatant was decanted and the cells were re-suspended completely in 4ml of Buffer P1 containing RNase A (100µg/ml). The bacterial cells were then lysed by addition of 4ml Buffer P2 and incubation at room temperature for

5 mins. Following incubation, 4ml of pre-chilled Buffer P3 was added (to precipitate genomic DNA, protein, cell debris and SDS), mixed gently by inverting the tube 5-6 times and incubated on ice for 15 mins. The mixture was then centrifuged for 1 hour 30 mins at 16,000 x g at 4˚C and the supernatant saved.

The Qiagen-tip 100 was equilibrated by applying 4ml of QBT buffer and allowing the column to empty by gravity. The column does not dry out at this stage, as the flow of buffer will stop when the buffer reaches the upper filter. Following equilibration, the supernatant from the previous centrifugation step was applied to the filter and allowed to flow through. The Qiagen-tip was washed with 2 x 10ml of Buffer QC. DNA was then eluted with 5ml of Buffer QF. DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol. The DNA/isopropanol mixture was then aliquoted and centrifuged immediately at 13,000 x g for 45 mins at 4˚C and the supernatant was carefully removed using pipettes. The resulting pellet was washed with 70% (v/v) ethanol (13,000 x g for 15 mins at 4˚C), allowed to air dry for 5 mins and re-dissolved in 30µl of TE or dH_2O. DNA was then quantified by spectrophotometric analysis, and quality of the DNA was checked by Agarose gel electrophoresis (Adapted from the manufactures protocol Qiagen® Plasmid Purification Handbook, 2005).

2.2.1.13 Qiagen® EndoFree plasmid purification protocol (Maxiprep)

Endotoxins are frequent contaminants in plasmid DNA prepared from bacteria. In order to improve transfection efficiency endotoxin free plasmid DNA was isolated and purified using the Qiagen® EndoFree Plasmid Midi Kit from Promega according to the manufacturers protocol (EndoFree® Plasmid Purification Handbook, 2005). Endotoxin free DNA was then quantified by spectrophotometric analysis, and quality of the DNA was checked by Agarose gel electrophoresis.

2.2.1.14 Determination of Nucleic Acid Sample Concentration.

The concentration of isolated nucleic acids can be determined spectrophotometrically. Nucleic acids absorbs UV light maximally at an abosorbance of 260nm thus, optical absorbance can be used as an accurate measurement of its concentration**.** Pure DNA at

50µg/ml in aqueous solution has an A_{260} of 1, while 40µg/ml of pure RNA also has an absorbance reading of 1 at this wavelength.

Absorbance is also useful as a measure of the purity of DNA. The ratio of A_{260}/A_{280} of a pure dsDNA preparation should be between 1.65 and 1.85. Higher values are often due to RNA contamination and lower values to protein and phenol contamination. Pure DNA and RNA have $A_{260/}A_{280}$ ratios of 1.8 and 2.0 respectively.

Shimadzu UV-160A Spectrophotometer

Absorbances were read on the Shimadzu UV-160A spectrophotometer using a quartz cuvette and nucleic acid concentrations were determined according to the following equations:

$$\text{Concentration of DNA (µg/µl)} = \frac{\text{Absorbance (260nm) x 50 x dilution factor}}{1000}$$

$$\text{Concentration of RNA (µg/µl)} = \frac{\text{Absorbance (260nm) x 40 x dilution factor}}{1000}$$

The NanoDrop® ND-1000 Spectrophotometer

The NanoDrop® ND-1000 Spectrophotometer was also used to determine nucleic acid sample concentrations. An undiluted 1µl sample was pipetted onto the end of a fibre optic cable (the receiving fibre). A second fibre optic cable (the source fibre) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends. (The gap is controlled to both 1mm and 0.2mm paths by the computer). A pulsed xenon flash lamp provided the light source and a spectrometer was used to analyse the light after passing through the sample. The instrument is controlled by special software run from a PC, and the data was logged in an archive file on the PC.

2.2.1.15 Agarose gel electrophoresis of DNA

DNA has a negative charge in solution, and the magnitude of this charge is relative to the DNA fragment length. DNA will migrate to the positive pole in an electric field and thus DNA fragments can be separated based on size by passing through a porous gel under an electrical current. This is the basis for agarose gel electrophoresis and it is the standard method used to separate, identify, and purify DNA fragments.

The appropriate quantity of agarose was added to 100ml 1X TAE buffer based on the percentage agarose gel required. Increasing the percentage agarose (1.8-2.0%) in the gel was generally used to improve resolution of smaller DNA fragments while separation of larger DNA molecules was best achieved by using lower percentage gels (0.7-1.0%). The agarose was completely dissolved by boiling with intermittent mixing and after sufficient cooling (~60˚C) the gel was cast into the Hybaid horizontal gel electrophoresis system. A comb was inserted for formation of the wells. The gel was left to polymerise before filling the chamber with 1X TAE and removing the comb. Sample buffer, containing bromophenol blue as a tracking marker (Appendix I), was added to each sample before loading up to 20µl of the sample containing the nucleic acid per well. DNA sample buffer was also added to 500ng of a 1Kb or 100bp ladder, which was loaded as a size marker. The gel was run at constant voltage (5V/cm, usually 100V), for 1 to 2 hours. After completion, the gel was stained in ethidium bromide (0.5mg/ml) for 30 mins, destained in dH_2O for 15 mins and viewed under UV illumination.

2.2.1.16 Isolation of DNA from Agarose Gels.

Agarose gels were prepared in 1X TAE buffer as described previously in 2.2.1.15. Ethidium Bromide was added to the samples before electrophoresis so as to minimise manipulation with the gel. After electrophoresis, the gel was viewed in a dark-room under 70% UV illumination. The time of exposure to UV light was kept to a minimum, as overexposure to UV leads to DNA damage. The DNA band of interest was excised from the gel using a clean scalpel blade, excess agarose was cut away

(without disrupting the fragment), to minimise the size of the gel slice, which was then placed in a pre-weighed sterile microfuge tube.

2.2.1.17 Eppendorf Perfectprep® Gel Cleanup

Extraction and Purification of dsDNA fragments from TAE agarose gels was achieved using the eppendorf Perfectprep® Gel Cleanup kit following the manufacturers' protocol (Perfectprep® Gel Cleanup Manual, 2002). The purified DNA was eluted in 30µl of molecular biology grade water and stored at 4˚C or -20˚C.

2.2.1.18 DNA Sequencing

Sequencing of plasmid DNA was performed to ensure the nucleic acid fidelity and 'in-frame' insertion of DNA fragments following cloning. Plasmid DNA as prepared in Section 2.2.1.12 was quantified (Section 2.2.1.14) and 1-2µg (typically 1-2µl) was transferred to a sterile 1.5ml eppendorf. The DNA was lyophilised in a Savant DNA110 speed vac on the low heat setting for 15-20 mins or until the solvent had evaporated. The lyophilised DNA was sent to MWG-Biotech, Ebersberg, Germany for sequencing. Sequencing forward and reverse primers were supplied by MWG-Biotech for most commercially available cloning systems. In the absence of existing sequencing primers (as in the case of the pRTS-1 vectors), a forward and/or reverse primer was designed and purchased and 100pmol of primer was transferred to a sterile eppendorf and sent with each sequencing reaction to MWG-Biotech. Sequencing results were obtained electronically as linear nucleotide sequences.

2.2.2 Cell Culture Methods

All cell culture techniques were performed in a sterile environment using a Holten laminar flow cabinet. Cells were visualised with an Olympus CK2 inverted phase contrast microscope.

2.2.2.1 Culture of cells in suspension

All media compositions and media supplements are given in Appendix I. The cell lines AG876, BJAB, BJABK3, BL41, BL41-B95.8, BL41K3, BL419A, BL72, DG75, DG75-tTA-EBNA2, DG75-tTA-LMP1, EREB 2.5, MUTU I, MUTU III, X50-7, IARC 171, IARC 290B, IB4, KEM BL, OKU BL, OKU LCL, RAEL BL were maintained in RPMI 1640 supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, 100µg/ml streptomycin and 100U/ml penicillin. Cultures were seeded at a density of 2 X 10^5 to 5 X 10^5 cells per ml in 25cm^2 flasks and expanded in 75cm^2 flasks. P493-6 was maintained in RPMI 1640 supplemented with 10% FBS, 2% L-glutamine, and 1% Sodium Pyruvate. These cells were seeded at a density of 2 X 10^5 to 5 X 10^5 cells per ml in 25cm^2 flasks. Additional supplements were added to some culture media as described in Section 2.2.2.3. Cells were sub-cultured two or three times per week by harvesting the cells into a sterile centrifuge tube and centrifuging at 1000 x g for 5 mins at room temperature. The cells were gently re-suspended in a suitable volume of fresh supplemented media and replaced into the tissue culture flask. All cell lines were incubated in a humid 5% CO_2 atmosphere at 37˚C in a Heraeus cell culture incubator.

2.2.2.2 Culture of adherent cells

The HEK293 cell line (Table 2.1-1; Human embryonic kidney cell line) was maintained in minimum essential medium (Eagle) with 2mM L-glutamine and Earles's BSS adjusted to contain 1.5g/L sodium bicarbonate, 0.1mM non-essential amino acids, and 1.0mM sodium pyruvate, 100µg/ml streptomycin and 100U/ml penicillin, 10% (v/v) FBS. HEK293 cells were seeded into 25cm^2 and 75cm^2 tissue culture flasks. Adherent cells were detached by treatment with Trypsin solution (Invitrogen). For trypsinisation the medium was decanted and the cells were washed

with 2ml of sterile 1X PBS to remove any residual serum. A volume of 2ml of 1X tyrpsin solution was added to each flask and the flasks incubated at 37˚C for 5 mins or until all the cells could be visualised as having detached form the flask surface. The cell suspension was then decanted into a sterile centrifuge tube containing 8ml of sterile supplemented media and centrifuged at 100 x g for 5 mins. Cells were resuspended in supplemented media at 2 to 5 X 10^5 cells/ml, using 5ml per 25cm^2 flask and 15ml per 75cm^2 flask and incubated in a humidified 5% CO_2 atmosphere at 37˚C in a Heraeus cell culture incubator.

2.2.2.3 Media supplements

Supplements were added to the growth media of certain cell lines either to select cells containing transfected plasmids, activate the function/or induce expression of a gene of interest or to improve cellular proliferation.

L-cysteine is essential for the proliferation and survival of the majority group 1 BL cell lines. However, L-cysteine is quickly oxidated under normal culture conditions. To improve proliferation of the group I Burkitt lymphoma cell lines α-thioglycerol was added to the growth media as a stable substitute for L-cysteine. The α-thioglycerol was dissolved in bathocuprine disulfonic acid (BCS). BCS inhibits autoxindation of thiols in liquid solutions. Sodium pyruvate was also added to the media to protect against any H_2O_2 which may be generated.

The DG75-tTA-EBNA2 and DG75-tTA-LMP1 cell lines were maintained in supplemented RPMI containing 1μg/ml of tetracycline. Every three weeks the transfected cells were reselected by the addition of 500μg/ml of hygromycin and 1,000μg/ml of geneticin (G418) to DG75-tTA-EBNA 2 and 800μg/ml of hygromycin and 2,000μg/ml of G418 to DG75-tTA-LMPI.
The stably transfected cell lines BL41K3, BL419A and BJABK3 were maintained under permanent selection with the addition of 800ug/ml geneticin to BL41K3 and BJABK3, and 1200ug/ml of geneticin to BL419A. In experiments where EBNA2 activation was required, cells were washed twice in PBS and placed in fresh

supplemented RPMI with the appropriate antibiotics for selection,) estrogen was then added to a final concentration of 1μM.

The estrogen responsive EREB 2-5 cell line was maintained in supplemented medium containing 1μM estrogen. To abolish functional EBNA2 activity, EREB 2-5 cells were washed 4 times in PBS, with a further 2 washes 24 hours later and replaced in estrogen-free supplemented medium.

In the P493-6, functional EBNA2 activity was induced by culturing the cells in supplemented medium containing 1μM estrogen. To abolish functional EBNA2 activity, cells were washed 4 times in PBS, with a further 2 washes 60 hours later and replaced in estrogen-free medium. Expression of c-*myc*, was repressed by treatment with 0.1μg/ml tetracycline was added to culture medium. For *myc* reinduction, cells were washed 3 times with tetracycline-free culture medium and reseeded in tetracycline-free medium.

2.2.2.4 Cell counts

Cell counts were carried out using an improved Neubauer haemocytometer slide. Trypan blue exclusion dye was used to establish cell viability. A volume of 10μl of trypan blue was added to 90μl of a cell suspension and mixed. A sample of this mixture was added to the counting chamber of the haemocytometer and cells were visualised by light microscopy. Viable cells excluded the dye and remained clear while dead cells stained blue. Cell numbers were determined by multiplying the average cell count (of 3-5 individual counts) by the dilution factor (usually 1.1) and again by the volume of the haemocytometer chamber (1 X 10^4 cells/ml). Thus, cell counts were expressed as the number of cells per ml.

2.2.2.5 Cell storage and recovery

In order to prepare stocks of suspension cells for long-term storage, 1 X 10^7 cells in exponential phase were centrifuged, the excess media was discarded and the cells were re-suspended in 750μl of supplemented RPMI to which 150μl of FBS was added, and then placed on ice for 10 mins. DMSO was added to a final concentration

of 10% (v/v), mixed gently and transferred to a sterile cryotube. In the case of adherent cells, one confluent 75cm^2 flask of cells was used per cell stock. Adherent cells were washed with 1X PBS followed by trypsinisation and re-suspended in 900µl of FBS and 100µl of DMSO. The cells were mixed gently and added to a sterile cryotube. The cryotubes were slowly lowered into the gas phase of liquid nitrogen and then immersed in liquid nitrogen in a cryofreezer (Cooper Cryoservices).

Cells were recovered from liquid nitrogen by thawing rapidly at 37˚C and transferring to a sterile centrifuge tube containing 5ml of pre-warmed supplemented media. The cells were centrifuged at 100 x g for 5 mins, the cells were re-suspended in 5-10ml of fresh supplemented media, transferred to a culture flask and incubated at 37˚C in 5% CO_2.

2.2.2.6 Transient transfections

Transient transfection of cells was performed by electroporation, nucleofection, Lipofectamine or using the DEAE-dextran method. In all cases, cells were seeded at a density of 2-5 X 10^5 per ml of media 24 hours prior to transfection. After this time in culture, cells were counted again as it was essential for cell numbers to have almost doubled before beginning the transfection, thus ensuring that cell growth was in logarithmic phase, allowing for optimal DNA uptake during transfection. The same quantity of total DNA was used per transfection.

2.2.2.7 Electroporation of B lymphocytes

Transfection of AG876, IB4, BL41-K3, BJAB and P493-6 cell lines (Table 2.1-1) was carried out by electroporation. During the electroporation method of transfection, the application of brief high voltage electric pulses to the cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of redistribution of membrane components that accompanies the closure of the pores.

Total DNA (10-20µg per transfection) dissolved in 30µl TE buffer (pH 8.0) was transferred into a sterile electroporation cuvette. A total of 1 X 10^7 cells were used per

transfection. Cells were centrifuged at 100 x g for 5 mins and the supernatant discarded. The cells were then washed in PBS, centrifuged at 100 x g for 5 mins and the supernatant again discarded. For each 1 X 10^7 cells, 220µl of serum-free media was used for re-suspension and the cells transferred to the DNA-containing electroporation cuvettes. The cell/DNA mix was incubated at room temperature for 5 mins. Each cell/DNA mix was then pulsed at 220-250V (BJAB, 250V; P493-6, AG876, IB4 and BL41K3 220V) with a capacitance of 960µF (using a capacitance extender) and resistance set to infinity using a Biorad Gene Pulser. Immediately after electroporation the cuvettes were placed on ice until the cell/DNA mix was transferred into 5ml supplemented media in a 6 well dish, and the contents were mixed by gently rocking back and forth. Care was taken to ensure cells were stored no longer than 10 mins after electroporation to avoid reduction in cell viability/gene transfer efficiency. Transfected cells were harvested 48 hours later.

2.2.2.8 Nucleofection of B lymphocytes

The Nucleofector technology is based on electroporation. It consists of the Nucleofector device II (Amaxa), which delivers a cell-specific combination of electrical parameters, and Nucleofector Solutions, in which the cells are contained while the unique electrical program is executed. Optimised conditions for nucleofection of BJAB and IB4 cells yielded higher percentage transfection efficiency, when compared to cells transfected using the electroporation technique.

The nucleofector solution specific to the particular cell line under investigation (Solution T/V for BJAB/IB4) was pre-warmed to room temperature and 50ml of supplemented media was pre-warmed to 37ºC. Supplemented medium (2ml) was aliquoted into the appropriate number of wells of a 6 well plate and pre-incubated at 37˚C/5% CO_2/humidified atmosphere. A total of ~7µg DNA was prepared in sterile 1.5ml eppendorfs for each sample. For each transfection, 2.5 X 10^6 (IB4)/ 5 X 10^6 (BJAB) cells were collected by centrifugation at 90 x g for 10 mins and the supernatant was discarded completely so that no residual medium remained. The cells were resuspended in room-temperature solution in question to a final concentration of 2.5 x 10^6 cells/100µl (IB4) or 5 X 10^6 /100µl (BJAB). Care was taken to ensure cells were stored in nucleofector solution no longer than 15 mins to avoid reduction in cell

viability/gene transfer efficiency. A volume of 100µl of cell suspension was mixed with the DNA and the nucleofection sample was transferred to an Amaxa-certified cuvette, taking care to avoid bubbles. The cuvette was placed in the nucleofection chamber and pulsed on program T-016 (IB4 and BJAB). Immediately after this, 500µl of pre-warmed medium was added to the cuvette. The contents were then gently transferred to the prepared 6-well plates, mixes gently and incubated at 37˚C/5% CO_2/humidified atmosphere until cells were harvested.

2.2.2.9 Lipofectamine-mediated transfection

Lipofectamine transfection reagent (Invitrogen) is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,Ndimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water was used to deliver DNA into the HEK293 cell line. Lipofectamine treatment alters the cellular plasma membrane, allowing nucleic acids to cross into the cytoplasm.

One day before transfection, 1 X 10^6 HEK293 cells were seeded in 2ml of supplemented growth medium in a six well plate to ensure that cells would be 90-95% confluent on the day of transfection. Antibiotics were excluded from the medium during transfection as they would be taken up with the lipofectamine, and thus cause cell death. On the day of transfection 4µg of DNA was diluted in 250µl of OptiMem medium and was mixed gently. Following this, 10µl of the Lipofectamine reagent was diluted in 250µl OptiMem and incubated for 5 mintues at room temperature. Care was taken to proceed to the next step with in 25 mins. The diluted DNA was then combined with the diluted lipofectamine and the mixture was incubated at room temperature for a further 20 mins. Following this, 500µl of the DNA:lipofectamine mix was added to the appropriate well of the six well plate and the contents were mixed gently by rocking the plate back and forth. Plates were left overnight at 37˚C under a 5% CO_2/humidified atmosphere. After 4-6 hours the medium was replaced with fully supplemented medium. Transfected cells were harvested 48 hours later.

2.2.2.10 DEAE-Dextran-mediated transfection

Transfection of DG75 and BJAB cells was performed using this method of transfection. During transfection, positively charged DEAE-dextran binds to the negatively charged phosphate groups of the DNA, forming aggregates. These complexes, when applied to cells, subsequently bind to the negatively charged plasma membrane. It is believed that cellular uptake of DNA is mediated by endocytosis, and further assisted by osmotic shock.

A maximum of 25µg of DNA dissolved in dH_2O was used per transfection. Total DNA was prepared in a sterile microfuge tube and brought to a volume of 600µl with TBS (Appendix I). Following this, 600µl of 1mg/ml DEAE-dextran solution (Appendix I) was mixed with the DNA solution. Meanwhile, 1 X 10^7 cells/transfection were centrifuged at 100 x g for 5 mins and the supernatant was discarded. The cells were then washed in PBS, centrifuged at 100 x g for 5 mins and the supernatant again discarded. The cells were then gently re-suspended in the DNA/DEAE-dextran mix. The transfection cocktails were incubated at 37 ˚C for 30 mins with gentle swirling every 5-10 mins to promote homogenisation. Transfections were terminated by the addition of 10ml supplemented medium to the tube and the cells were collected by centrifugation at 100 x g for 5 mins, washed in PBS as before and transferred to 6 well plates containing 10ml supplemented media for incubation. Cells were harvested at 48 hours post transfection.

2.2.2.11 Microscopic analysis following gene transfer

Following some transfections gene transfer efficiency was monitored by fluorescence microscopy for green fluorescent protein (GFP)-expressing cells at 4-72 hours post transfection. A volume of 600µl of suspension cells was removed to a 1.5 ml sterile eppendorf and centrifuged at 100 x g for 5 mins. The supernatant was discarded and the cells were resuspended in 20µl of PBS/0.5% BSA and applied to a glass microscope slide. A coverslip was applied with care taken to avoid air bubbles and the slide was examined under bright field and fluorescent field microscopy using an Olympus DP-50 fluorescent microscope [excitation 450–500 nm, emission 515–565 nm] with a camera and StudioLife software (Olympus Optical Company).

2.2.2.12 Harvesting cells post-transfection for luciferase/β-gal assays

Suspension Cells

Cells were collected by centrifugation at 100 x g for 5 mins at room temperature. The cells were washed once in sterile PBS and centrifuged again at 100 x g for 5 mins. The supernatant was discarded, insuring all traces of PBS were removed using pipettes. The cells were re-suspended in 50µl of 1X reporter lysis buffer (diluted with dH_2O from a 5X stock, Promega) and the cell suspension transferred to a microfuge tube. The tubes were vortexed for 10-15 seconds. The lysates were clarified by centrifugation at 13,000 x g for 5 mins and the supernatant saved in a fresh tube. Samples were stored at –80 ˚C until luciferase/β-galactosidase assays were performed. Transfected cells were also harvested for the purposes of FACS analysis and Western Blotting. Details pertaining to the protocol followed in each case is given in Sections 2.2.2.16 and 2.2.4.3 respectively.

Adherent Cells

The supernatant was removed from each well and placed in a universal tube. 1ml of PBS was added to each well and the cells were scraped. The cell suspension was transferred into the corresponding universal tube containing the supernatant. Each well was rinsed with 2ml PBS and added to the corresponding universal tube. Cells were collected at 1,800rpm for 5 mins at room temperature. The cells were washed once in sterile PBS and centrifuged again at 1,800rpm for 5 mins. From this point the samples were processed as for suspension cells above.

2.2.2.13 Luciferase assay

Following transfection, promoter activity was determined by means of the luciferase assay. Firefly luciferase from the firefly *Photinus pyralis*, a monomeric 61 kDa protein, catalyses luciferin oxidation using ATP-Mg^{++} as a co-substrate. In this luminescent reaction light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin.

In the conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined.

Luciferase assay reagent was prepared by reconstituting luciferase assay substrate with luciferase assay buffer (Promega) and stored in aliquots in the dark at –70 ˚C. At the time of assay, it was important to allow sufficient time for the luciferase detection reagent to equilibrate to room temperature. A volume of 20µl of cell lysate was dispensed into individual wells in a white 96 well plate, along with 20µl of 1X lysis buffer to act as a blank. Subsequently, 100µl of detection reagent was added to the lysate immediately prior to measuring, in order to initiate enzyme activity, mixed by repetitive pipetting (3 times) and light emission integrated over a period of 60 seconds, after lag period of 10 seconds, was measured on a luminometer (Labsystems Luminoskan 391A). Luciferase activity levels were adjusted for transfection efficiencies, estimated using *β*-galactosidase assay.

2.2.2.14 β-Galactosidase assay

When measuring the effect of promoters or enhancers on gene expression, it is essential to include an internal control that will distinguish differences in the level of transcription from differences in the efficiency of transfection or in the preparation of extracts. This is best achieved by co-transfecting the cells with two plasmids: one that carries the construct under investigation, and another that constitutively expresses an activity that can be assayed in a separate experiment. An enzyme frequently used for this is *E. coli* β-galactosidase (β-gal). This assay is a convenient method for assaying β-gal activity in lysates prepared from cells transfected with β-gal reporter vectors, in this case pCMV-LacZ.

The β-gal assay was performed using a sample from the same lysates assayed for luciferase activity. Cell extract (30µl) was added to 3µl 100X Mg solution, 66µl 1X *o*-nitrophenyl-β-D-galactopuranoside (ONPG) and 201µl 0.1M sodium phosphate (all described in Appendix I) and incubated at 37˚C for >30 mins or until a faint yellow colour developed. This yellow colour development is the result of hydrolysis of ONPG by β-gal to form *o*-nitrophenyl. A reaction tube was included containing 1X

lysis buffer instead of cell lysate in order to obtain a background reading. Reactions were terminated by addition of 500µl 1M Na_2CO_3 (Appendix I). Optical densities were read at 420nm using a Shimadzu spectrophotometer over a linear range of 0.2-0.8.

2.2.2.15 Stable Transfections

Stable cells lines were established by transfection (as in Section 2.2.2.15) followed by selection with the appropriate drug 48 hours later. Within 2-4 weeks drug resistant cells grew out. In some cases, specific drug concentrations for selection in individual cell lines were known from previous publications/projects, otherwise drug curves were prepared in advance of transfection to determine the minimum drug concentration to cause cell death.

2.2.2.16 Flow cytometric analysis

Flow cytometry may be defined as a technology to measure properties of cells as they move, or flow, in liquid suspension. Most flow cytometers can measure two kinds of light from cells, light scatter and fluorescence. Light scatter is the interaction of light and matter. All materials, including cells, will scatter light. In the flow cytometer, light scatter detectors are located opposite the laser (relative to the cell), and to one side of the laser, in-line with the fluid-flow/laser beam intersection. The measurements made by these detectors are called forward light scatter and side light scatter, respectively. Forward light scatter provides information on the relative size of individual cells, whereas side light scatter provides information on the relative granularity of individual cells. Fluorescence is the property of a molecule to absorb light of a particular wavelength and re-emit light of a longer wavelength. The wavelength change relates to an energy loss that takes place during the process.

2.2.2.17 Annexin V/ vital dye staining

In normal live cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to

the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, annexin V, is a 35–36 kDa Ca2+-dependent phospholipid-binding protein that has a high affinity for PS and can be used to identify apoptotic cells by binding to PS. Fluorescent dyes that bind to nucleotides and penetrate only damaged cellular membranes were used (vital dyes). Intercalation complexes are formed by propidium iodide or 7-Amino-actinomycin (7-AAD) with double-stranded DNA, which effect an amplification of the fluorescence.

2.2.2.18 Annexin V-PE/7-AAD

The Annexin-PE Apoptosis Detection Kit (Becton Dickinson) contains recombinant annexin V conjugated to the fluorochrome, phycoerythrin (PE) and the vital dye 7-AAD. Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. Cells that stain positive for Annexin V-PE and negative for 7-AAD are undergoing apoptosis. Cells that stain positive for both Annexin V-PE and 7-AAD are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both Annexin V-PE and 7-AAD are alive and not undergoing measurable apoptosis.

To perform the assay, cells were harvested and washed twice in ice-cold PBS. The cells were resuspended in 1X annexin-binding buffer at a concentration of ~1 X 10^6 cells/ml. A volume of 5µl of Annexin V-PE solution and 5µl of 7-AAD solution were added to 100µl of cell suspension. The cells were incubated at room temperature for 15 mins in the dark and 400µl of 1X annexin-binding buffer was then added and the samples were analysed within one hour by flow cytometry.

2.2.2.19 PI/FACS (cell cycle) analysis

Cells were harvested and washed in ice-cold PBS (typically 1-5 X 10^6 cells). The cells were resuspended in 500µl 1X PBS containing 0.1% Glucose. A volume of 5ml of ice cold 70% ETOH was added immediately, by slowly running down the side of the tube and the solution was mixed promptly. The cells were then stored at 4^oC for between 1 hour and 1 week to fix. Two to three hours prior to FACS analysis the cells were

collected by centrifugation at 1,800rpm for 5 mins, and washed once in 1X PBS. The cells were then centrifuged for 2 mins and any residual PBS was removed using pipettes. The cells were suspended in 300µl propidium iodide solution (Appendix I) and 20µl of 10mg/ml RNase was added. The cells were mixed gently and incubated at 37^{o}C for 30-45 mins. Samples were then analysed by flow cytometry.

2.2.3 RNA Analysis

2.2.3.1 RNase-free environment

Because RNA is easily degraded by ubiquitous RNases, standard procedures were employed to avoid this potential hazard (Sambrook *et al*, 1989). Prior to working with RNA, any apparatus or surfaces to be used were treated to remove RNase. The apparatus/surface was washed in detergent and rinsed well in DEPC-treated H_2O, then in 100% (v/v) ethanol and finally allowed to air dry. Any apparatus to be used were also immersed in a 3% (v/v) solution of hydrogen peroxide (Sigma-Aldrich) for 15 mins, and were then rinsed thoroughly in DEPC treated upH_2O and allowed to dry. Because hands are a major source of RNase contamination, gloves were used at all times and changed frequently.

2.2.3.2 RNA analysis by gel electrophoresis

In order to examine RNA transcripts/ascertain the integrity of RNA, isolated samples were run on 1.5% (w/v) agarose gels. The appropriate amount of agarose was dissolved in DEPC-treated H_2O and prepared according to Section 2.2.1.15. The RNA samples (1µl) were prepared for electrophoresis by adding 3µl of RNA sample buffer (Appendix I) and made up to 15µl in DEPC-treated H_2O. The samples were heated to 65 °C for 10 mins prior to loading on the gel. The gel was run in 1X TAE as described in Section 2.2.1.15. As ethidium bromide is included in the RNA sample buffer the gels did not require further staining and could be visualised directly on a UV trans-illuminator.

2.2.3.3 RNA extraction from cultured cells

Prior to RNA isolation the condition of cells was reviewed by phase contrast microscopy. A cell count was performed as described in Section 2.2.2.4.

2.2.3.4 RNA extraction using Tri-reagent

Cells grown in suspension were centrifuged at 100 x g for 5 mins, washed once in PBS and the cells were lysed by repeat pipetting in Tri Reagent (Sigma-Aldrich). A volume of 1ml of Tri reagent was used per 1 X 10^7 cultured cells. The lysate was left at room temperature for 5 mins after which time the procedure could be halted by storing samples at –80 ˚C.

Phase separation was achieved by adding 200µl of chloroform per 1ml of lysate. The samples were covered and shaken gently but thoroughly for 15 seconds or until completely emulsified. Samples were incubated at room temperature for 15 mins. The resulting mixture was centrifuged at 13,000 x g for 20 mins at 4 ˚C. During centrifugation the mixture separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase, which contained the RNA, was removed to a fresh tube and RNA was precipitated by addition of 500µl of ice-cold isopropanol per ml of Tri-reagent used initially. The samples were incubated for 10 mins on ice and then centrifuged at 13,000 x g for 15 mins at 4 ˚C. The resulting RNA pellet was washed using 1ml of 75% (v/v) ethanol by inverting the tube 5 times. The pellets were then centrifuged at 13,000 x g for 5 mins at 4 ˚C, and the 75% (v/v) ethanol was aspirated. Pellets were air dried and dissolved in DEPC treated upH_2O. The resulting RNA preparation was heated at 60˚C and mixed gently to ensure a homogeneous solution prior to aliquoting. An aliquot was removed for spectrophotometric (Section 2.2.1.14) and gel electrophoretic analysis (Section 2.2.3.2) and the remainder of the purified RNA was stored at – 80˚C.

2.2.3.5 Total RNA isolation from cells using QIAgen RNeasy™ kit

RNA was extracted from small numbers of cultured cells (<5 X 10^5) using an RNeasy kit (QIAgen) according to the manufacturer's protocol (RNeasy Mini Handbook, 2006). An aliquot of purified RNA was removed for spectrophotometric (Section 2.2.1.14) and gel electrophoretic analysis (Section 2.2.3.2) prior to RT-PCR.

2.2.3.6 Quantification of mRNA from cultured cells by RT-QPCR

The quantification of mRNA purified from cultured cells was performed in a two-step procedure. In the first step, cDNA was prepared from RNA by reverse transcription (RT) with random hexamers serving as primers. During the second step, cDNA was amplified by real time quantitative PCR (QPCR). Real-time QPCR is increasingly being adopted for RNA quantification based on its ability to detect the amount of PCR product present at every cycle (i.e. in real time), as opposed to the endpoint detection by conventional PCR methods, thus allowing the real-time progress of the reaction, especially its exponential phase, to be viewed. The real-time PCR approach is based on the detection and quantification of a fluorescent reporter, where the signal increases in direct proportion to the amount of PCR product in a reaction. TaqMan gene expression assays were used in this study for comparative gene expression analysis, normalising with *gapdh* endogenous control mRNA levels (Applied Biosystems, Table: 2.1-7).

2.2.3.6.1 Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase

In this process mRNA was transcribed into cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. Initially, 2µl of random hexamers was added to 2µg RNA and the volume brought up to 10µl with DEPC H_2O. The mixture was heated to 70˚C for 5 mins, to destabilise secondary mRNA structures, and then placed on ice. Then, the RT reactants were added in the order listed in Table 2.2-3.

Table 2.2-3 MMLV RT reactants

Component	Volume
Reverse Transcriptase buffer (5X)	8µl
dNTP mix (20mM)	1µl
$MgCl_2$ (25mM)	4µl
BSA (4µg/µl)	1µl
DEPC H_2O	13µl
RNasin ribonuclease inhibitor	2µl
M-MLV reverse transcriptase (200U/µl)	1µl

The reactions were placed in a Hybaid thermocycler at 37˚C for 1 hour and 95˚C for 2 mins followed by storage at 4˚C.

2.2.3.6.2 Real time relative PCR (QPCR)

cDNA generated by RT was quantified by real time PCR using the TaqMan gene expression assays (Applied Biosystems), which consisted of two unlabeled primers for amplifying the sequence of interest (final concentration of 900nM each) and one dual-labeled TaqMan MGB probe (6-FAM dye- and TAMRA-labeled) for detecting the sequence of interest (final concentration of 250nM). PCR exploits the 5'-3' nuclease activity of the DNA polymerase system to cleave a TaqMan probe during PCR as illustrated in Figure 2.2.1.

Quantification of a cDNA target was normalised for differences across experiments/samples using an endogenous control as an active reference (*gapdh*). The PCR reaction mix was prepared for each sample (in triplicate) by addition of the reagents listed in Table 2.3-2 to the individual wells of a 96-well reaction plate (Applied Biosystems) followed by the addition of 2µl of cDNA to give a total reaction volume of 25µl.

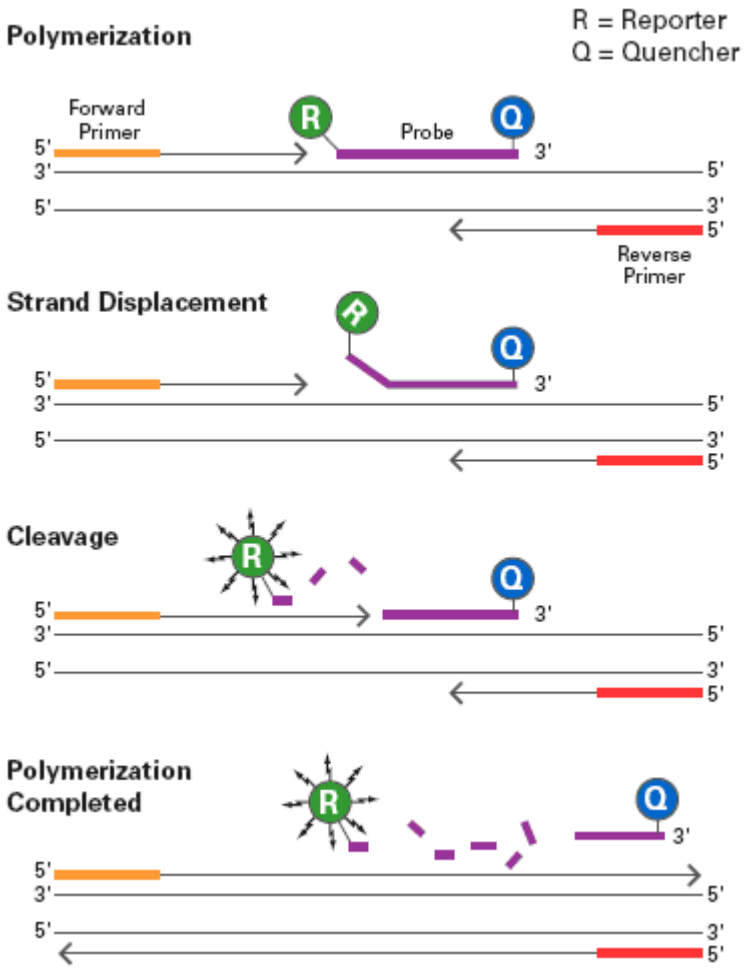


Figure 2.2.1 Principles of the TaqMan assay primers and probes

When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Lakowicz, 1983). During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5'–3' nucleolytic activity of the DNA polymerase system cleaves the probe between the reporter and the quencher only if the probe hybridises to the target. The probe fragments are then displaced from the target, and polymerisation of the strand continues. The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any non-specific amplification is not detected (From Applied Biosystems White Paper on TaqMan gene expression assays http://docs.appliedbiosystems.com/pebiodocs/00106737.pdf).

Table 2.2-4 Real time QPCR reactants

Component	Volume/Reaction
Assay	1.25µl
20X TaqMan PCR master mix*	12.5µl
Nuclease Free H_2O	9.25µl
Total	23µl

The plate was covered with an optical adhesive cover and centrifuged at 2,000 x g for 2 mins to eliminate air bubbles. Amplification and detection were performed with an ABI Prism 7500 sequence detection system under the following conditions: 10 mins at 95 °C to activate AmpliTaq Gold DNA polymerase, and 45 cycles of 15 seconds at 95 °C and 1 min at 60 °C. Following QPCR, the products were analysed by agarose gel electrophoresis according to Section 2.2.1.15 to check for migration of fragments at the expected size.

During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analysing fluorescence emissions. The reporter dye (FAM) signal was measured against the internal reference dye (ROX) signal to normalise for non-PCR-related fluorescence fluctuations occurring from well to well. The threshold cycle represented the refraction cycle number at which a positive amplification reaction was measured and was set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 15. The results were analysed the Comparative C_T method ($\Delta\Delta C_T$) as described by (Livak and Schmittgen, 2001).

The equation below shows the $2^{-\Delta\Delta CT}$ equation, which was used to compare the gene expression in two different samples (e.g. samples A and B); and in every case each sample was related to an internal control gene (*gapdh*). During analysis the treated sample was designated as 'sample A' and the untreated control as 'sample B'.

* TaqMan universal PCR Master mix contained AmpliTaq gold polymerase, dNTPs and the passive reference, ROX

Fold Change = $2^{-\Delta\Delta CT}$ = [(C_T of the gene of interest- C_T internal control gene) sample A –

(C_T of the gene of interest- C_T internal control gene) sample B]

Equation used for analysis of real-time PCR data by the comparative C_T method.

Results were interrupted as the expression of the gene of interest (relative to the internal control) in the treated sample compared to the untreated control.

2.2.4 Protein Analysis

2.2.4.1 Preparation of cellular protein

Proteins were isolated from both suspension and adherent cells for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by western blotting. Prior to isolation, cells were washed in PBS. Suspension cells were centrifuged at 100 x g for 5 mins and the supernatant removed. Ice-cold PBS (10ml) was added; the cells were centrifuged again at 100 x g and all of the supernatant removed. Adherent cells were washed twice with 10ml of ice-cold PBS, and the cells were scraped into 1ml of PBS. The crude cell suspension was centrifuged at 100 x g for 5 mins. For both suspension and adherent cells, the cells were re-suspended in ice-cold suspension buffer (Appendix I) using 200µl of suspension buffer for every 1 X 10^7 cells and the cell suspension transferred to a microfuge tube. An equal volume of 2X SDS gel loading buffer (Appendix I) was immediately added to the cell suspension, after which the sample became extremely viscous. The sample was then subjected to sonication for 1 min on full power (using a Vibra Cell Sonicator) to shear the DNA. The lysate was clarified by centrifugation at 12,000 x g for 10 mins at room temperature. The supernatant was aliquoted and stored at –20 ˚C until required for analysis.

2.2.4.2 SDS-polyacrylamide gel electrophoresis of proteins

During polyacrylamide gel electrophoresis (PAGE), proteins are driven by an applied current through a polyacrylamide gel. PAGE is carried out in the presence of the negatively charged detergent sodium dodecylsulphate (SDS), which binds to all types of protein molecules. Electrostatic repulsion between the bound SDS molecules causes the proteins to unfold into a similar rod-like shape, and since the amount of SDS bound is proportional to the molecular weight of the polypeptide and is sequence independent, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

2.2.4.2.3 Preparation of SDS-polyacrylamide gels

SDS-PAGE was performed using 10% (v/v) resolving gels and 5% (v/v) stacking polyacrlyamide gels prepared as detailed in Appendix I.

An ATTO protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water, then with dH_2O and finally wiped in one direction with tissue soaked in 100% (v/v) ethanol. The gasket was placed around the ridged plate; the plates were assembled and secured with clamps. The resolving gel was then poured to within 2cm of the top of the larger plate and overlaid with 100% (v/v) ethanol. When set, the ethanol was removed and the stacking gel was poured. A clean comb that had been wiped in 100% (v/v) ethanol was inserted and the gel was allowed to polymerise for at least 20 mins. The electrophoresis tank was filled with 1X Tris-glycine running buffer (Appendix I) to a level of about 5cm deep. After polymerisation, the gaskets and clamps were removed and the pre-poured gels were lowered into the buffer at an angle to exclude air bubbles from the gel-buffer interface. The tank was completely filled with 1X running buffer and the comb removed from the gel. Un-polymerised gel was removed by gently rinsing the wells with 1X running buffer and the wells were then straightened using a loading tip. The gel plates were fixed firmly in place with the pressure plates. The chamber formed by the inner plates (notched plate facing inwards) was filled with 1X running Buffer, the samples were loaded and the electrodes attached. The gels were electrophoresed at a constant current of 30mA per gel until the blue dye front had reached the bottom of the gel. When complete the plates were removed, separated and the gel was placed in transfer buffer prior to Western blotting (Section 2.2.4.3).

2.2.4.3 Western blotting

During western blotting, electrophoretically separated proteins were transferred from the polyacrylamide gel to a solid support, usually a nitrocellulose membrane, and probed with antibodies that reacted specifically with antigenic epitopes displayed by the target protein attached to the solid support. The bound antibody, unless conjugated, was detected by a secondary immunological reagent, conjugated to either the alkaline phosphatase (AP) or horseradish peroxidase (HRP) enzyme for detection.

2.2.4.3.4 Transfer of protein to nitrocellulose filters

Following gel electrophoresis, gels were equilibrated in transfer buffer (Appendix I) for at least 15 mins. Equilibration facilitated the removal of electrophoresis salts and detergents. Salts if not removed, increase the conductivity of the transfer and the amount of heat generated during transfer. Nitrocellulose membrane was cut to the dimensions of the gel, along with 6 pieces of 3MM filter paper that were required for the gel/membrane sandwich. Protein transfer was carried out on the Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell. A pre-soaked sheet of filter paper was placed onto the platinum anode. A pipette was rolled over the surface of the filter paper to exclude all air bubbles. This step was repeated with two more sheets of filter paper. Then, the pre-wetted blotting membrane was placed on top of the filter paper and all bubbles rolled out. The equilibrated gel was carefully placed on top of the nitrocellulose membrane, aligning the gel on the centre of the membrane. Any air bubbles were again rolled out. Another 3 sheets of pre-wetted filter were placed on top of the gel, with care taken to remove air bubbles. The cathode was placed on top of the stack and the safety cover replaced on the transfer unit. Gels were transferred for 30 mins at 17V.

2.2.4.3.5 Staining of proteins immobilised on nitrocellulose filters

Ponceau S is a negative stain, which binds to positively charged amino acid groups of proteins. It also binds non-covalently to non-polar regions of proteins. Ponceau S staining was employed to determine whether uniform transfer of proteins to the nitrocellulose membrane had taken place. Transferred protein was detected as red bands on a white background. This staining technique is reversible to allow further immunological analysis. Following electrophoretic transfer, the nitrocellulose membrane was immersed in 20ml Ponceau S solution (Sigma-Aldrich) and stained for 5 mins with constant agitation. After proteins were visualised, the membrane was washed in several changes of dH_2O until all the stain had been washed away. The membrane was then used for immunological probing.

2.2.4.3.6 Immunological probing

Following Ponceau S staining, the membrane was incubated in blocking buffer (Appendix I) for 3 hours at room temperature followed by incubation with the appropriate primary antibody (diluted in blocking buffer, see Table 2.2-5) at 4 ˚C overnight.

Table 2.2-5 Incubation Conditions for Antibodies Used in Western Blotting

Primary Antibody	Name	Dilution In Blocking Buffer	Secondary Antibody	Dilution In Blocking Buffer
Anti-β-actin	Anti-actin AC-15	1/10,000	AP-conjugated Anti-mouse IgG	1/20,000
Goat-Anti-Bik	NBK (N-19): sc-1710	1/200	Anti-Goat-HRP	1/5000
Mouse-Anti-Bik	Mouse Anti-Human Bik Clone C33-1 (557040)	1/500	AP-conjugated Anti-mouse IgG	1/5000
Anti-Bax-HRP	Bax (N-20): sc-493	1/100	n/a	n/a
Anti-cMyc	*c*-Myc (N-262) :sc-764	1/1000	Anti-Rabbit-AP	1/1000
Anti-HA-peroxidase	Anti-HA-peroxidase, high affinity	1/1000	n/a	n/a
Anti-p53	Anti-P53 OP43	1/500	AP-Conjugated Anti Mouse IgG	1/1000
Anti-EBNA2	PE2	1/100	AP-Conjugated Anti Mouse IgG	1/5000
Anti -LMP1	CS1-4	1/1000	AP-Conjugated Anti Mouse IgG	1/5000

After overnight incubation, the membrane was washed three times in TBS-T (0.1% (v/v) Tween-20 in TBS (Appendix) for 15 mins. The filter was then incubated with the appropriate secondary antibody (Table 2.2-5) for 1 hour 30 mins at room temperature, followed by three 15-min washes in TBS-T. (Conjugated primary

antibodies were developed following three washes in TBS-T, and needed no further incubation step). All of the above incubations were carried out with constant agitation. The membrane was then placed in a clean container and covered with 5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT, Sigma) or 3,3',5,5'-tetramethylbenzidine (TMB, Sigma), which are used for the colourimetric detection of alkaline phosphatase- or hydrogen peroxidase-conjugated molecules respectively. When incubated with alkaline phosphatase or hydrogen peroxidase, bound enzyme catalysed the production of a coloured product that was easily observable. The membrane was then rinsed in distilled water to stop the reaction and photographed.

CHAPTER 3 :

EBV-associated changes in *bik* expression in BL-derived and Lymphoblastoid cell lines.

3.1 Introduction

Central to the infection strategy of EBV is its ability to inhibit the cellular apoptotic programme (Klein, 1994). Suppression of apoptosis *in vivo* is an essential feature contributing to the persistence of the latent virus in infected B-cells (Gregory *et al*, 1991). The manner in which EBV can protect B-cells from apoptosis has long been attributed to cellular alterations caused by the interactions between the virus and the various members of the cellular apoptotic programme (Gregory *et al*, 1991; Snow *et al*, 2006). To this end, EBV encodes genes that can prevent or delay the death of a host cell (Henderson *et al*, 1993).

There are two phenotypically distinct EBV-carrying B-cell types that can be grown in culture, exhibiting different patterns of EBV gene expression and regulation (Figure 3.1.1). In studies of freshly isolated BL tumour cells and cell lines from cases of EBV-positive African BL, the tumour biopsy cells were found to display a group I phenotype and were found to express only the EBNA1 protein plus the EBERs (Middeldorp *et al*, 2003). These cells readily undergo apoptosis in response to various triggers (Gregory *et al*, 1991). In some cell lines following their establishment *in vitro* the group I phenotype is either retained or the cell populations fluctuate between group I and its subcategory group I/II, during continuous culture. Other cell lines, however, undergo a spontaneous 'drift' or phenotypic change from group I, through the transient group II stage to group III. This propensity for phenotypic change was found to be a feature of EBV-positive, rather than EBV-negative cases of BL (Perera *et al*, 1999). These cells (group-III BL cells and LCLs) are phenotypically altered due to the presence of the EBV genome and express the complete viral repertoire including six EBV nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP) three virus-encoded latent membrane proteins (LMP1, LMP2A, LMP2B) and the EBERs (Middeldorp *et al*, 2003). Type III cells also display enhanced cell survival (Gregory *et al*, 1991). A comparison of the phenotype of BL type I cells directly to that of the proliferating EBV-immortalised LCL or BL cells with group III latency, reveals that the virus induces dramatic changes in the gene expression programme of the cell during infection.

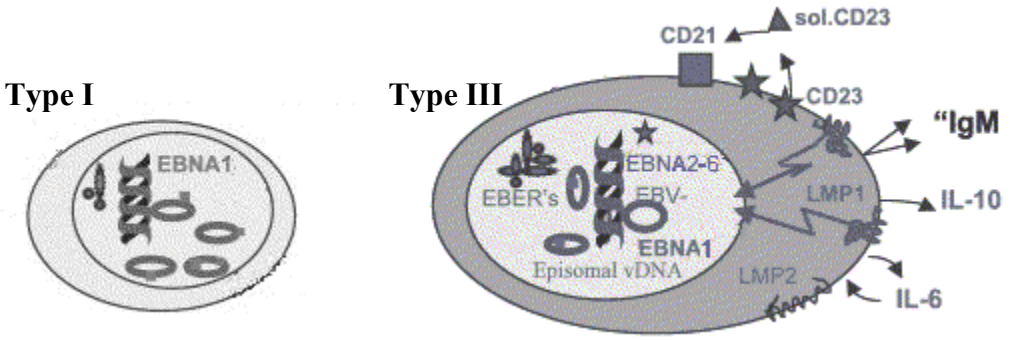


Figure 3.1.1:Type I and Type III latency

Phenotypically distinct human B-cell lines display three transcriptionally distinct forms of EBV latency frequently described as Type I (or latency I/group I or the latency programme), Type II (or latency II/group II) and Type III (or Latency III/group III, or the growth programme) (Kerr *et al*, 1992). In BL Type I, only EBNA1 and BARF-0, as well as large copy numbers of EBER1 and EBER2 are expressed. EBV-infected LCLs express six EBV nuclear antigens [EBNAs -1, -2, -3A, -3B, -3C and –LP] and three latent membrane proteins [LMPs 1, 2A and 2B] and show high levels of expression of the B-cell activation markers CD23, CD30, CD39 and CD70, and of the cell adhesion molecules lymphocyte-function-associated antigen 1 (LFA1; also known as CD11a/18), LFA3 (also known as CD58) and intercellular cell adhesion molecule 1 (ICAM1; also known as CD54) (Young and Rickinson, 2004). The cell surface changes that characterise the Type I and Type III phenotypic drift are accompanied by changes in morphology (Rowe *et al*, 1987; Gregory *et al*, 1990). Adapted from Middeldorp *et al*, 2003.

The apoptotic threshold of a cell is determined by the balance in the expression of the levels of pro- and anti-apoptotic dimerisation partners available and in this regard the ability of EBV to regulate various members of the Bcl-2 family to shift the balance of cell fate has long been realised (Henderson *et al*, 1991). Suppression of apoptosis in EBV-infected cells has been associated with the induction of anti-apoptotic factors including Bcl-2, A20, and Mcl-1 (Henderson *et al*, 1991; Finke *et al*, 1992; Laherty *et al*, 1992; Rowe *et al*, 1994; Silins and Sculley, 1995; Wang *et al*, 1996; Kenney *et al*, 1998; Sheng *et al*, 2003) and the inhibition of pro-apoptotic molecules such as Bax and Bim (Clybouw *et al*, 2005; Grimm *et al*, 2005; Anderton *et al*, 2007). The regulation of the cellular *bfl-1* gene has been studied extensively in our laboratory, and findings indicate that, *bfl-1* is anti-apoptotic in BL cells and is regulated by EBV via two different pathways with roles for both NF-κB and Notch nuclear proteins (D'Souza *et al*, 2000; D'Souza *et al*, 2004; Pegman *et al*, 2006). While the expression pattern of viral genes during EBV infection is well defined, the spectrum of cellular

target genes and the potential contribution of these target genes to B-cell immortalisation and EBV pathogenesis remains an interesting area of research which has implications for the biology of EBV and malignant disease.

Preliminary experiments in our laboratory, examining EBV-associated changes in the level of expression of apoptosis-related genes indicated that the pro-apoptotic *bik* gene may be a target of EBV regulation. Thus, these observations prompted an investigation to further current understanding of the molecular mechanisms that cause EBV-infected cells to survive by elucidating the status of the *bik* gene during EBV-infection.

3.2 EBV associated down-regulation of the *bik* gene.

3.2.1 Expression of *bik* gene in EBV-infected cell lines.

A preliminary experiment was undertaken in the lab to examine previously unreported EBV-associated changes in the expression of *bcl-2* related genes using two established isogenic EBV-positive BL cell lines, group I MUTU-BL (MUTU I) and group III MUTU-BL (MUTU III). MUTU I cells are an early passage BL cell line expressing EBNA1 as the only viral gene (Gregory *et al*, 1990). MUTU III cells are a stable clone of MUTU I which has drifted upon serial passage to express the full compliment of EBV latent genes (Gregory *et al*, 1990). mRNA levels from the apoptosis related genes $bcl_{XL/S}$, *bfl-1, bik, bak, bax, bcl-2* and *mcl-1* were analysed by Ribonuclease Protection Assay (RPA) and it can be seen in Figure A (Appendix II) that significantly elevated expression of *bfl-1* and *bcl-2* were present in MUTU-III cells relative to MUTU-I cells, as has been previously reported (Henderson *et al*, 1991; D'Souza *et al*, 2000). It is also evident that repression of *bik* mRNA appears to be a feature of the BL cell line expressing a group III phenotype (MUTU-III) but not the BL group I phenotype (MUTU-I). These cell lines are isogenic clones, allowing the study of phenotypic differences in an identical genetic background. The result seems concurrent with a model for a differential expression of *bik*, with decreased levels of *bik* mRNA expressed in EBV-infected cell lines expressing the full compliment of EBV latent genes. Thus, it was indicated for the first time that the pro-apoptotic *bik* gene could potentially be an important target for down-regulation by EBV latent genes in the mediation of an increased anti-apoptotic threshold of EBV infected cells.

This novel finding prompted an investigation to elucidate if *bik* down-regulation is a general feature of EBV-infected B-cells, and to this end *bik* expression was examined in a range of Burkitt's lymphoma (BL)-derived cell lines and B lymphoblastoid cell lines (LCLs).

On this basis total protein and RNA were prepared simultaneously from a range of cell lines and were used in Western blot analysis and Reverse Transcription (RT) Real Time PCR (QPCR) respectively (Section 2.2.4.3 and 2.2.3.6). The level of Bik protein was detected by Western blot analysis using an affinity purified goat polyclonal IgG raised against a peptide mapping at the N-terminus of Bik and each lane was normalised for β-Actin expression levels (endogenous control) using a mouse monoclonal IgG generated from an N-terminal peptide of β-Actin.

Bik protein expression was studied in 7 BL-derived cell lines with group I phenotype [BJAB, AKATA-4E3, MUTU I, DG75, OKU BL, KEM BL and BL41K3], 5 EBV positive cell lines with a group III phenotype [AG876, MUTU III, BL41-B95.8, DAUDI and BL41-P3HR1] and in 6 EBV-immortalised LCLs [IB4, IARC 171, IARC 290B, X50-7, OKU LCL, EREB2.5]. From this experiment it was clearly seen that Bik is highly expressed in the cells expressing the EBV latency programme (Lat I) and has little or no expression in those BL cells expressing the EBV growth programme (Lat III) and LCLs as indicated in Figure 3.2.1A. Two exceptions to this general observation were the DAUDI and BL41-P3HR1 cell lines, two long-established BL Lat III cell lines. These cell lines contain EBV genomes that carry different deletions spanning the viral EBNA2 coding sequence, and both of which express high levels of Bik (Figure 3.2.1A). DAUDI, originally described by (Klein *et al*, 1968) contains a 7.4kb deletion corresponding to the EBNA2/EBNA-LP coding sequence, which is larger than but similar to the deletion found in a non-transforming strain of the virus; P3HR-1 (Jones *et al*, 1984). BL41-P3HR1 is a BL cell line infected with this EBNA2-deficient isolate (Jones *et al*, 1984; Kempkes *et al*, 1996). Consequently, since both cell lines are deficient for EBNA2 and both express elevated levels of Bik protein, in contrast to their EBNA2-expressing counterparts, a potential role for EBNA2 in the modulation of the *bik* gene in this B-cell background was considered.

Isogenic MUTU-BL clones show either a group I or a group III phenotype and thus allow the study of phenotypic differences due to EBV in an identical genetic background. Bik is expressed in group I clones of MUTU I and is not expressed in the isogenic group III counterpart, MUTU III (Figure 3.2.1A) thus confirming the correlation between Bik expression and EBV latent viral gene expression in an isogenic cell system. In addition to this, OKU BL is a group I BL cell line expressing only EBNA1 whereas OKU LCL was established upon infection of peripheral lymphocytes from the same patient from whom OKU BL was derived with the EBV strain B95.8.

Having established that suppressed Bik protein levels was a consistent feature of LCLs and BL cells expressing a type III latency, QPCR was then used to ascertain if down-regulation was at the transcriptional level in this context. Firstly, RT reactions using random primers were carried out to generate cDNA, which was subsequently used in a QPCR assay to determine the level of *bik* mRNA present. mRNA levels of the internal control gene *gapdh* were also monitored, and the resulting data obtained was used to normalise data acquired for *bik*. The relative level of *bik* mRNA was calculated using the Comparative C_T method [$\Delta\Delta C_T$] as described by (Livak and Schmittgen, 2001). *Bik* mRNA level was studied in 6 BL cell lines with group I phenotype [BJAB, MUTU I, DG75, OKU BL, KEM BL and RAEL], 4 cell lines with a group III phenotype [AG876, MUTU III, BL41-B95.8, DAUDI and BL41-P3HR1] and in 5 EBV-immortalised LCLs [IB4, IARC 171, IARC 290B, X50-7, EREB2.5]. Overall it was found that significantly less levels of *bik* mRNA were present in BL cells expressing a group III phenotype and LCLs when compared to BL type I. (Figure 3.2.1B). Relative quantitation of the levels of *bik* transcript after normalising for *gapdh* mRNA levels in KEM BL cells had comparatively high *bik* expression at an approximately 100-fold greater level than that observed in the LCL X50-7, and at a level 63-fold above the mean expression level seen in LCLs. *Bik* expression was also relatively high in the RAEL, BJAB and DG75 BL cell lines with approximately 71, 40 and 34-fold respectively increased mRNA levels observed than in LCL X50-7. The mean expression level for BL type I in general was 46-fold greater than that observed in LCLs and an average 3-fold more than that observed in Lat III cells. *Bik* mRNA levels observed in the MUTU I cell line were similar to those observed in MUTU III. This inconsistency is possibly an indication that these Type I BL cells had drifted on

serial passage to a group III phenotype, prior to RNA isolation in the QPCR experiment. In general however in the cell lines examined, *bik* mRNA was generally expressed to a greater extent in the BL cell lines with a group I phenotype than in those expressing a group III phenotype or in EBV-immortalised B-cells. However, upon closer examination of Figure 3.2.1, it can be seen that expression of *bik* in Type III cell lines is elevated relative to the expression level seen in LCLs. These results indicate that the *bik* gene may be down-regulated by EBV at a transcriptional level in LCLs, however transcriptional regulation of *bik* by EBV in BL type III cell lines is less in evidence.

Interestingly, whereas the Bik protein expressed in BL type I cells always appeared as 27/26-kDa double bands during Western blotting, any low levels of Bik detected in LCLs and in BL Type III cell lines was always evident as a single band. In principle there are a several possible explanations for this double band pattern. Assuming the correct specificity of the antibody, some form of post-tranlational modification may account for the two-band pattern observed, possibly phosphorylation or glycosylation. Since the regulation of Bik by phosphorylation has already been verified (Verma *et al*, 2001) these observations may suggest that the phosphorylated form of Bik may predominate in BL Type I cell lines. However, in order to determine if phosphorylation accounts for this post-translational modification and if one of the bands may represent the phosphorylated form of Bik, it would be necessary to determine whether Bik is a phosphoprotein and if it can serve as a substrate for protein kinases in this particular context.

Thus, Bik repression appears to be a common feature of LCLs and BL cells expressing all the viral latency-associated proteins. Taken together these results suggest that *bik* is a negative transcriptional target of the EBV latent proteins.

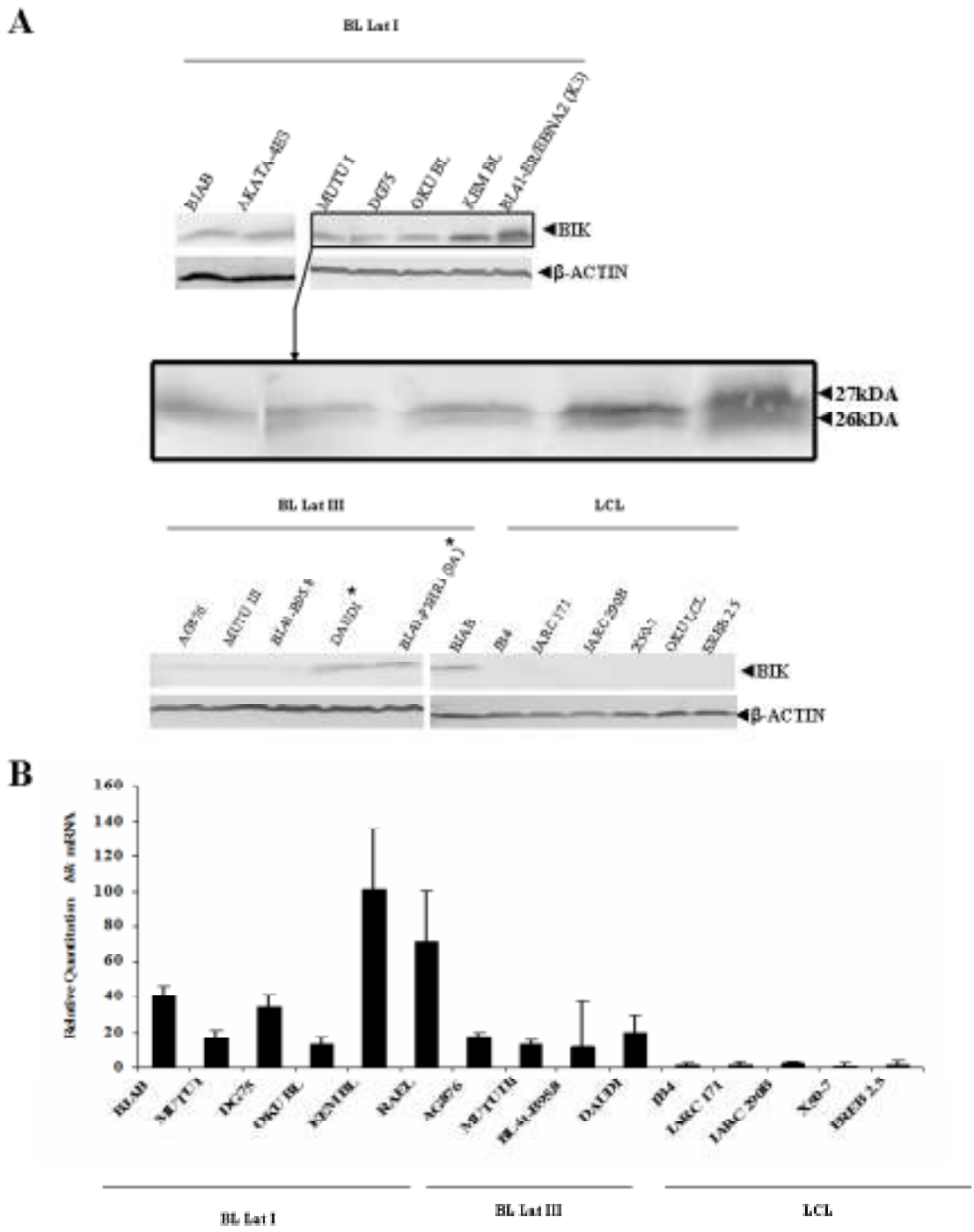


Figure 3.2.1: Analysis of a range of EBV-related cell lines for Bik expression.

(A) Protein extracts prepared from a range of BL cell lines with a group I phenotype, a group III phentotype, and LCLs (as indicated above) were analysed by Western blot analysis. The level of Bik protein was detected using an affinity purified goat polyclonal IgG (Santa Cruz, sc-1710) (Upper Panel) and was normalised for β-Actin expression levels (endogenous control) using a mouse monoclonal IgG (Lower Panel). (B) The level of *bik* mRNA expression present was quantitated by QPCR. DNA was monitored in real-time by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were recorded using

the ABI Prism 7500 Sequence Detection System. *Bik* levels were assayed in triplicate and normalised using *gapdh*. Relative quantification levels were calculated relative to the control (X50-7; arbitrarily assigned a value of 1). (Data are Mean±SD, n=2).

* Both cell lines carry deletions spanning the EBNA2 coding sequence.

3.2.2 Bik is not mutated in EBV negative BL cell lines.

Considering that Bik possesses an already exposed BH3 domain and is thus predicted to be constitutively active (McDonnell *et al*, 1999) and also given the fact that Bik is known to trigger apoptosis in various cell types (Han *et al*, 1996a; Tong *et al*, 2001; Germain *et al*, 2002; Hur *et al*, 2004; Oppermann *et al*, 2005) the detection of Bik in BL type I cell lines was not consistent with a pro-apoptotic role for Bik in this cell context. Interestingly, inactivating mutations have been found in other lymphomas but no data has yet been reported for *bik*.

In order to investigate the possibility that mutations leading to functionally redundant forms of Bik might be present in BL type I cells, *bik* cDNAs derived from two EBV-positive (AKATA and MUTU I) and two EBV-negative BL-derived cell lines (BL41 and DG75) were sequenced across the *bik* open reading frame (ORF) located on exons 2-5 of the gene (Verma *et al*, 2000). As LCLs are derived from normal B-lymphocytes it was not deemed necessary to examine putative mutations in this context. Total RNA was prepared from all four cell lines (Section 2.2.3.4) and RT reactions were carried out to generate cDNA (Section 2.2.3.6.1), which was subsequently used for PCR (Section 2.2.1.5). Conditions used for PCR and the primer sequences used for the amplification of the *bik* ORF (BIKORF F, BIKORF R) from the four different cell lines are indicated in Table 2.1-5. Following PCR, the products were analysed by agarose gel electrophoresis (Section 2.2.1.15) to check for amplification of fragments of the expected size (Figure 3.2.2A). The PCR products were subsequently purified using the Promega Wizard® DNA purification system (Section 2.2.1.6), and the individual, purified PCR products were then sequenced (Section 2.2.1.18). All stages of the process were carried out in isolation for each individual cDNA, ensuring no cross-contamination of cDNA or PCR products could occur. Sequencing results, which were obtained as linear nucleotide sequences, were compared with the nucleotide sequence of the human *bik* gene (accession number

U34584) using the multiple-sequence alignment program MultAlin (Table 2.1-6). The sequence of the four cDNAs for *bik*, demonstrated 100% homology with the published *bik* gene sequence. (Figure 3.2.2B). Thus, no mutations leading to changes in the primary amino acid sequence were found, implying that the selection of loss-of-function mutations in the *bik* gene is unlikely to be a feature of BL cells.

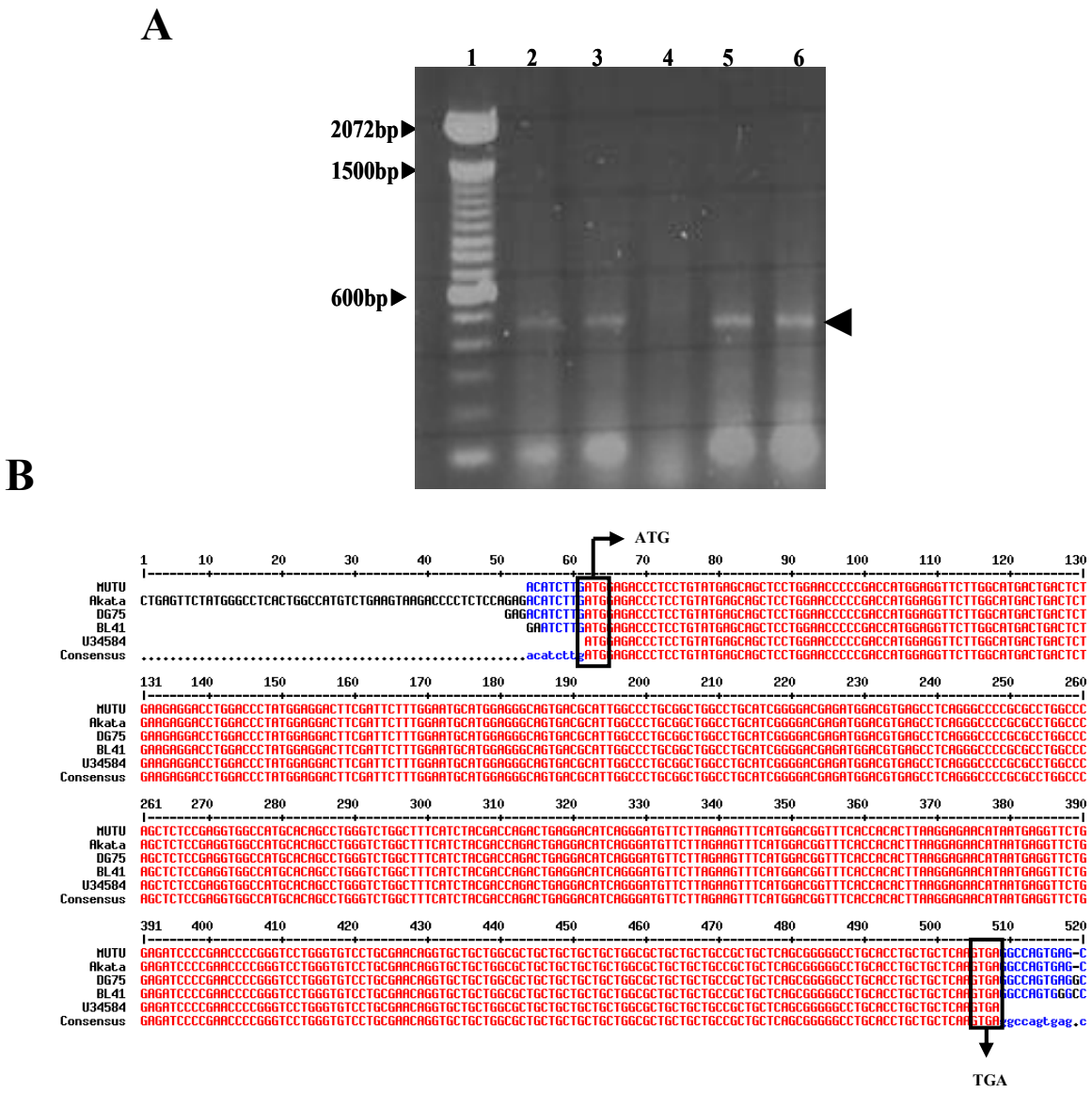


Figure 3.2.2 Sequencing of *bik* cDNA from EBV-positive and EBV-negative BL-derived cell lines.

(A) PCR products amplified from two EBV-positive (AKATA and MUTU I) and two EBV-negative BL-derived cell lines (BL41 and DG75) using forward and reverse primers; BIKORF F and BIKORF R, were analysed by agarose gel electrophoresis on a 1.2% agarose-1X TAE gel and electrophoresis at 100V for 1 hour in 1X TAE. Amplification of the *bik* cDNA fragment resulted in a band at 483bp (lane 2

[AKATA], lane 3 [BL41], lane 4 [negative control], lane 5 [DG75] and lane 6 [MUTU I]). A 100bp DNA ladder (Invitrogen) is shown in lane 1. (B)Bik ORF sequencing results highlighting the ATG codon and the TGA Stop codon. The nucleotide sequence of *bik* cDNAs from (A) above were sequenced across the *bik* open reading frame using Bik ORF specific forward and reverse primers (BIKORF F and BIKORF R). Sequence results were aligned with the published *bik* sequence (GenBank Accession number: U34584) using MultAlin software. Sequences highlighted in red demonstrated 100% homology.

3.2.3 Status of Bax in EBV derived cell lines

Recent evidence has suggested that pro-apoptotic BH3 only members of the Bcl-2 family couple upstream death signals to downstream activation of the death effectors Bax and Bak, resulting in cell death (Wei *et al*, 2001; Zong *et al*, 2001). Indeed, specifically in the case of *bik,* reports indicate that induction of cell death is mediated by an entirely Bax-dependent mitochondrial pathway (Gillissen *et al*, 2003; Mathai *et al*, 2005). The absence of Bax expression might therefore explain the failure of type I BL cells to succumb to apoptosis. Therefore in an attempt to indicate a role for *bik* in cells from EBV associated malignancies, *bax* expression was investigated in a range of cell lines including BL Type I, BL type III and LCLs.

Total protein and RNA were prepared simultaneously from a range of cell lines and were used in Western blot analysis and RT Real Time PCR (QPCR) respectively as before. The level of Bax protein was detected by Western blot analysis using affinity purified rabbit polyclonal antibody raised against a peptide mapping at the N-terminus of Bax (Table 2.1-2). Bax protein expression was examined in 6 BL cell lines with group I phenotype [DG75, MUTU I, BL41K3, KEM BL, BJAB, and OKU BL], 5 BL-derived cell lines with a group III phenotype [BL72 III, AG876, BL41-B95.8, DAUDI and BL41-P3HR1] and in 4 EBV-immortalised LCLs [IB4, X50-7, IARC 171, and IARC 290B]. It can be seen that Bax was generally expressed in cells expressing the EBV latency programme (Lat I) and in those cells expressing the EBV growth programme (Lat III and LCLs) (Figure 3.2.3A). Bax expression in BL cell lines was independent of EBV status and, with the exception of DG75 cells, was expressed in every cell line examined and did not vary significantly (Figure 3.2.3A).

In addition it was found that *bax* mRNA was present in all BL cells expressing a group I phenotype (including DG75), in BL group III cell lines and in LCLs and no trend in expression levels was evident (Figure 3.2.3B). Interestingly, DG75 cells express *bax* mRNA, and the absence of Bax protein is due to single base deletions and additions in a poly-guanine tract within the *bax* ORF. These frameshift mutations are known to result in premature termination of translation (Brimmell *et al*, 1998). Overall, the expression of Bax protein and mRNA was confirmed in a range of cell lines including BL Type I (except DG75), BL type III and LCLs.

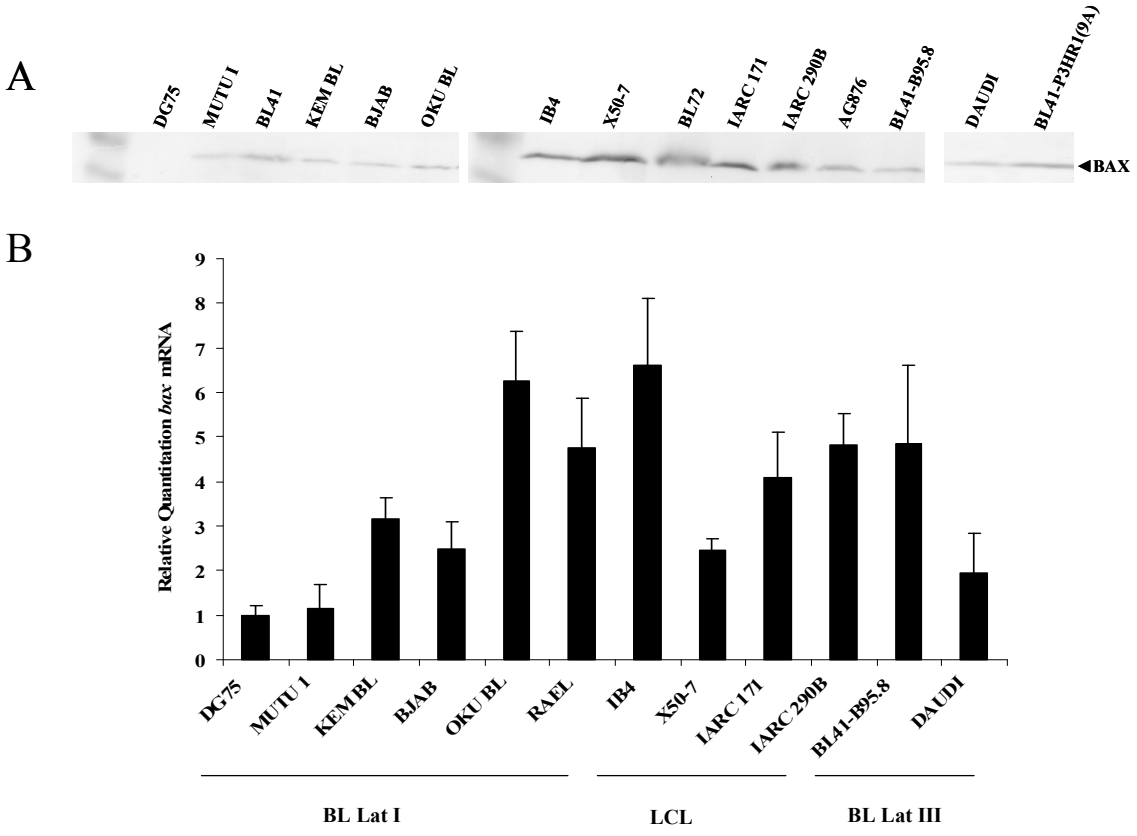


Figure 3.2.3: Analysis of a range of EBV-related cell lines for Bax expression.

(A) Protein extracts were prepared from a range of BL cell lines with a group I phenotype, a group III phentotype and LCLs (as indicated above [panel A]), and were analysed by Western blot analysis as before. The level of Bax protein was detected using an affinity purified Rabbit polyclonal IgG. (B) RT-Real Time PCR analysis for *bax* expression. Total RNA was reverse transcribed using random primers oligonucleotides and amplification of DNA was monitored in real-time using FAM-labelled TaqMan probes for *bax* and *gapdh*. *Bax* levels were assayed in triplicate and normalised using *gapdh* as before. Relative quantification level of *bax* mRNA was plotted as a function of the control (DG75; arbitrarily assigned a value of 1). (Data are Mean±SD).

3.2.4 Effect of culture conditions on Bik expression in the AG876 cell line.

The results presented in Section 3.2.1, indicated *bik* mRNA was expressed at relatively elevated levels in Type III cells compared to LCL cells. It was therefore a possibility that *bik* levels may vary during serial passaging in BL Type III cell lines and it was decided to examine expression of Bik protein and mRNA in AG876 (Type III BL) during passaging. A time course experiment was performed in which cells that had been passaged three times a week were seeded into multiple flasks at a density of 5 X 10^5 cells/ml and were subsequently left unpassaged, or alternatively were passaged every 24 hours or every 48 hours. Total mRNA and protein were harvested simultaneously from cells at various time points after the initial passage and were used in Western blot analysis and QPCR respectively.

Western blotting was used to examine the level of Bik protein over the course of the experiment and levels of the endogenous control β-Actin were also monitored and confirmed equal loading in each case (Figure 3.2.4A). A second Bik antibody was acquired from BD Biosciences (557040; Table 2.1-2) at a later stage in this study. In order to validate this antibody for use a control experiment was undertaken (see Chapter 4; Section 4.23). The BD antibody was used in the following experiment. Analysis of Bik protein revealed that no significant fluctuation in the level of Bik protein was evident over the course of the experiment (Figure 3.2.4A). Of note, whereas the Bik protein expressed in DG75 (BL type I) appeared as 27/26-kDa double bands during Western blotting, the low levels of Bik detected in the AG876 cell line (BL type III) were detected as a single band at 26kDa (Figure 3.2.4A).

QPCR was subsequently used to examine the expression of the *bik* gene during the same experiment. As shown in Figure 3.2.4B, fluctuations in the quantity of *bik* transcript were evident. The level of *bik* mRNA increased approximately 2-fold after 96 hours of continuous culture without passaging, and *bik* mRNA levels approached the level of *bik* transcript seen in DG75 cells. The same observation was made in the cells that were passaged every 24 hours. In cells which were passaged every 48 hours however, a small transient increase was observed after 24 hours in culture, which dropped thereafter returning to a similar level that was observed at 0 hours.

Considering the level of protein evident in DG75 on the Western blot, a higher level of mRNA expression would have been expected.

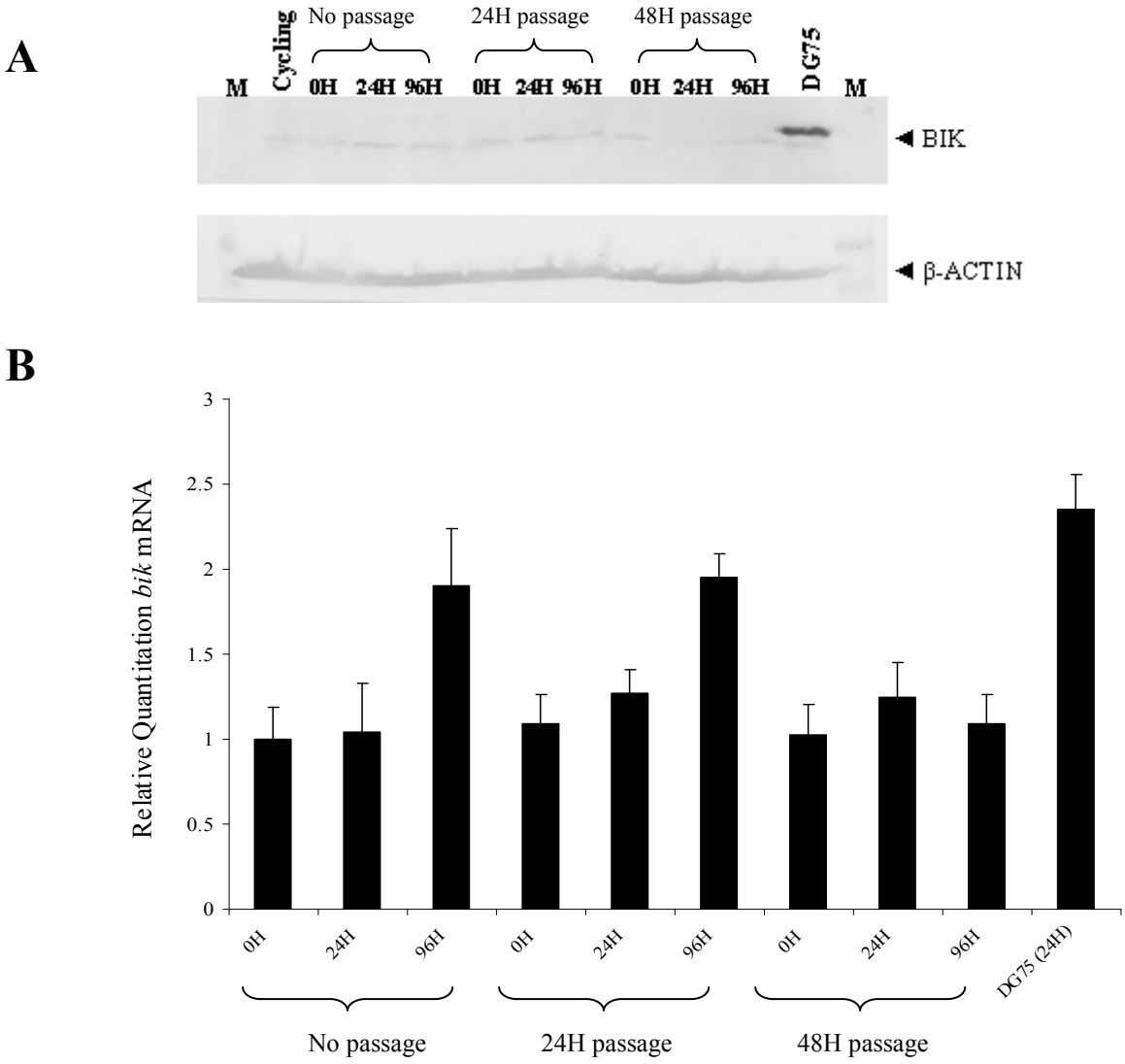


Figure 3.2.4: Effect of passaging on Bik expression in the AG876 cell line.

(A) AG876 cells were monitored for Bik expression during passaging. Total protein was prepared at various time points and used in Western blot analysis. The level of Bik protein was detected using a monoclonal IgG (BD Biosciences 557040). DG75 cells were used as a positive control. (B) RT-Real Time PCR analysis for the level of *bik* expression in (A) above was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labelled TaqMan probes for *bik* and *gapdh.* Flourescent signals were detected using an ABI Prism 7500 Sequence detection system. After normalisation for *gapdh,* relative quantitation of *bik* mRNA was plotted as a function of the control (0h, no passage; arbitrarily assigned a value of 1). (Data are Mean±SD).

3.3 Discussion

The results presented here identify *bik* as a possible target for down-regulation in EBV-infected B-cells. Elevated levels of *bik* mRNA and protein in two EBV-positive type III cell lines (DAUDI and BL41-P3HR1), which harbour different deletions spanning the viral EBNA2 coding sequence indicated a possible role for this viral protein and also identify EBNA2 as a possible candidate protein with the ability to mediate this effect. Since the apoptotic threshold of a cell is determined by the balance of protein expression partners, both pro- and anti-apoptotic, the ability of EBV to down-regulate the expression of a pro-apoptotic protein may raise the apoptotic threshold of the infected, EBNA2-expressing cell.

The detection of Bik in BL type I cell lines, was not consistent with a pro-apoptotic role for this protein in this cell context. Considering Bik behaves as a pro-apoptotic component of the apoptosis machinery, this apparent resistance of Lat I cells to Bik expression is not clear. The detection of some somatic mutation that would impair its apoptotic function in BL cells may explain this inconsistency. However, no mutations leading to changes in the primary amino acid sequence were identified, (in 2 EBV positive and 2 EBV negative BLs) implying that the selection of loss-of-function mutations is unlikely to be a feature of BL cells. Inactivating mutations of the *bik* gene have previously been reported in human peripheral B-cell lymphomas (Arena *et al*, 2003). Interestingly, those mutations were only found in peripheral B–cell lymphomas with germinal centre/post-germinal centre origin in which a severe impairment in the control of apoptotic death plays an essential role in B-cell transformation. Another report rules out *bik* mutations in colorectal carcinomas (Abdel-Rahman *et al*, 1999), and yet another in Non-Hodgkins Lymphomas, gastric cancers, colon cancers and urinary bladder cancers (Lee *et al*, 2006). It is of interest that mutational inactivation of the gene is not observed in BL cells in which mutational events in other genes are common (Lindstrom and Wiman, 2002). Since Bik-induced apoptosis has been shown to be dependent on Bax, it was therefore confirmed that Bax was expressed in a range of cell lines including BL Type I (except DG75), BL type III and LCLs, and this is consistent with the findings of others (Brimmell *et al*, 1998). Of note, Bax in the DAUDI cell line is non-functional, expressing a mutant *bax* allele that contains a missense mutation in the BH1 domain,

which leads to abnormal dimerisation characteristics and loss of pro-apoptotic activity (Meijerink *et al*, 1998).

Interestingly, BL type I cells do not express high levels of Bcl-2 (Finke *et al*, 1992) which is known to inhibit Bik-induced apoptosis (Letai *et al*, 2002). However, several BL type I cells have been shown to express high levels of Bcl-$_{XL}$ and Mcl-1 (Wang *et al*, 1996; D'Souza *et al*, 2000), both of which are known to interfere with Bik-induced apoptosis (Shimazu *et al*, 2007). In particular, several lines of evidence suggest that the balance between pro-apoptotic Bik and the anti-apoptotic Bcl-$_{XL}$ protein may play a key role in determining B-cell fate (Saltzman *et al*, 1998; Jiang and Clark, 2001). The relative proportion of Bik not associated with Bcl-$_{XL}$ may therefore be an important factor in the ability of these cells to tolerate the expression of this pro-apoptotic molecule.

In BL type I cells (which express only EBNA1), Bik appears as a doublet compared to LCLs and BL type III cells, (which express the full compliment of EBV latent genes). Since the regulation of Bik by phosphorylation has been verified elsewhere (Verma *et al*, 2001), these observations may be suggestive that constitutive phosphorylated Bik is the dominant form in BL type I, but not in the BL type III or LCL cell lines. Dephosphorylation of Bik may provide an "off" switch for Bik function. Indeed, BH3 proteins are known to be regulated by post-translational modifications, resulting in conformational changes to cause their release from an inactive complex and increased affinity for anti-apoptotic *bcl-2* homologues (Huang and Strasser, 2000). In cells stimulated by growth factors, Bad is phosphorylated at several serine residues and this allows its sequestration in the cytoplasm by binding to the 14.3.3 chaperon protein (Zha *et al*, 1996). Phosphorylation of two residues, serine 112 and 136, depends on several kinases, including AKT, RSK, PKA or Raf-1 (Datta *et al*, 1997; Bonni *et al*, 1999; Bertolotto *et al*, 2000). Studies have also established that Bim is regulated by phosphorylation (Shinjyo *et al*, 2001; Biswas and Greene, 2002) and incubation of Ramos cells with either anti-IgM or the phorbol ester PMA, two conditions that efficiently activate the Erk1/2 pathway, led to Bim-EL mobility shift, indicative of phosphorylation and correlated with induction of apoptosis (Luciano *et al*, 2003). Importantly as discussed in Chapter 1 phosphorylation of Bik is required for eliciting

efficient apoptotic activity and does not significantly affect its ability to heterodimerise with Bcl-2 (Verma *et al*, 2001).

The levels of *bik* mRNA were found to be relatively stable when AG876 cells were passaged every 48 hours. The fluctuation in *bik* mRNA levels in cells which were left unpassaged and in cells which were passaged every 24 hours, may suggest that the available amounts or activities of cell factors required for synthesis varied during cell growth, possibly due to the effects of passaging/the addition of fresh serum to the cells. The differences in *bik* mRNA levels between Type III cell lines and LCLs may indicate a dissimilarity of regulatory mechanisms involved in the expression of the *bik* gene between the different cell types. A potential role for EBNA2 in the regulation of the *bik* gene is to be considered (Section 3.2.1). Interestingly, EBNA2 has cell-context dependent effects on its target gene *c-myc*. In this regard EBNA2 negatively regulates *c-myc* causing a down-regulation in its expression in those BL-derived cell lines carrying the t(8;14) chromosomal translocation (Maier *et al*, 2005) while in lymphoblastoid B-cell lines that do not carry this translocation, *c-myc* expression is transiently up-regulated in response to EBNA2 (Maier *et al*, 2005).

Thus, repression of *bik* at the level of transcription, with an associated loss of Bik protein appears to be a feature of LCLs relative to BL-derived cell lines. This indication that the *bik* gene may be regulated by EBV at the level of transcription is in line with the findings of others, since cytokine mediated changes in the level of expression of a number of the BH3 only proteins is regulated at a transcriptional level (as reviewed in Puthalakath and Strasser, 2002).

CHAPTER 4：

Regulation of *bik* by EBNA2 in BL-derived cell lines

4.1 Introduction

Nine latent viral proteins act together to initiate and maintain B-cell proliferation upon EBV infection *in vitro* (Young and Rickinson, 2004). Of these LMP1, EBNA2 and EBNA3C have long be been realised to make the most significant contribution to the promotion of cell survival in EBV-infected cells (Wang *et al*, 1990). In BL-derived cell lines, LMP1 is known to activate many of the phenotypic changes that are seen in the EBV infected B-cell, including the up-regulation of B-cell activation markers and adhesion molecules and an increased resistance to apoptosis (Wang *et al*, 1988; Cordier *et al*, 1990; Wang *et al*, 1990; Hatzivassiliou and Mosialos, 2002). EBNA2, is the first protein expressed after infection of primary B-cells (Alfieri *et al*, 1991) and all the viral genes expressed in immortalised B-cells (including LMP1) are under the control of this viral transcription factor (Zimber-Strobl and Strobl, 2001). In addition EBNA2 modulates the expression of many cellular genes, either directly or indirectly via c-Myc modulation (Kaiser *et al*, 1999; Zimber-Strobl and Strobl, 2001). In this way, EBNA2 instigates the transcription of a plethora of target genes, which regulate the activation of the resting B-cell, cell cycle entry, and proliferation of growth-transformed cells (Cohen *et al*, 1989; Sinclair *et al*, 1994; Kempkes *et al*, 1995a; Kaiser *et al*, 1999; Zimber-Strobl and Strobl, 2001).

It has previously been demonstrated in our laboratory that both EBNA2 and LMP1 can independently up-regulate the anti-apoptotic cellular *bfl-1* gene (D'Souza *et al*, 2000; Pegman *et al*, 2006). Understanding the importance of EBNA2, in particular, as a key effector of phenotypic change in EBV-infected cells, and considering the finding that the BL cell lines DAUDI and BL41-P3HR1 express high levels of Bik, (in contrast to the other BL latency III cells), while also harbouring deletions spanning the EBNA2 coding sequence, it was decided to examine EBNA2 as a significant participant in the down-regulation of the *bik* gene during EBV infection. Accordingly, this study set out to investigate if EBNA2 was capable of regulating the expression of *bik*. Stable expression of EBNA2 in EBV-negative BL cell lines leads to cytostatis (Floettmann *et al*, 1996). Here, several established cell lines were used in which EBNA2 expression could be induced as the sole EBV protein, the first of these being the well established tetracycline-regulated cell line DG75-tTA-EBNA2.

4.2 Bik is down-regulated by EBNA2 in cell lines derived from BL tumours.

4.2.1 Induction of EBNA2 leads to decrease in Bik protein and mRNA levels in the EBV-negative cell line DG75-tTA-EBNA2.

Initially, the DG75tTA-EBNA2 cell line was employed to obtain inducible expression of EBNA2 as the exclusive EBV protein under investigation. This cell line is a stably-transfected derivative of the EBV-negative BL cell line DG75, in which the expression of EBNA2 is tightly repressed by low, non-toxic concentrations of tetracycline (≤1μg/ml) in the growth medium (Floettmann *et al*, 1996). In this cell line, DG75 was stably transfected with two plasmids, pJEF-3, a plasmid containing a tetracycline regulated transactivator (tTA) that is constitutively expressed from a CMV promoter, and pJEF-31, the responsive plasmid, which expresses wild type EBNA2. Stable integration of these plasmids was then selected for during cell culture using hygromycin, geneticin and tetracycline. The inducible promoter that drives EBNA2 expression contains several binding sites for the tetracycline-regulated trans-activator (tTA). Tetracycline binds to the tTA preventing binding to the promoter sequences which remains inactive. Upon removal of tetracycline the tTA binds to the promoter activating the transcription of EBNA2 (Floettmann *et al*, 1996).

EBNA2 expression was induced over a 96 hour time period in the DG75-tTA-EBNA2 cell line, by the removal of tetracycline from the cell culture medium. Total protein and RNA were prepared simultaneously from cells harvested at various time-points after EBNA2 induction, (Figure 4.2.1) and used in Western blot analysis and QPCR respectively. Western blotting was used to demonstrate the induction of EBNA2 over the course of the experiment and to measure Bik and c-Myc protein levels in response to EBNA2 (Figure 4.2.1A). Levels of the endogenous control, β-Actin, were also examined and confirmed equal loading in every lane (Figure 4.2.1A). Induction of EBNA2 was monitored using the anti-EBNA2 murine monoclonal antibody, PE2. c-Myc protein levels were investigated using an anti-c-Myc rabbit polyclonal antibody raised against an N-terminal peptide. In this experiment, EBNA2 was detected in induced DG75-tTA-EBNA2 cells at 24 hours after the removal of tetracycline and

remained detectable 96 hours after induction (Figure 4.2.1A). A significant decrease in the level of Bik protein can be seen to coincide with the induction of EBNA2 expression over the time course. Bik levels are repressed in conjunction with EBNA2 expression at 48 hours, and are completely abolished at the 72 and 96 hour time points (Figure 4.2.1A). Thus, Bik is down-regulated in response to EBNA2 induction in this cell context. c-Myc repression correlated with EBNA2 induction and was significantly down-regulated by 72 hours (Figure 4.2.1A), as has been described previously for this cell line (Maier *et al*, 2005). This is to be expected as EBNA2 represses transcription regulation from the Igμ enhancer which drives expression of the translocated *myc* gene in BL cells (Jochner *et al*, 1996).

RT reactions were carried out to generate cDNA, which was subsequently used in real-time PCR assays to measure *bik* mRNA levels in response to EBNA2 activation over the same 96-hour time period. mRNA levels of the endogenous control gene *gapdh* were also monitored, and the resulting data obtained was used to normalise data acquired for *bik*. Differences in *bik* mRNA levels over time in the DG75-tTA-EBNA2 cell line in response to EBNA2 induction can be seen in Figure 4.2.1B. As was the case with the Bik protein, *bik* mRNA levels in DG75 decreased in response to EBNA2 activation. A significant decline in *bik* levels (approximately 2.6-fold) was observed by 48 hours following the removal of tetracycline from the culture medium. EBNA2 was expressed at high levels at this time point (Figure 4.2.1A). Levels of *bik* mRNA remained stably repressed from this point on, with the level of *bik* transcript at 96 hours similar to that observed at 48 hours. These results indicate that expression of EBNA2 as the sole EBV protein led to a sustained decrease in the level of *bik* mRNA and protein in the EBV-negative BL cell line DG75-tTA-EBNA2.

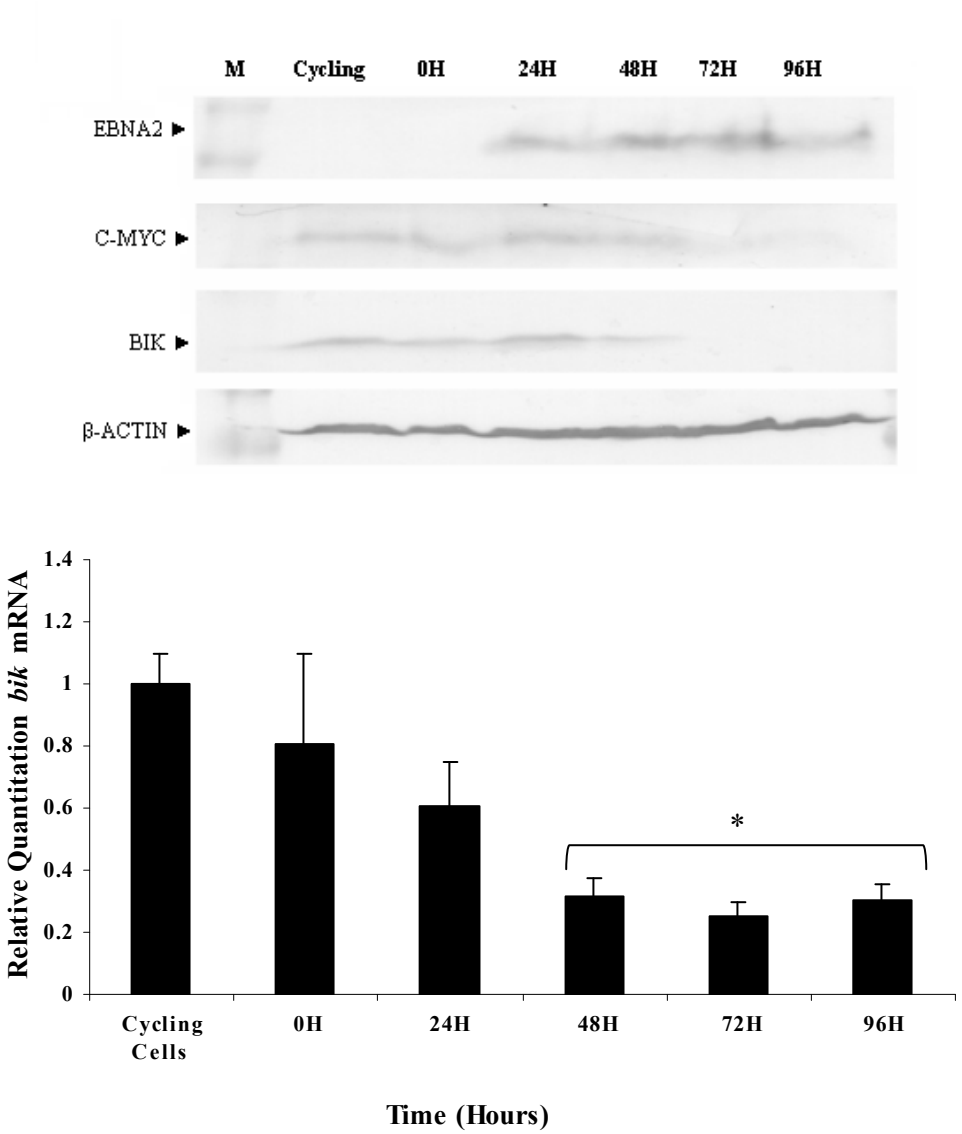


Figure 4.2.1: Induction of EBNA2 leads to decrease in *bik* protein and mRNA levels in the EBV-negative cell line DG75-tTA-EBNA2.

EBNA2 was induced in DG75-tTA-EBNA2 by removal of tetracycline from the growth media and both RNA and protein extracts were prepared at the indicated time points (hours) following induction. (A) Western Blot analysis of Bik protein expression in response to induction of EBNA2 was examined. The level of Bik protein was detected using an affinity purifed goat polyclonal IgG (Santa Cruz, sc-1710). Analysis of EBNA2 induction in DG75-tTA-EBNA2 cells is illustrated in the top panel. The second and third panels show c-Myc and Bik protein expression in response to the induction of EBNA2 respectively. The lower panel shows the same blot probed for β-Actin. (B) RT-Real Time PCR analysis for the level of *bik* expression was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. After

normalisation for *gapdh*, relative quantitation level of *bik* mRNA was plotted as a function of the control (Cycling cells; arbitrarily assigned a value of 1) (Data are Mean±SD,* $p < 0.05$).

4.2.2 Bik down-regulation by EBNA2 is a general feature of BL-derived cell lines.

Experiments with a similar objective were performed using established transfectants of two further well-studied EBV-negative B-cell lymphoma cell lines, BL41 and BJAB. These transfectants also used a conditional system, in which the function of EBNA2 was dependent on the presence of estrogen.

The fusion of a protein with the hormone-binding domain of a steroid receptor can cause the function of that particular protein to become dependent on the presence of that specific hormone and thus to become inducible (Picard *et al*, 1988; Eilers *et al*, 1989). In accordance with this, an EBNA2-estrogen receptor fusion construct p554-4 (ER/EBNA2) has been generated elsewhere by fusing the hormone-binding domain of the estrogen receptor to the N terminus of EBNA2 and subsequently cloning this chimeric gene into a mini-EBV vector (Kempkes *et al*, 1996). The mini-EBV vector carries a viral episomal and lytic origin of replication together with terminal repeats, encompassing a packaging signal for encapsidation of the DNA into virions (Kempkes *et al*, 1996). EBNA2 functionality and its ability to interact with CBF1, was thus made dependent on the presence of estrogen. Kempkes *et al*., (1996) subsequently transfected the EBV-negative B-cell lymphoma cell lines BL41 and BJAB (Burkitt-like lymphoma cell line) with the conditional ER/EBNA2 expression plasmid. Stably-transfected clonal derivatives were then selected during cell culture using geneticin, giving rise to the cell lines BL41-ER/EBNA2-K3 (BL41K3) and BJAB-ER/EBNA2-K3 (BJABK3) respectively. Addition of estrogen to the cell culture medium activates the function of ER/EBNA2, and affects the electrophoretic mobility of the ER/EBNA2 fusion protein. B-cells transformed by ER/EBNA2 express EBNA2 fusion proteins at an apparent molecular weight of 120kDa in contrast to the 86kDa protein of wild type EBNA2 (Kempkes *et al*, 1995a). This activation of ER/EBNA2 function is thought to be associated with post-translational modifications or profound changes in the tertiary structure of ER/EBNA2 (Kempkes

et al, 1995a). In addition, ER/EBNA2 enhances its own transcription from the *BamH IC* promoter (Kempkes *et al*, 1996), leading to an increase in the level of expression of ER/EBNA2 e.g. compare lanes "cycling" and "6H" in Figure 4.2.2A. EBNA2-estrogen receptor fusion proteins are capable of substituting for wild type EBNA2 in primary B-cell transformation and in the *trans*-activation of viral and cellular genes (Kempkes *et al*, 1995b) and thus because of the specific way in which EBNA2 activity could be regulated, these cell lines were a useful model to study the regulation of *bik* by EBNA2 in a BL cell background.

In order to monitor Bik levels in the presence of EBNA2 a series of time courses were undertaken with the BL cell lines, cultivated in the presence of estrogen. All cell lines were washed twice in PBS and β-estradiol was added to the culturing media to a final concentration of 1μM to activate EBNA2, in accordance with previously published studies (Kempkes *et al*, 1996) (Section 2.2.2.3).

EBNA2-mediated changes in Bik protein expression and mRNA levels observed during the time courses in both cell lines BL41K3, BJABK3 are shown in Figure 4.2.2. Western blot analysis (Figure 4.2.2 A, C) confirms that the stably-transfected cells grown in the presence of estrogen showed an increase in chimeric EBNA2 expression over the duration of the time-course (6 hours in BL41K3, and at 24 hours in BJABK3). Cells grown in the presence of estrogen expressed chimeric EBNA2 with an apparent molecular weight of 120kDa (in contrast to the molecular weight of wild type EBNA2, which is 86kDa). Activated ER/EBNA2 remained detectable for the duration of the time course in all cases. Bik levels in response to the activation of ER/EBNA2 were monitored as before.

The BL41K3 cell line demonstrated a substantial down-regulation of Bik protein in response to EBNA2 activation (Figure 4.2.2A). Bik protein expression was repressed by 48 hours but was not completely eliminated. The decrease in Bik expression again correlated with an increase in the expression of functional EBNA2 (Figure 4.2.2A). Furthermore, in BJABK3, induction of ER/EBNA2 expression also led to a decrease in Bik protein levels in conjunction with EBNA2 activation and was totally undetectable by 72 hours in response to EBNA2 (Figure 4.2.2C).

These results indicated that activation of functional EBNA2, led to a substantial decrease in the level of Bik protein in both cell lines investigated. Overall these experiments show that Bik levels decrease in response to EBNA2, and are significantly diminished at 48-hours in all cases. Importantly, c-Myc down-regulation correlated with EBNA2 induction and was significantly repressed by 72 hours in BL41K3 (Figure 4.2.2A) as was anticipated (Maier *et al*, 2005). In the BJABK3 cell line c-*myc* expression was transiently up-regulated at 24 hours in response to EBNA-2 induction as has been previously reported (Maier *et al*, 2005), declining to pre-induced levels by 48 hours (Figure 4.2.2C).

The total RNA harvested (from the same cell pool, and at the same time points as protein lysates) was used to assay *bik* mRNA levels in response to EBNA2 activation for each time course. The down-regulation of *bik* mRNA in response to EBNA2 activation in the BL41K3 and the BJABK3 cell lines is shown in Figure 4.2.2 B and D. Consistently, *bik* mRNA was repressed in response to EBNA2 activation in both cell types. As was the case for the other cell lines examined, *bik* mRNA levels were seen to diminish over time in response to the levels of EBNA2. A considerable reduction was observed by 9 hours (approximately 4-fold decrease), which was additionally reduced at the time points up to 24 hours (maximum 10-fold decrease). The amount of *bik* mRNA was seen to increase gradually over time after this point, with the level at 48 and 72 hours, similar to that observed at 12 and 6 hours respectively. These results indicated that turning on functional EBNA2 led to a significant transient decrease in the level of *bik* mRNA in the BL41 derived cell line BL41K3.

Results obtained from the time course undertaken with BJABK3 are illustrated in Figure 4.2.2D. As was the case previously, steady state levels of *bik* mRNA in the BJAB derivative were repressed in response to EBNA2 activation. A reduction in *bik* levels (approximately 1.6-fold) was observed by 24 hours following the addition of estrogen to the culture medium. The amount of *bik* decreased gradually over time, with the level of *bik* mRNA at 72 hours approximately 2.4-fold less than the zero hour time point. Thus, a repression in *bik* mRNA levels coincides with EBNA2 induction in this context.

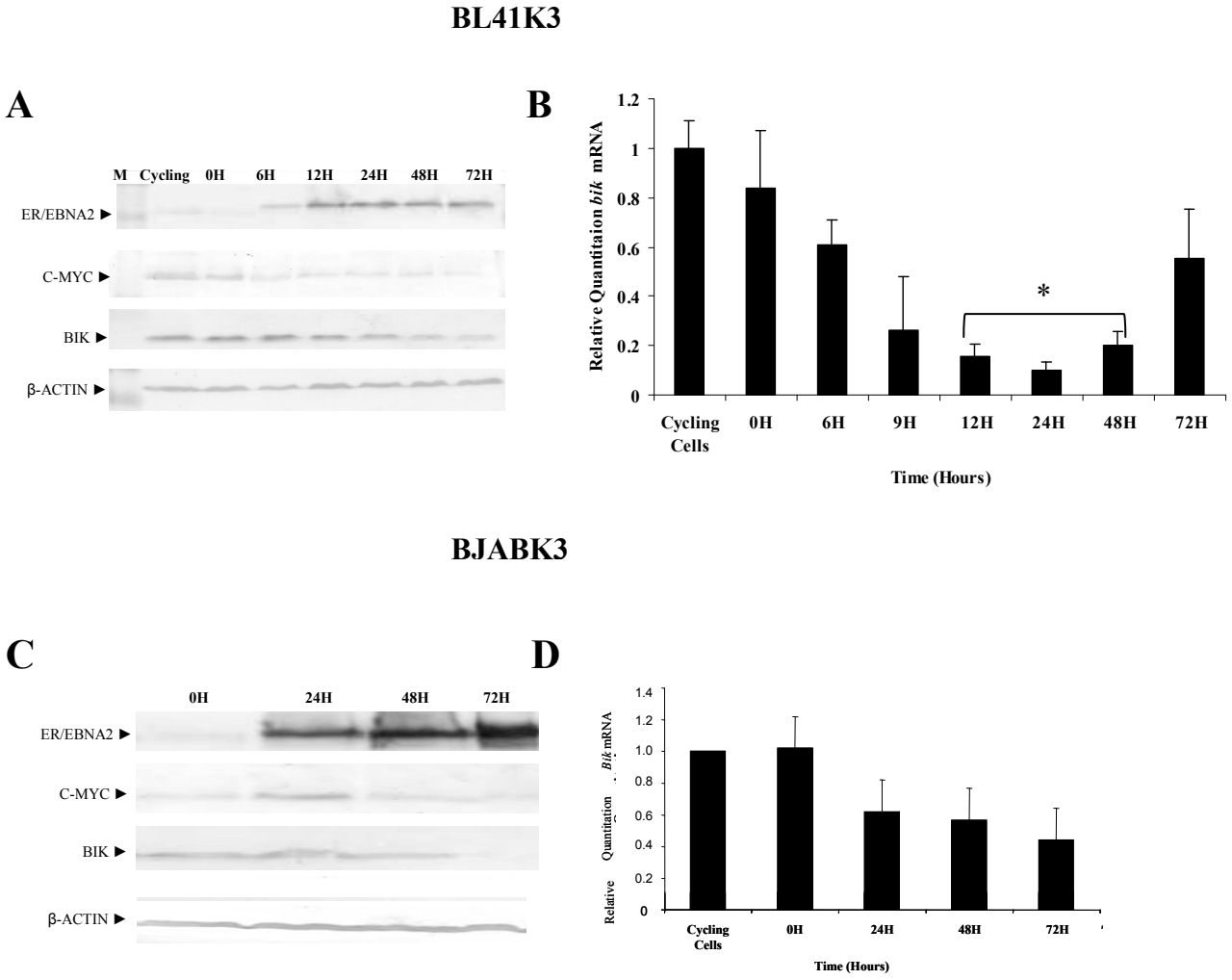


Figure 4.2.2: Down-regulation of Bik expression by EBNA2 in EBV-negative cell lines.

The EBV-negative cell lines BL41K3 and BJABK3 were induced for EBNA2 by re-culturing in the presence of estrogen. Cells were harvested and both RNA and protein extracts were prepared at the indicated time points after activation (hours). The level of Bik protein was detected using an affinity purified goat polyclonal IgG (Santa Cruz, sc-1710). Western Blot analysis of Bik protein expression in response to activation of EBNA2 was monitored. (A) EBNA2 activation in the EBV-negative cell line BL41K3. (C) Estrogen-Regulated activation of EBNA2 expression in the EBV-negative cell line BJABK3. Induction of EBNA2 protein is illustrated in the upper panel. Analysis of c-Myc and Bik in response to EBNA2 activation is shown in the second and third panels respectively. The lower panel in each figure shows each blot probed for β-Actin. RT-Real Time PCR analysis for the level of *bik* expression was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. After normalisation for *gapdh*,

relative quantitation level of *bik* was plotted as a function of the control (Cycling cells; arbitrarily assigned a value of 1). QPCR analysis both cell lines; BL41K3 and BJABK3 can be seen in (B), (D) respectively (Data are Mean±SD,* $p < 0.05$).

Another similar cell line was also used in this study, namely BL41P3HR1-ER/EBNA2-9A (BL419A). This cell line was generated by infection of the BL41 cell line with the EBV-P3HR1 virus genome which is a non-transforming EBNA2-deficient strain of EBV (Kempkes *et al*, 1996), and subsequent transfection and selection as for each of the previous cell lines (Section 4.2.2).

Since BJABK3 and BL41K3 were established by infecting B-cells with a plasmid that just expresses ER/EBNA2, these cell lines express EBNA2 as the sole EBV protein. BL419A, in contrast, was co-infected with the mini-EBV plasmid together with the EBV P3HR1 virus. This is a non-immortalising viral strain that is deleted for its own EBNA2 gene. The activated ER/EBNA2 on the mini-EBV plasmid *trans*-activates the full EBV compliment on the P3HR1 viral genome in the cell, thus complementing the EBNA2 deletion on the P3HR1 virus, and so through activation of EBNA2 in BL419A, the entire EBV profile of EBV latent genes should be expressed. This cell line thus allows the examination of ER/EBNA2 expression against the background of the complete EBV genome. However, although EBNA2 expression generally results in the transcriptional up-regulation of LMP1, this is not the case for the BL419A cell line. The molecular basis for this has yet to be investigated.

Changes to Bik protein and mRNA levels observed upon activation of ER/EBNA2 in BL419A are shown in Figure 4.2.3. Western blot analysis (Figure 4.2.3A) confirms that cells grown in the presence of estrogen showed an increase in chimeric EBNA2 expression by 12 hours and remained detectable for the duration of the time course. A considerable and sustained reduction in Bik protein levels was observed 48 hours after the addition of estrogen to the BL419A cells (Figure 4.2.3A), again indicating that activating EBNA2 expression down-regulates the level of Bik protein in BL cells. Akin to what was seen in BL41K3, Bik protein expression was not completely abolished (Figure 4.2.3A).

EBNA2-mediated changes in *bik* mRNA levels over time in BL419A are illustrated in Figure 4.2.3B. A decrease in the level of *bik* transcript (approximately 1.8-fold) was

observed by 6 hours following the addition of estrogen to the culture medium. Further, significant reductions in *bik* mRNA were seen in the subsequent time points up to 72 hours, where a maximal 6-fold repression was recorded, and the down-regulation of *bik* is greatest. There was no further decrease in *bik* mRNA by 96 hours, and interestingly, by 144 hours the levels of *bik* mRNA increased approximating the level of expression observed at the 6 hour time point.

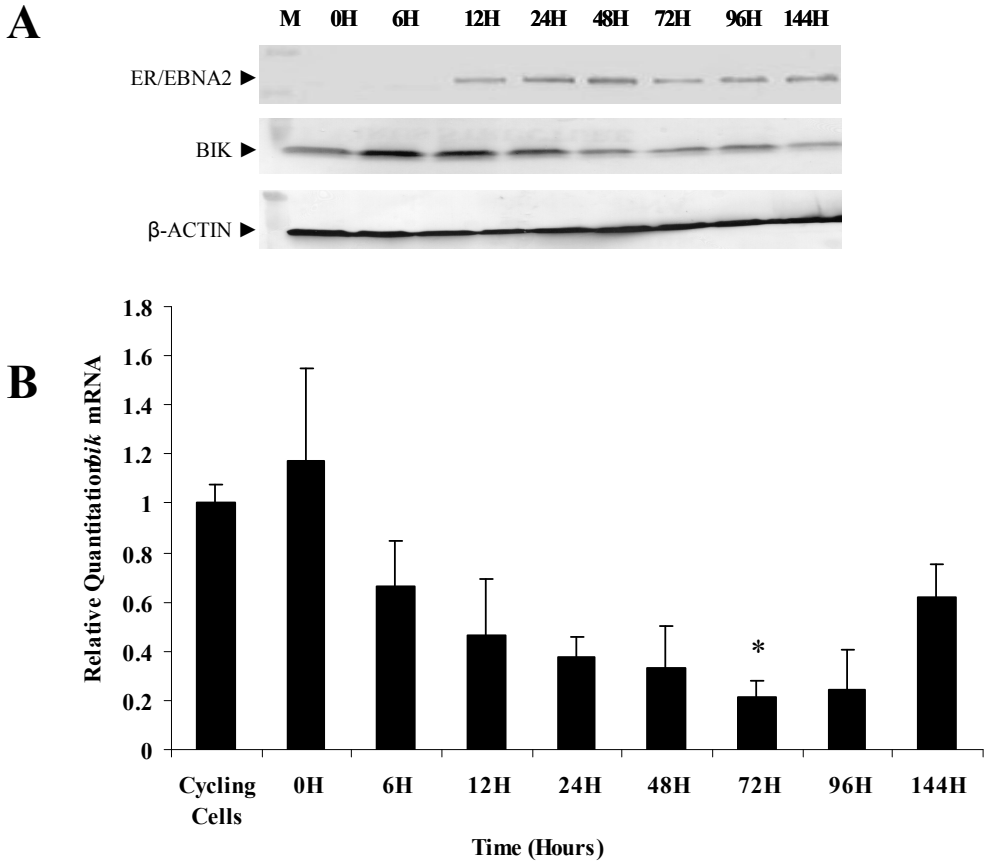


Figure 4.2.3: Down-regulation of Bik expression by EBNA2 in the EBV-positive cell line BL419A.

The EBV-positive cell line BL419A was induced for EBNA2 by re-culturing in the presence of estrogen. Cells were harvested and both RNA and protein extracts were prepared at the indicated time points after induction (hours). The level of Bik protein was detected using an affinity purifed goat polyclonal IgG (Santa Cruz, sc-1710). (A) Western Blot analysis of Bik protein expression in response to induction of EBNA2 was monitored. The upper panel illustrates EBNA2 induction. The middle panel shows Bik protein expression in response to EBNA2 expression and the lower panel shows the same blot probed for β-Actin (B) RT-Real Time PCR analysis for the level of *bik* expression was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of

fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. After normalisation for *gapdh*, relative quantitation level of *bik* was plotted as a function of the control (Cycling cells; arbitrarily assigned a value of 1) (Data are Mean±SD, * $p < 0.05$).

Interestingly, expression of c-Myc correlates with Bik expression in the BL type I cells examined, and a reduction in c-Myc expresssion mediated by the activation of EBNA2 corresponded to a decrease in the level of Bik protein in all of the cell lines tested (BL41K3, BJABK3 and DG75-tTA-EBNA2).

Overall, a direct role for EBNA2 in *bik* repression has thus been investigated using four established BL-derived cell lines DG75-tTA-EBNA2, BL41K3, BJABK3 and BL419A and in all cases, induction of EBNA2 led to the repression of both *bik* mRNA (10, 6 and 2.4-fold reduction respectively) and protein (Figure 4.2.1, Figure 4.2.2 and Figure 4.2.3). These results showed that *bik* is transcriptionally down-regulated by EBNA2 in cell lines derived from BL tumours. Together this data suggests that EBNA2 contributes to the down-regulation of *bik* in BL cells.

4.2.3 Down-regulation of Bik expression by EBNA2 in the EBV-negative cell line BL41K3: BD Biosciences antibody.

A second Bik antibody was acquired from BD Biosciences (557040; Table 2.1-2) at a later stage in this study. Extracts prepared during the time course using the BL41K3 cell line (detailed in Section 4.2.2) were used in Western blot analysis to monitor EBNA2-mediated changes in Bik protein expression (Figure 4.2.4). Western blot analysis confirmed that a significant decrease in Bik protein level was observed in response to ER/EBNA2 activation (Figure 4.2.4), thus validating this antibody for future use. The levels of the endogenous control β-Actin were also monitored and confirmed equal loading in each case (Figure 4.2.4).

It was observed in Chapter 3 that Bik protein expressed in BL Type I cells in the absence of EBNA2 expression always appeared as 27/26-kDa double bands during Western blot analysis. Interestingly, the EBNA2-mediated Bik repression in BL41K3

seen here led to the gradual disappearance of the 27kDa band. These observations may suggest that the larger band is preferentially down-regulated by EBNA2.

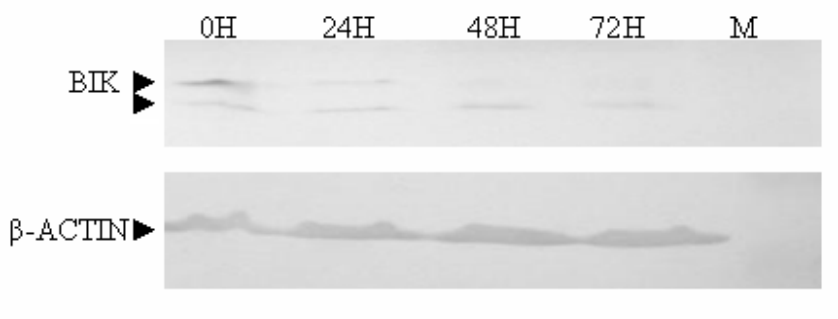


Figure 4.2.4: Down-regulation of Bik expression by EBNA2 in the EBV-negative cell line BL41K3: BD Biosciences antibody.

The EBV-negative cell line BL41K3 was induced for EBNA2 by re-culturing in the presence of estrogen as before. Cells were harvested and protein extracts were prepared at the indicated time points after induction (hours). Western Blot analysis of Bik protein expression in response to induction of EBNA2 was monitored. The upper panel illustrates Bik protein expression in response to EBNA2 expression and the lower panel shows the same blot probed for β-Actin.

4.2.4 EBNA2 mediated repression of *bik* in BL Cell Lines does not require CBF1.

Since EBNA-2 lacks an intrinsic DNA binding function (discussed in Chapter 1), it gains access to target genes through various cellular adaptor proteins including CBF1, Spi-1/PU.1 and Spi-B-related proteins, or ATF-CRE (as reviewed in Zimber-Strobl and Strobl, 2001). The best-studied cellular DNA adapter protein of EBNA-2 to date is the CBF1 protein. It has also recently shown in our laboratory that the anti-apoptotic *bfl-1* gene is a CBF1-dependent transcriptional target of EBNA2 (Pegman *et al*, 2006).

Hence, in order to determine if the *bik* gene is a CBF1-dependent target of EBNA2, an investigation as to whether EBNA2 could suppress *bik* in DG75 cells in which the CBF1 gene had been inactivated was undertaken. Accordingly, *bik* mRNA levels were examined by QPCR in the DG75 clones SM296D3 and SM295D6. These cell

lines are stably-transfected derivatives of the EBV-negative BL cell line DG75, in which the expression of ER/EBNA2 is regulated by the addition of estrogen to the growth medium, and in which the human CBF1 gene has (SM296D3)/ or has not (SM295D6) been inactivated by the deletion of exon 4 (Maier *et al*, 2005).

Total mRNA extracts were received as a gift from Dr. Bettina Kempkes (GSF-National Research Centre for Environment and Health, Munich, Germany) and the corresponding protein extracts were unavailable. EBNA2-mediated changes in *bik* mRNA levels were monitored by QPCR. Activation of ER/EBNA2 in the SM295D6 cells led to a sustained decrease in the level of *bik* mRNA (greatest repression 2.4-fold after 6 hours) (Figure 4.2.5), and this effect was also emulated in the cell line containing the knockout of the CBF1 gene (SM296D3) (1.4-fold repression after 6 hours, greatest repression 1.9-fold after 48 hours) although not to the same extent. The down-regulation of *bik* mRNA levels in SM295D6 and SM296D3 was comparable to the effect seen in DG75-tTA-EBNA2 and BJABK3, where suppressed *bik* mRNA levels were sustained and did not fall below a 3.2 -fold reduction (Figure 4.2.1A and Figure 4.2.2C).

In summary*, bik* expression was down-regulated in both SM295D6 and in SM296D3 cells, indicating that EBNA2-mediated *bik* repression is not CBF1 dependent. While it is clear that neither the Notch pathway associated protein, CBF1, nor the EBNA2-CBF1 interaction are absolutely required for EBNA2-mediated repression of *bik,* some role for the CBF1 gene in this effect cannot be absolutely excluded at this point. These experiments further corroborate the finding that EBNA2 down-regulates *bik* expression.

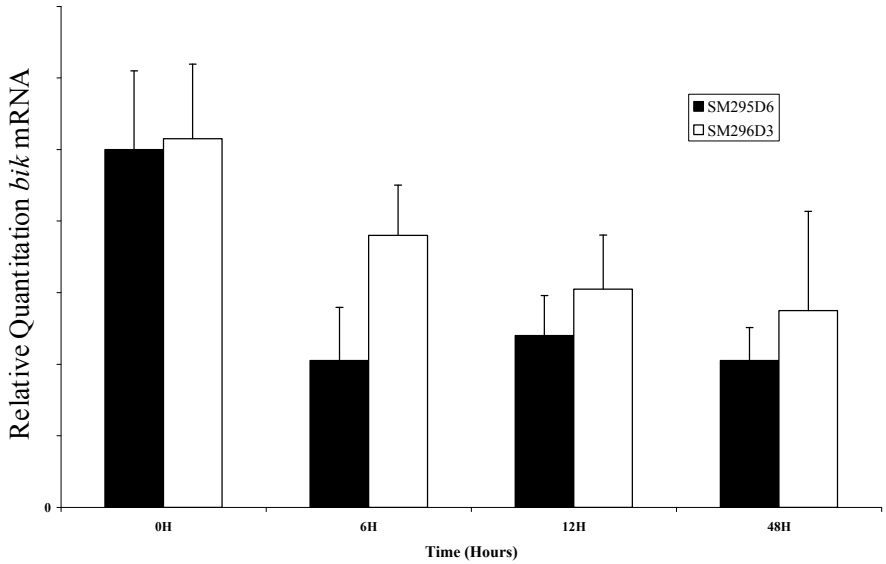


Figure 4.2.5: EBNA2 mediated repression of *bik* in BL Cell Lines is not CBF1-dependent.

QPCR analysis was performed to determine the level of *bik* mRNA. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Detection System as before. After normalisation for *gapdh*, the level of *bik* transcript was plotted as a function of the control (0H SM295D6; arbitrarily assigned a value of 1) (Data are Mean±SD).

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4.2.5 Induction of LMP1 in the EBV-negative cell line DG75-tTA-LMP1 does not affect Bik expression.

As discussed in Chapter 1, LMP1 is also required for immortalisation of resting B lymphocytes (Kaye *et al*, 1993), and studies in rodent fibroblast cell lines have demonstrated that LMP1 is oncogenic (Wang *et al*, 1985). LMP1 has been shown to up-regulate the expression of anti-apoptotic proteins including Mcl-1 and A20 (Laherty *et al*, 1992; Wang *et al*, 1996). Indeed, in our laboratory, using a system in which the expression of LMP1 was inducibly regulated by tetracycline (Floettmann *et al*, 1996), it was shown that LMP1 expression alone can up-regulate transcription from the *bfl-1* gene in a B-cell specific manner. Further studies using the *bfl-1* promoter also revealed that LMP1 *trans*-activates the *bfl-1* promoter and that this

trans-activational effect may be mediated by the NF-κB signaling pathway (D'Souza *et al*, 2000). Considering that both EBNA2 and LMP1 can independently up-regulate the anti-apoptotic cellular *bfl-1* gene it was decided to determine if LMP1 could also down-regulate *bik*. The well established tetracycline-regulated cell line DG75tTA-LMP1 was used for this purpose. This cell line was employed to obtain inducible expression of LMP1 as the exclusive EBV protein. As was the case for DG75-tTA-EBNA2, the DG75-tTA-LMP1 cell line is a transfected clonal derivative of the EBV-negative BL cell line DG75 in which the expression of LMP1 is tightly repressed by low concentrations of tetracycline (≥1μg/ml). The inducible promoter driving LMP1 expression contains several binding sites for the tetracycline-regulated *trans*-activator (tTA) and is continuously expressed. Elimination of tetracycline from the cell culture medium thus leads to binding of the *trans*-activator to the promoter and the induction of LMP1 expression (Floettmann *et al*, 1996).

DG75-tTA-LMP1 cells were induced to express LMP1 by the removal of tetracycline from cell culture medium. Cells were washed in PBS three times, and resuspended in tetracycline free media. Total protein and RNA were prepared simultaneously from cells harvested at the indicated time-points after LMP1 induction, (Figure 4.2.6) and used in Western blot analysis and QPCR respectively. Western blot analysis on samples isolated during the induction time course is shown in Figure 4.2.6A. LMP1 expression and Bik protein levels in response to LMP1 induction were analysed by Western blot analysis as before (Figure 4.2.6A). β-Actin levels were also monitored (Figure 4.2.6A). DG75-tTA-LMP1 cells grown in the absence of tetracycline expressed LMP1 at a molecular weight of 62kDa as expected, with some additional glycosylation/degradation evident at the later time points. LMP1 was detected in induced DG75tTA-LMP1 cells at 6 hours and large amounts remained detectable up to 72 hours (Figure 4.2.6A). An increase in LMP1 expression was observed over time. No significant alteration in Bik protein levels can be seen to correspond to the induction of LMP1 expression over the time course (Figure 4.2.6A). Thus, it appears that LMP1 is not directly involved in Bik regulation in this cell context.

Total RNA harvested was used to measure *bik* mRNA levels in response to LMP1 activation over the same 72-hour time period. Differences in *bik* mRNA levels over time in the DG75-tTA-LMP1 cells are shown in Figure 4.2.6B. It is clear that *bik* mRNA was not repressed in response to LMP1 activation, in fact, a transient increase

in *bik* levels (approximately 2-fold) was observed 6 hours following the removal of tetracycline from the culture medium (Figure 4.2.6B). This experiment indicates that expression of LMP1 in the EBV-negative BL cell line DG75 does not appear to be a contributor in the regulation of the level of *bik* mRNA in the absence of other EBV proteins. Of note, it was observed that the Bik protein expressed in the DG75-tTA-LMP1 cell line appeared as 27/26-kDa double bands throughout the course of the experiment. This analysis was carried out using the BD Bik Antibody (Table 2.1-2).

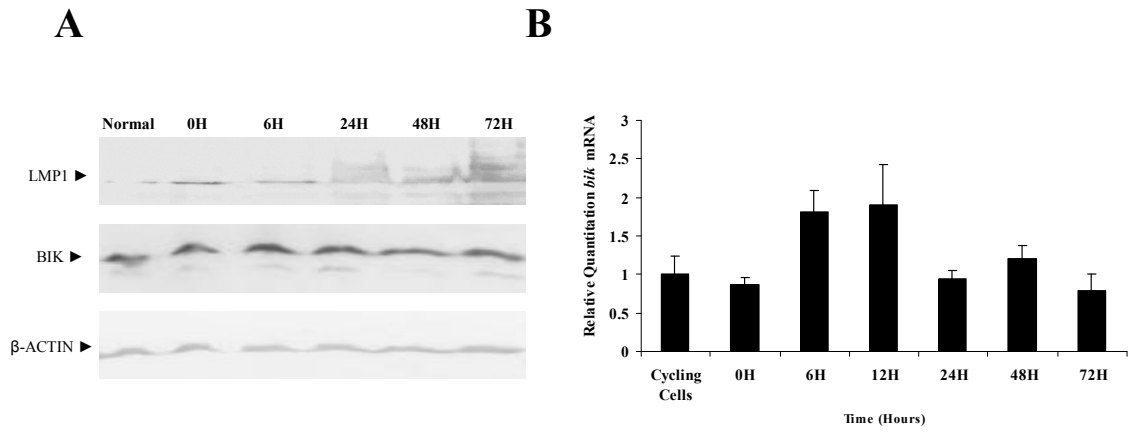


Figure 4.2.6: Induction of LMP1 in the EBV-negative cell line DG75-tTA-LMP1 does not effect Bik expression.

Total protein and RNA were prepared simultaneously at the indicated time-points post- LMP1 induction. The experiment was carried out over 72 hours. The level of Bik protein expression was detected using a mouse anti-Bik antibody (BD Biosciences 557040) and LMP1 protein levels were monitored using the anti-LMP1 murine monoclonal antibody CS1-4. (A) Western blot of DG75-tTa-LMP1 cells induced to express LMP1. LMP1 induction was carried out over 72 hours. The upper panel shows Western blot analysis of LMP1 protein levels in response to induction. Analysis of Bik protein expression in response to induction of LMP1 is illustrated in the middle panel, and the corresponding β-Actin blot is shown in the lower panel. (B) QPCR analysis for the level of *bik* expression was also analysed. RNA was harvested from the same induction as that in panel A. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI 7500 Detection System. After normalisation for *gapdh*, relative quantitation level of *bik* was plotted as a function of the control (Cycling cells; arbitrarily assigned a value of 1). (Data are Mean±SD).

4.3 Discussion

Of all the EBV latent proteins LMP1, EBNA2 and EBNA3C are long established as the most significant contributors to the promotion of cell survival in EBV-infected cells and (Wang *et al*, 1990). Previous studies in the laboratory have shown that the anti-apoptotic, *bfl-1* gene, is a CBF1-dependent transcriptional target of EBNA2 (Pegman *et al*, 2006) and that LMP1 expression alone can up-regulate transcription from the *bfl-1* gene in a B-cell specific manner (D'Souza *et al*, 2000; D'Souza *et al*, 2004).

The experiments in this chapter demonstrate the novel finding that EBNA2 down-regulates expression of the cellular *bik* gene at the protein and the mRNA level. The fact that both native EBNA2 (expressed in the Dg75-tTA-EBNA2 cell line) and chimeric ER/EBNA2 (in the BL41K3 and BJABK3 cell lines) down-regulated the *bik* gene in the absence of other EBV latent proteins provides direct evidence for EBNA2 regulation of the *bik* gene. Additionally, the observation of decreased *bik* mRNA and protein in an EBV positive but LMP1 negative cell line (BL419A), further substantiates the evidence that *bik* can be independently regulated by the expression of EBNA2. These results clearly demonstrate that *bik* is transcriptionally down-regulated by EBNA2 in cell lines derived from BL tumours. To date EBNA2 is known to transcriptionally repress the activity of only one cellular gene, IgM (Jochner *et al*, 1996). This is the first indication of the down-regulation of a pro-apoptotic gene by this viral protein.

Bik is induced in estrogen-dependent MCF7 breast cancer cells in response to inhibition of estrogen signaling (Hur *et al*, 2004). Indeed, in that study the suppression of Bik protein revealed that Bik induction was necessary for the antiestrogen-induced apoptosis of this cell line. Any correlation between the expression of *bik* and the presence of estrogen however can be ruled out after considering the results obtained using the tetracycline-inducible system, DG75-tTA-EBNA2, which confirms that the repression of *bik* is due to the induction of EBNA2, and is not an artefact of estrogen-dependency in this context.

Inactivation of CBF1 in DG75 cells did not abrogate the ability of EBNA2 to down-regulate the expression of the *bik* gene in these cells. *Bik* expression was significantly

down-regulated in SM295D6 and to a similar degree in SM296D3 cells, indicating that EBNA2-associated down-regulation of *bik* is independent of its CBF1-binding ability. In contrast, it has previously been shown in the laboratory that EBNA2-mediated *trans*-activation of the *bfl-1* gene is abolished by the inhibition of EBNA2/CBF1 signaling, proving that this signal transduction pathway is essential in EBV-mediated regulation of this anti-apoptotic gene (Pegman *et al*, 2006). This finding is significant, as CBF1 is the major rate-limiting factor for EBNA2 activation of cellular target genes (Maier *et al*, 2006). The promoters of several EBV latent genes including *EBNA Cp*, *LMP1*, *LMP-2A*, and *LMP-2B* (Ling *et al*, 1993a; Zimber-Strobl *et al*, 1993; Johannsen *et al*, 1995) and that of the cellular genes *CD21*, *CD23*, *CCR7* and *FcRH5* (Ling *et al*, 1994; Maier *et al*, 2005; Mohan *et al*, 2006) have been shown to contain functional CBF1-binding motifs and to be targets of EBNA2-CBF1 signaling. While it is clear that EBNA2-mediated repression of *bik* does not require CBF1, some role for CBF1 in *bik*-regulation cannot be ruled out.

Repression of Bik by EBNA2 was readily seen in all cell lines tested. However, overall, it could be argued that the BL41 cell system supports the modulation of cellular target genes by EBNA2 more efficiently than in DG75 or BJAB. In support of this theory, it is illustrated in Figure 4.2.1, Figure 4.2.2 and Figure 4.2.3 that mRNA levels in the BL41-derived cell lines, demonstrate a significant reduction at least 24 hours in advance of that seen in the DG75 and BJAB cell lines, and the overall level of repression at the RNA level in BL41 derivatives was not equalled. Also, down-regulation of the protein is evident at much earlier time points in the BL41-derived cells (Figure 4.2.2 and Figure 4.2.3). In concurrence with this, it has previously been demonstrated that the cellular context can significantly influence target gene modulation in response to EBNA2 (Wang *et al*, 1990; Cordier-Bussat *et al*, 1993). Significantly in DG75 cells, the levels of CBF1 protein levels are known to equal the levels seen in BJAB, while only reaching about half the levels observed in BL41 (Maier *et al*, 2005). Thus, although there is no absolute requirement for CBF1 in this interaction, it is plausible that EBNA2 may mediate Bik regulation partially through CBF1 and a limiting amount of CBF1 might somewhat contribute to the weaker EBNA2 responses seen in DG75 and BJAB. Other epigenetic factors can also not be ruled out. mRNA levels in BL41K3 and BL419A are seen to be rapidly degraded (Figure 4.2.2 and Figure 4.2.3), this decrease in the level of *bik* mRNA could be

attributable either to a decreased rate of transcription from the promoter of the *bik* gene and/or a de-stabilising effect of EBNA2 on *bik* mRNA. The delayed decrease of *bik* mRNA levels in the DG75 and BJAB cells (Figure 4.2.1B and Figure 4.2.2D) could therefore also be attributable to its higher stability in this cell background.

EBNA2-induced down-regulation of *bik* mRNA levels in the BL41K3 and BL419A cell lines appears transient at the transcriptional level, in contrast to what was seen in the DG75 and BJAB derivatives. It would seem that this de-repression of *bik* is not reflective of subsiding EBNA2 expression levels at the relevant time points, as indicated by Western Blot analysis (Figure 4.2.1A and Figure 4.2.2C). However, it should be considered that these cells have entered growth arrest/growth retardation in response to EBNA2 activation (Kempkes *et al*, 1996), and the increase in the level of *bik* could be attributable to the effects of other complex pathways within the cell, triggered in response to an apoptotic signal. Conceivably this response could also be attributed to the spurious effect of EBNA2 over-expression and thus may be a stress-induced artefact caused by high levels of protein in the cell. Alternatively, the discrepancy may be due to factors particular to the specific cell type, in this case particular characteristics of the BL41 cell line, causing a distinct response to EBNA2 expression in this context.

In all BL cells, the fundamental growth-transforming event appears to be constitutive activation of the *c-myc* oncogene through its translocation into an Ig gene locus (Brady *et al.*, 2007). EBNA2 directly regulates c-Myc causing a down-regulation in its expression in those BL cell lines carrying the t(8;14) chromosomal translocation (Maier *et al*, 2005) (Figure 4.2.1A and Figure 4.2.2A). A significant subset of EBNA2-regulated genes will therefore be targets of c-Myc. Accordingly, considering c-Myc as a direct target gene of EBNA2 and an important mediator of cell proliferation, it was of interest to consider a possible role for c-Myc in EBNA2-associated repression of *bik*. In DG75-tTA-EBNA2 and BL41K3, EBNA2 negatively regulates both Bik and c-Myc. However, in BJABK3, a non BL cell line derived from a lymphoblastoid B-cell line that does not carry the t(8;14) translocation, c-Myc expression is transiently up-regulated in response to EBNA2 (Maier *et al*, 2005 and

Figure 4.2.2C). Since c-Myc is not down-regulated by EBNA2 in that cell line and yet Bik is still down-regulated, it appears that Bik is down-regulated by EBNA2 independently of c-Myc (at least in that cell line). The results presented here therefore, indicate that EBNA2 mediated repression of *bik* is not dependent on c-Myc although this does not rule out a role for Myc in *bik* regulation during the complete EBV growth programme where all the EBV latent proteins are expressed.

In BL cell lines carrying the chromosomal translocation juxtaposing the c-*myc* and the immunoglobulin M (IgM) heavy-chain locus, the expression of IgM and c-*myc* is co-regulated and repressed (Jochner *et al*, 1996). Interestingly, it has previously been demonstrated that EBNA2 can down-regulate IgM expression (Jochner *et al*, 1996). In BJAB, IgM repression and c-*myc* expression are uncoupled, and it is thus made clear that IgM is an EBNA2 target not requiring Myc (Maier *et al*, 2005). Significantly, increased expression of both *bik* mRNA and protein is induced by sIgM ligation in B-cells (Jiang and Clark, 2001). Conceivably, therefore, EBNA2 induction may repress IgM expression which in turn affects the down-regulation of expression of the *bik* gene. On this note EBNA-2 mediated down-regulation of both IgM and c-Myc is only partially CBF1 dependent (Maier *et al*, 2005) and a putative role for either of these genes in the modulation of the *bik* gene is supported by the finding that *bik* down-regulation by EBNA2 is CBF1 independent.

The activated Notch receptor is considered to be the functional equivalent of the viral EBNA2 protein (Zimber-Strobl and Strobl, 2001). As discussed in Chapter 1, upon ligand binding, the transmembrane receptor Notch is proteolytically processed, and an intracellular fragment (NotchIC) is translocated to the nucleus, where it binds to the transcriptional repression domain of CBF1, activating target gene expression by means of replacing co-repressor complexes, (as reviewed in Zimber-Strobl and Strobl, 2001). EBNA2 then, acts through shortcutting CBF1 signaling and activating a set of viral and cellular target genes, which at least partially overlap the target genes of activated Notch (Hofelmayr *et al*, 1999; Strobl *et al*, 2000; Gordadze *et al*, 2001; Hofelmayr *et al*, 2001; Hubmann *et al*, 2002; Johansen *et al*, 2003). It has been shown that constitutively activated Notch can also down-regulate IgM expression (Stuart *et al*, 1995; Strobl *et al*, 2000; Morimura *et al*, 2001) and Myc is also a direct transcriptional target of Notch1 (Efstratiadis *et al*, 2007) a further indication that

EBNA2 is mimicking the Notch cellular pathway. Since both EBNA-2 and activated Notch can repress IgM and c-Myc, it is plausible that EBNA2 interacts with and subverts the cellular Notch signaling pathway, executing Notch-like functions involved in IgM/c-Myc regulation, consequently affecting *bik* repression and the ensuing disturbance of the apoptotic balance in the cell that is consistent with virus survival. It should be noted that there are additional common interaction partners of both Notch and EBNA2, such as SKIP, Nur77, PU.1, Spi-1 or Spi-B (Laux *et al*, 1994a; Johannsen *et al*, 1995; Zhou *et al*, 2000a; Zhou *et al*, 2000b; Lee *et al*, 2002), which could potentially contribute to this function.

Results presented here demonstrate that the induction of LMP1 did not influence the expression of the *bik* gene in response to tetracycline-regulated induction of this protein indicating no role for LMP1 in its regulation. Recent findings in the laboratory have shown that LMP1 drives *bfl-1* promoter activity through interactions with components of the TNFR/CD40 signaling pathway. Activation of CD40 receptor also led to increased *bfl-1* mRNA levels and an NF-κB-dependent increase in *bfl-1* promoter activity in BL-derived cell lines (D'Souza *et al*, 2004). In a recent study CD40 stimulation enhanced the constitutive anti-apoptotic profile of B-cell chronic lymphocytic leukaemia (B-CLL) cells by up-regulation of Bcl-$_{XL}$ and Bfl-1 and down-regulation of the BH3-only protein Harakiri, with no change in the level of expression of the *bik* gene (Kater *et al*, 2004) adding further support to the contention that LMP1/CD40 signaling is not involved in Bik down-regulation, while also reinforcing its role in *bfl-1* regulation.

Of interest, EBNA2-mediated Bik repression in BL41K3 led to the gradual disappearance of the slower moving band (27kDa) which has previously been identified as the phosphorylated form of Bik (Verma *et al*, 2001). These observations may suggest that the larger band is preferentially degraded by EBNA2. Interestingly, it is thought that phosphorylation of Bik is required for eliciting efficient apoptotic activity (Verma *et al*, 2001). Thus, while this model is consistent with the role for EBV in protecting infected cells from apoptosis, it does not explain the apparent resistance of BL Type I cell lines to Bik expression. As suggested in Chapter 3, the relative proportion of Bik not associated with Bcl-$_{XL}$ may be a crucial factor in the ability of these cells to tolerate the expression of this pro-apoptotic molecule. Bik may

also be counteracted by a so far undefined inhibitor in type I cells (EBV latent programme) as compared with type III cells and LCLs (EBV growth programme).

It was shown previously that the infection of BL cells with EBV results in a significant down-regulation in the expression of the BH3-only *bim* gene (Clybouw *et al*, 2005; Leao *et al*, 2007). It has been reported that EBV infection may lead to post-translational modulation of Bim expression, involving the phosphorylation of BimEL by the EBV-activated kinase ERK1/2, followed by its degradation through the proteasome pathway (Clybouw *et al*, 2005). However, a pathway in which EBNA3A and EBNA3C co-operate to regulate Bim expression at the level of transcription; has also been described (Anderton *et al*, 2007) and it is formally claimed that EBV does not regulate Bim post-translationally; that there is no direct correlation between ERK phosphorylation and Bim expression in B-cells. In addition a recent report indicates that pro-apoptotic Bax is an important target of the anti-apoptotic activity of LMP-1. LMP-1 inhibits *bax* promoter activity, through activation of NF-κB. Moreover, in cells with impaired Bax function or expression the anti-apoptotic activity of LMP-1 is significantly reduced (Grimm *et al*, 2005). The data presented here indicates that down-regulation of pro-apoptotic *bik* by EBV is also at the level of transcription and is mediated by EBNA2. This is the first indication of down-regulation of a pro-apoptotic protein by this viral protein. Thus, the novel discovery that EBNA2 is down-regulating transcription from the pro-apoptotic *bik* gene in combination with the up-regulation of the anti-apoptotic *bfl-1* gene suggests that EBNA2 expression may have a protective effect on BL cells during apoptosis.

CHAPTER 5 :

Regulation of *bik* by EBV in Lymphoblastoid Cell Lines.

5.1 Introduction

Since EBV-negative BL cells are immortalised by a mechanism not requiring EBNA2 expression, it is difficult to assess the contribution of this gene to cell survival in the context of this cancer cell. A critical question is whether *bik* repression during the activation of EBNA2 in BL cells occurs during infection of primary B-cells by EBV. In EBV-immortalised lymphoblastoid cell lines (LCLs), EBNA2 maintains the EBV growth programme (Kempkes *et al*, 1995a) and consequently, in contrast to the investigation of a gene in the background of a pre-existing tumour cell line, the use of the conditionally immortalised LCL EREB 2-5 enables the study of EBNA2 in its physiological context; i.e. in B-cells that are induced to proliferate due to EBV (Schlee *et al*, 2004). The EREB 2-5 cell system imitates fundamental steps in the EBNA2-driven immortalisation of primary B-cells (Schlee *et al*, 2004) allowing one to determine if *bik* repression is a cellular response that occurs due to the EBV growth programme.

It has been shown elsewhere that enforced c-Myc expression is sufficient to sustain B-cell proliferation independently of both EBNA2 and LMP1 (Polack *et al*, 1996). The proliferation programme imposed by enforced expression of c-Myc in BL cells differs considerably from that triggered in EBV-immortalised cells, driven by the expression of EBNA2. EBV-immortalised cells grow in clumps, expressing an array of activation markers and adhesion molecules, are particularly resistant to apoptosis, and are immunogenic. In contrast, c-Myc-driven cells grow as single cells in suspension, do not express the various cell surface molecules expressed on EBV-immortalised cells, are highly susceptible to apoptosis, and are non-immunogenic (Schlee *et al*, 2004). The immense differences in the growth programmes between cells driven into proliferation by EBV and c-Myc indicate that there must be considerable differences in the target genes of EBNA2 and c-Myc. Thus, EBNA2 targets, which include viral growth programme genes, comprise additional genes that are independent of c-Myc, and are not involved in proliferation, but rather play a role in protection against apoptosis, or cell-cell communication.

This study set out to investigate if *bik* down-regulation was a feature of the EBV growth programme in B-cells proliferating due to EBV latent proteins, and to determine if c-Myc, itself a positive EBNA2 target in this context, played a role in *bik* repression. The P493-6 cell line was a particularly appropriate tool to use for this purpose (Table 2.1-1). This cell line is an EBV-infected LCL that is conditional for both EBNA2 and c-Myc. When P493-6 cells are starved of estrogen, EBNA2 function is lost leading, in turn, to loss of the EBV growth programme and the cessation of cell growth. In this setting, Myc expression can be turned on, as it is tetracycline regulated, and the cells therefore approximate EBV-negative BL cells, proliferating due to the over-expression of c-Myc (Staege *et al*, 2002).

5.2 Bik is down-regulated by EBNA2 in EBV-immortalised cell lines.

5.2.1 Bik is down-regulated by EBNA2 in EREB 2-5 cells.

EREB 2-5, a conditional LCL in which the function of EBNA2 can be controlled by the presence or absence of estrogen has previously been developed (Kempkes *et al*, 1995a). This was achieved by the fusing of the N-terminus of EBNA2 to the hormone binding domain of the estrogen receptor. Essentially, this ER/EBNA2 fusion gene was then used to compliment the EBNA2 deletion in the EBV P3HR1 viral genome (Kempkes *et al*, 1995a). The resulting 'conditional' LCL, designated EREB 2-5, generated after immortalising resting B-cells with this recombinant EBV, therefore required the presence of estrogen for EBNA2 function and hence cell proliferation. Addition of estrogen results in EBV-infected cells with a phenotype similar to those found in healthy individuals upon primary infection with the virus, or EBV-infected cells found in immuno-suppressed patients. Cells growing in the absence of estrogen cease to proliferate due to the expression of only non-functional ER/EBNA2 and the shutoff of the EBV growth programme (see Figure 5.2.1).

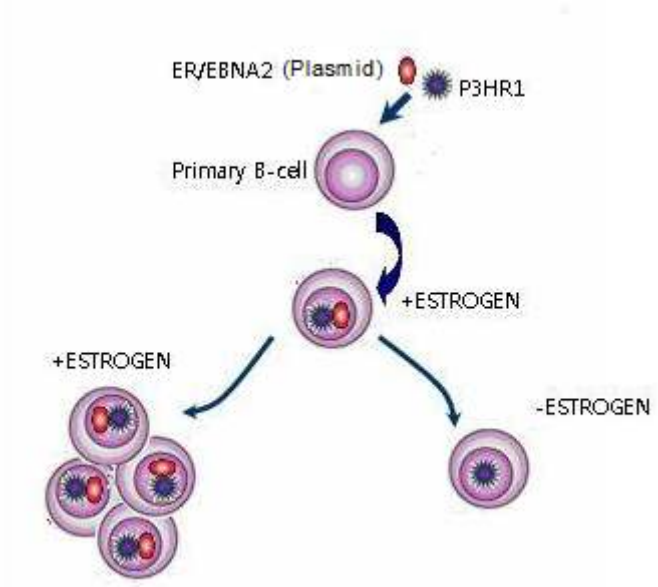


Figure 5.2.1: Schematic diagram of EREB 2-5.

EREB 2-5 is an LCL that contains a mini-EBV plasmid expressing the chimeric EBNA2 protein as an autonomously replicating plasmid, plus the P3HR1 viral genome, thus complementing the EBNA2 deletion of the P3HR1 virus. EBNA2 expression and proliferation are dependent on the presence of estrogen.

In order to examine changes in *bik* expression due to the expression of EBNA2 a time course was performed with the EREB 2-5 cell line cultivated in the presence and absence of estrogen (Section 2.2.2.3). Cells were washed four times in PBS to remove estrogen from the cell culture medium and seeded into estrogen-free medium, followed by another two washes 24 hours later. This was considered time 0. Protein and RNA extracts were prepared at various time points over a 120 hour period. After this time, these cells were recultured in medium supplemented with 1μM estrogen (Kempkes *et al*, 1995a) and protein and RNA extracts were prepared over the subsequent 72 hours. The levels of EBNA2, Bik and β-Actin protein expressed during the time-course were analysed by Western blot, and *bik* mRNA levels were assayed by RT followed by QPCR as before.

EBNA2-associated changes in Bik protein expression and mRNA levels observed during the time courses in the EREB 2-5 induction are shown in Figure 5.2.2. Western blot analysis (Figure 5.2.2A) confirms that EREB 2-5 cells grown in the absence of estrogen, only expressed non-functional EBNA2, while cells grown in the presence of estrogen expressed chimeric EBNA2 with an apparent molecular weight of 120kDa, confirming the activation of EBNA2. It is clear from Figure 5.2.2A that a considerable and sustained induction of Bik protein levels occurred 48 hours following the removal of estrogen from the EREB 2-5 culture medium. Accordingly after the re-activation of EBNA2, Bik expression was completely abolished (72-hours) (Figure 5.2.2A).

Changes in *bik* mRNA levels due to EBNA2 expression over time in EREB 2-5 are shown in Figure 5.2.2B. *Bik* mRNA levels were seen to increase dramatically over time (7-fold at 24 hours) in EREB 2-5 cells upon inactivation of EBNA2. The amount of *bik* mRNA was seen to increase dramatically by 120 hours, with a 92-fold increase in the level of *bik* mRNA compared to time 0.

A reduction in *bik* levels (approximately 1.7-fold) was observed 24 hours following the subsequent addition of estrogen to the culture medium (Figure 5.2.2B). A further decrease was seen at 48 hours (2.5-fold), and levels of *bik* mRNA remained stably-repressed from this point on, with the level of *bik* at 96 hours similar to that observed at 48 hours.

These results establish that *bik* is transcriptionally repressed by the EBNA2-driven growth programme in EBV-immortalised B-cells. In EREB 2-5 cells, EBNA2 function is dependent on estrogen, and the proliferative and growth transformation effects of EBV are reversed upon withdrawal of this hormone from the medium. It can be seen that the removal of estrogen led to de-repression of *bik* mRNA and protein (Figure 5.2.2), co-incident with the established observation of the onset of apoptotic death in the absence of hormone (Kempkes *et al*, 1995a). The reactivation of EBNA2, was also concurrent with a reduction in the level of *bik* expression.

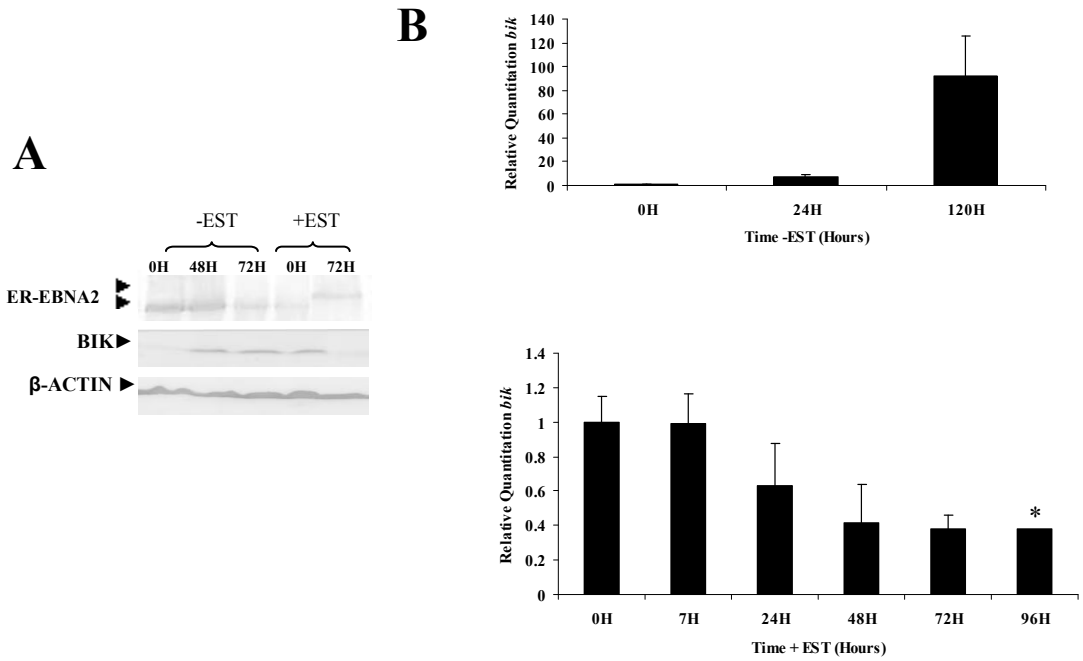


Figure 5.2.2: Modulation of *bik* expression by EBNA2 in the EBV-infected cell line EREB 2-5.

EREB 2-5 cells which had been cultivated in the absence of estrogen for the preceding 120 hours, were cultivated in the presence of estrogen for a further of 72 hour period in order to activate EBNA2. Both RNA and protein extracts were prepared at various time points (hours) after activation/repression. (A) Western Blot analysis of Bik protein expression in response to activation of EBNA2 was monitored. The level of Bik protein expression was detected using a mouse anti-Bik antibody (BD Biosciences 557040). Analysis of EBNA2 activation in EREB 2-5 is shown in the upper panel. The middle panel illustrates Western blot analysis of Bik protein expression in response to activation of EBNA2. The lower panel shows the same blot probed for β-Actin (B) QPCR analysis for the level of *bik* expression was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. After normalisation for *gapdh*, relative quantitation level of *bik* was plotted as a function of the control (0H; arbitrarily assigned a value of 1). The lower plot illustrates the changes in *bik* expression in response to activation of EBNA2, while the upper plot shows differences in *bik* expression coincident with EBNA2 repression. (Data are Mean±SD, *$p < 0.05$)

It was also observed that the Bik protein band pattern that emerges upon EBNA2 repression is similar to that observed in the DG75 BL type I cell line, and contrasts with the low level of Bik seen in the BL type III cell line AG876 and the LCL IB4 where only the lower Bik band is apparent (Figure 5.2.3). These results show that *bik* mRNA and protein levels are repressed due to the EBV growth programme.

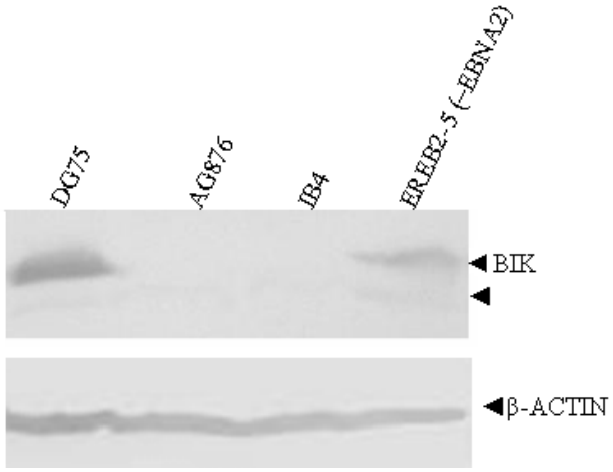


Figure 5.2.3: De-repression of *bik* following estrogen withdrawl in the conditional LCL EREB 2-5

Western Blot analysis of Bik protein expression in the EREB 2-5 cell line in response to repression of EBNA2 for 48 hours, relative to that expressed in DG75, AG876 and IB4 was monitored. The level of Bik protein expression was detected using a mouse anti-Bik antibody (BD Biosciences 557040). The upper panel illustrates Western blot analysis of Bik protein expression and the lower panel shows the same blot probed for β-Actin

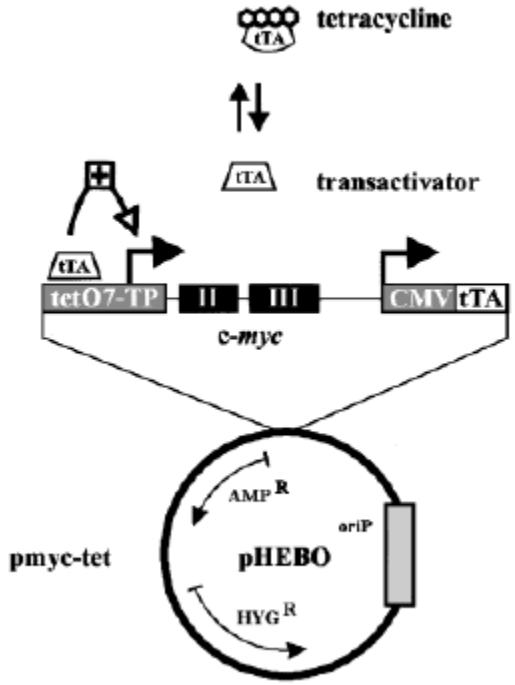
5.2.2 Modulation of *bik* expression in the EBV-infected cell line P493-6.

In LCLs, *c-myc* is a positive transcriptional target of EBNA2. In addition, constitutive expression of *c-myc* can substitute for EBNA2 in the maintenance of proliferation in EREB 2-5 cells (Pajic *et al*, 2000). It was therefore of interest to determine if Bik repression also occurred in B-cells driven to proliferate due to c-Myc. For this purpose, the P493-6 cell line was employed. This cell line was clonally derived elsewhere from EREB 2-5 and conditionally expresses both EBNA2 (regulated by estrogen) and ectopic c-Myc (negatively regulated by tetracycline) (Schuhmacher *et al*, 1999; Pajic *et al*, 2000; Schuhmacher *et al*, 2001). P493-6 is therefore an EBV-infected LCL, which when starved of estrogen resembles a resting B-cell and upon

induction of Myc the cells approximate BL cells, in that they proliferate due to the over-expression of *c-myc* (Staege *et al*, 2002).

In brief, the B-cell line P493-6 was established by stable transfection of a tetracycline-regulatable *c-myc* gene into EREB 2-5 cells using an episomal vector; (tet-off system) (Pajic *et al*, 2001). In this vector the two coding exons of *myc* are under the control of a tetracycline regulated promoter, TP-tetO7. pMyc-tet also carries the gene coding for the tetracycline repressor-VP16 *trans*-activator fusion protein, tTA on the same molecule (Gossen and Bujard, 1992). Hygromycin resistant and estrogen independent cell clones were then selected for during cell culture (Pajic *et al*, 2001). Addition of tetracycline to the cell culture medium abolishes promoter activation by direct interaction with tTA (Figure 5.2.4).

A



B

Estrogen	Tetracycline	EBNA2	c-Myc
+	+	YES	NO
+	-	YES	YES
-	+	NO	NO
-	-	NO	YES

Figure 5.2.4: Conditional expression of *myc* or the EBV growth programme in the B-cell line P493-6, a clonal derivative of EREB 2-5.

(A) Schematic illustration of the *myc* expression construct pMyc-tet. The two coding exons of *myc* (black boxes II and III) were cloned under the control of the promoter TP-tetO7. The construct also includes an expression cassette for the *trans*-activator tTA (CMV-tTA). Tetracycline abolishes *trans*-activation of TP-tetO7 by interfering

with tTA DNA-binding. pMyc-tet is based on the EBV-derived vector pHEBO. Selection marker genes are depicted as arrows, and the origin for latent replication by oriP (adapted from Pajic *et al*, 2001). (B) P493-6 cells were induced to grow on an EBV-driven programme; where the cells either proliferate due to the expression of EBNA2 (in the presence of estrogen and tetracycline), or on the c-Myc growth programme (when estrogen and tetracycline are withdrawn), with the cells proliferating due to the over-expression of c-Myc. In order to activate EBNA2 in its functional form, estrogen is added to the culturing media to a final concentration of 1μM, in agreement with previously published studies (Pajic *et al*, 2000; Pajic *et al*, 2001). Expression of *c-myc* may be suppressed by treatment with 0.1μg/ml tetracycline again in accordance with previously published studies (Pajic *et al*, 2000; Pajic *et al*, 2001).

5.2.2.1 *Bik* is regulated by EBNA2 in the B-cell line P493-6

In order to examine EBNA2-associated changes in the level of *bik* expression a time course was undertaken with the P493-6 cell line cultivated in the presence of estrogen and tetracycline as per Section 2.2.2.3 (Figure 5.2.4B). Under these conditions the cells resemble EREB 2-5, since inducible *c-myc* expression is switched off, and the cells express ER/EBNA2 and endogenous c-Myc. P493-6 cells which had been previously been cycling on the EBV growth programme (in the presence of tetracycline and estrogen), were cultivated in the absence of estrogen for a period of 60 hours. After this time cells were washed twice with PBS and were cultivated in the presence of estrogen. Protein and RNA extracts were prepared at various time points over a 72 hour period. At this point the cells were washed twice with PBS and re-seeded in the absence of estrogen and RNA extracts were prepared over the subsequent 72 hours. The levels of EBNA2, Bik c-Myc and β-Actin protein expressed were analysed by Western blot, and *bik* and *gapdh* mRNA levels were assayed by RT followed by QPCR.

Western blotting was undertaken to demonstrate the activation of ER/EBNA2 over the course of the experiment and to analyse Bik and c-Myc protein levels in response to EBNA2 expression (Figure 5.2.5A). Levels of the endogenous control, β-Actin, were also analysed and confirmed equal loading in each case (Figure 5.2.5A). In this experiment, functional ER/EBNA2 was detected in induced P493-6 cells at 24 hours and remained detectable up to 72 hours (Figure 5.2.5A). A significant decrease in the level of Bik protein can be seen to coincide with the activation of EBNA2 expression

over time, Bik levels are repressed in conjunction with EBNA2 expression at 24 and 48 hours, and are completely abolished at the 72 hour time point. Thus, Bik down-regulation is again governed by EBNA2/EBV growth programme as was seen in the parental cell line EREB 2-5. Endogenous c-Myc expression correlated with EBNA2 induction and was up-regulated by 24 hours, and remained up-regulated at 72 hours (Figure 5.2.5A) as expected.

Steady state levels of *bik* mRNA in P493-6 were also repressed in response to EBNA2 activation, reminiscent of the result obtained with the parental EREB 2-5 cell line (Figure 5.2.5). A significant and sustained decrease in *bik* mRNA levels (approximately 10-fold) was observed by 24 hours following activation of EBNA2. Chimeric EBNA2 was expressed at high levels at this time point (Figure 5.2.5A). After removal of estrogen, (and the resulting withdrawal of EBNA2 function), the amount of *bik* mRNA was seen to increase gradually over time after this point, with the level at 72 hours approximately 3.4-fold greater than the levels observed upon removal of estrogen from the cell culture medium (0H–EBNA2). The levels of *bik* mRNA however, never reached the levels originally observed upon induction of EBNA2 (0H+EBNA2). These results indicate that the activation of ER/EBNA2 and consequently the EBV growth programme led to a sustained decrease in the level of *bik* mRNA in P493-6, and subsequent removal of EBNA2 function was concurrent with a de-repression in *bik* mRNA levels.

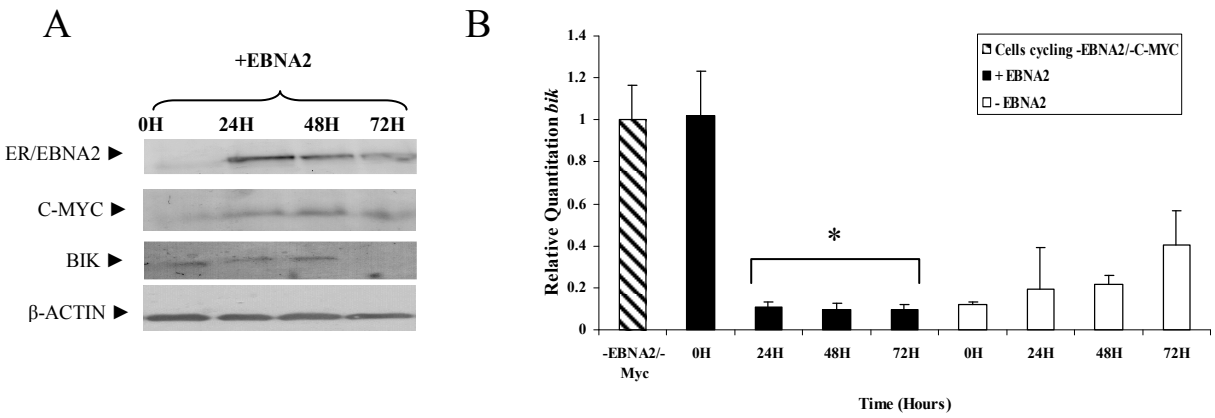


Figure 5.2.5: Regulation of *bik* by EBNA2 in the EBV-immortalised cell line P493-6.

P493-6 cells which had previously been cycling on EBV growth programme for 72 hours were cultivated in the absence of estrogen for a further of 72 hour period in order to inhibit EBNA2. Total protein and RNA were prepared simultaneously from cells harvested at the indicated time-points after ER/EBNA2 activation/repression. The level of Bik protein was detected using an affinity purified goat polyclonal IgG (Santa Cruz, sc-1710). (A) Western Blot analysis of Bik protein expression in response to ER/EBNA2 in the P493-6 cell line. The upper panel illustrates EBNA2 protein upon activation by reculturing in the presence of estrogen and tetracycline. Analysis of c-Myc in response to EBNA2 induction in P493-6 is shown in the second panel. Western Blot analysis of Bik protein expression in response to induction of EBNA2 expression is shown in third panel. The lower panel shows the same blot probed for β-Actin. (B) QPCR analysis for the level of *bik* expression in response to EBNA2 activation/repression was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. After normalisation for *gapdh*, relative quantitation level of *bik* mRNA was plotted as a function of the control (Cells cyling –EBNA2/-Myc; arbitrarily assigned a value of 1). (Data are Mean±SD, * $p<0.05$)

5.2.2.2 P493-6 cells express lower levels of Bik when cycling due to EBV than when cycling due to c-Myc.

Bik expression in P493-6 cells which were driven to proliferate on the EBV and c-Myc programmes were next compared. Accordingly, cells that had been previously been cycling due to c-Myc were cultivated in medium containing tetracycline for 60 hours. After this time, cells were washed four times in PBS and re-seeded in the absence of tetracyline and estrogen. This was considered time 0. c-Myc expression was induced over the 72 hour period and protein and RNA extracts were prepared at 72 hours. At the same time, cells that had been cycling on the EBV growth programme were cultivated in the absence of estrogen for 60 hours preceding induction. After this time cells were washed four times in PBS, and subsequently seeded into medium containing estrogen. This was considered time 0. EBNA2 expression was induced over a 72 hour period and protein and RNA extracts were prepared at 72 hours. The levels of Bik and β-Actin proteins expressed were analysed by Western blot, and *bik* and *gapdh* mRNA levels were assayed by RT followed by QPCR.

It can be seen that Bik protein was detectable in P493-6 cells proliferating due to the c-Myc in comparison to those cells expressing the EBV growth programme, wherein Bik was in fact undetectable (Figure 5.2.6A). Levels of the endogenous control β-Actin were also monitored and confirmed equal quantity loaded in each case (Figure 5.2.6A, lower panel).

Differences in *bik* mRNA expression due to regulation by either c-Myc or the EBNA2 programmes are shown in Figure 5.2.6B. Relative quantitation of the levels of *bik* transcript after normalising for *gapdh* indicated that c-Myc-driven P493-6 cells had a comparatively high *bik* mRNA level, approximately 8-fold higher than that observed in the EBNA2-driven cells (Figure 5.2.6B). Thus, the steady state level of *bik* mRNA and Bik protein is significantly lower when cells are cycling due to EBV growth (Lat III) programme compared to cells proliferating on the c-Myc programme.

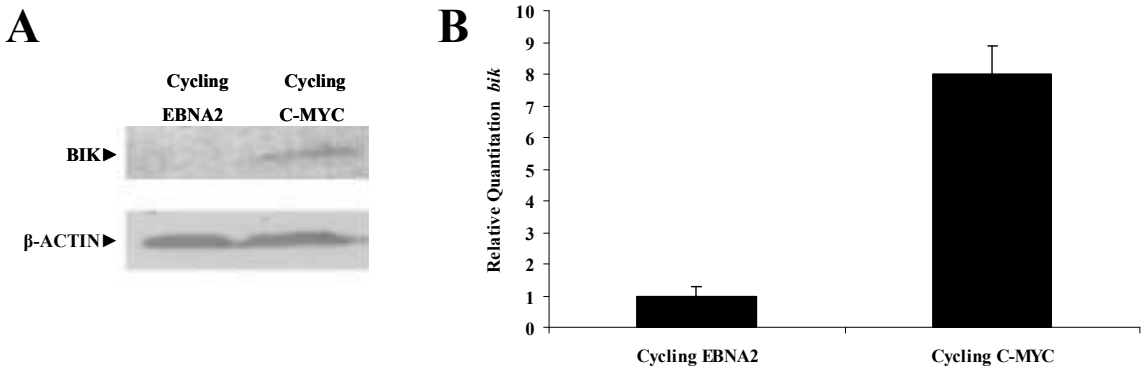


Figure 5.2.6: Cells cycling on EBV lat III express less Bik than cells proliferating on the Myc programme.

P493-6 cells which had been starved of estrogen for the preceding 60 hours were cultivated in the presence of estrogen for 72 hours in order to activate EBNA2. At the same time, P493-6 cells which had been maintained in the absence of c-Myc expression for the preceding 60 hours were cultivated in the absence of tetracycline in for a further 72 hours in order to induce c-Myc expression. Total protein and RNA were prepared simultaneously from cells harvested at 72 hours. (A) Western Blot analysis of Bik protein expression in response to either EBNA2 or c-Myc expression in the EBV-positive cell line P493-6. The level of Bik protein was detected using an affinity purified goat polyclonal IgG (Santa Cruz, sc-1710). Western Blot analysis of Bik protein expression in response to ER/EBNA2/c-Myc expression can be seen in the upper panel. The lower panel shows the same blot probed for β-Actin. (B) QPCR analysis for the level of *bik* expression was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. After normalisation for *gapdh*, relative quantitation level of *bik* was plotted as a function of the control (Cycling EBNA2 cells; arbitrarily assigned a value of 1). (Data are Mean±SD**)**

5.2.2.3 *Bik* is negatively regulated by c-Myc in the LCL P493-6.

In order to directly investigate *bik* expression in response to c-Myc P493-6 cells which had been previously been cycling on the c-Myc growth programme (in the absence of tetracycline and estrogen) for the preceding 60 hours, were cultivated in the presence of tetracycline in for a further 72 hours in order to suppress c-Myc expression (Section 2.2.2.3). Protein and RNA extracts were prepared at various time points over a 72 hour period. At this point the cells were washed twice with PBS and

re-seeded in the absence of tetracycline and RNA extracts were prepared over the subsequent 72 hours. The levels of EBNA2, Bik c-Myc and β-Actin protein expressed were analysed by Western blot, and *bik* and *gapdh* mRNA levels were assayed by RT followed by QPCR.

Western blot analysis (Figure 5.2.7A) confirmed that cells grown in the absence of estrogen and tetracycline showed a decrease in c-Myc expression over the duration of the 72 hour period. c-Myc expression was undetectable after 24 hours, and remained undetectable up to 72 hours after the-addition of tetracycline. A considerable, if transient increase in the level of Bik protein was observed in response to c-Myc repression at the 24 and 48 hour time points. Although decreased, Bik still remained detectable at 72 hours. These results showed that loss of c-Myc led to a significant transient increase in the level of Bik in the LCL P493-6. Inactivated ER/EBNA2 protein and β-Actin levels were also monitored by Western blot analysis, and neither showed any significant variation over the time period (Figure 5.2.7A).

Similar to the result observed with Bik protein, *bik* mRNA levels were also transiently induced in P493-6 cells 24 hours after *c-myc* repression. Upon removal of tetracycline from the cell culture medium, a reduction in the level of *bik* mRNA in response to ectopic c-Myc was again to be seen (Figure 5.2.7B). *Bik* mRNA levels were seen to diminish over time in response to the levels of cMyc. A considerable reduction in *bik* levels was observed by 24 hours (approximately 2.5-fold decrease from the levels observed upon induction of c-Myc, which persisted at the subsequent time points up to 72 hours. The amount of *bik* mRNA was seen to decrease to a level similar to that seen upon withdrawal of c-Myc expression (0H-Myc).

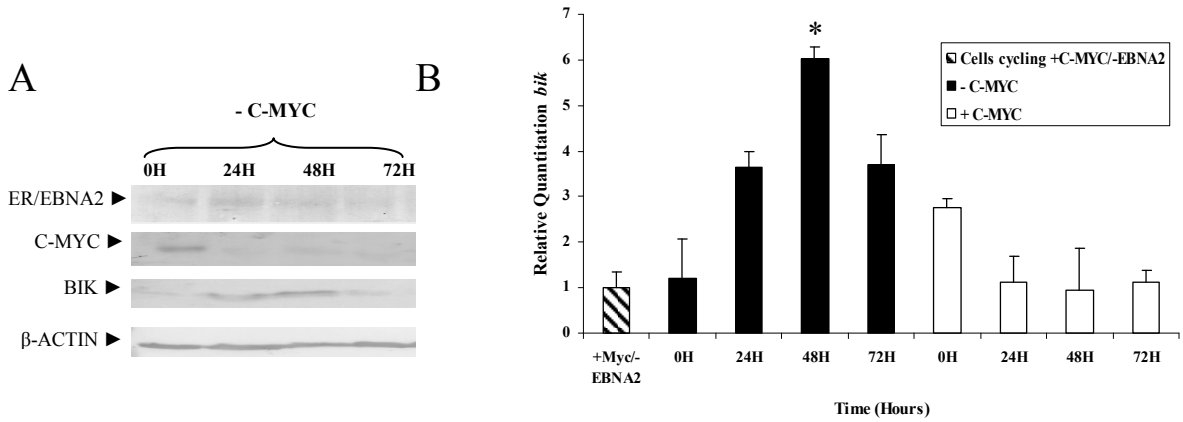


Figure 5.2.7: Regulation of *bik* by c-Myc in P493-6 cells

P493-6 cells which had been previously been cycling on the c-Myc growth programme for the preceding 60 hours, were cultivated in the presence of tetracycline in for a further 72 hours in order to suppress c-Myc expression. Protein and RNA extracts were prepared at various time points over a 72 hour period. At this point the cells were re-seeded in the absence of tetracycline and RNA extracts were prepared over the subsequent 72 hours. Protein and RNA extracts were prepared at various time points over a 72 hour period. The level of Bik protein was detected using an affinity purified goat polyclonal IgG (Santa Cruz, sc-1710). (A) Western Blot analysis of Bik protein expression in response to repression of c-Myc in the P493-6 cell line. Analysis of EBNA2 protein upon in response to c-Myc inhibition by reculturing in the absence of estrogen and the presence of tetracycline is illustrated in the upper panel. Repression of c-Myc in P493-6 is shown in the second panel. Western Blot analysis of Bik protein expression in response to c-Myc repression can be seen in the third panel. The lower panel shows the same blot probed for β-Actin. (B) QPCR analysis for the level of *bik* expression in response to c-Myc induction/repression was also examined. Isolated RNA was reverse transcribed using random primers and amplification of DNA was monitored by detection of fluorescence intensity using FAM-labeled TaqMan probes for *bik* and *gapdh*. Fluorescent signals were detected using an ABI Prism 7500 Sequence Detection System. After normalisation for *gapdh*, relative quantitation level of *bik* mRNA was plotted as a function of the control (Cells cyling –EBNA2/+Myc; arbitrarily assigned a value of 1). (Data are Mean±SD, *$p<0.05$)

5.3 Discussion

It is difficult to assess whether EBNA2-associated changes in *bik* expression in BL tumour-derived cell lines reflect events which take place during infection of primary B-cells by EBV. Since the lack of detectable *bik* expression (Chapter 3; Figure 3.2.1) was an observation consistent with a potential tumour suppressor role for *bik*, and could have pro-survival consequences during EBV infection of B-cells, it was decided to investigate whether *bik* down-regulation was the result of the EBV growth programme in an LCL background. Accordingly experiments were carried out using the EREB 2-5 cell line in which the induction of functional EBNA2 activates the EBV growth programme and results in an LCL phenotype, similar to that seen in infected B-cells found in individuals experiencing EBV primary infection, or EBV-infected B-cells found in immuno-suppressed patients (Kempkes *et al*, 1995a).

The experiments presented here demonstrate that an induction of functional EBNA2 expression coincides with a decrease in the level *bik* mRNA and protein in the LCL cell lines EREB 2-5 and its clonal derivative P493-6. Significantly removal of estrogen from the culture medium, and the consequent inhibition of EBNA2 function resulted in a de-repression of *bik*. This novel observation implies that the *bik* gene is directly down-regulated by the EBV growth programme in virus-infected B-cells. This is compatible with an anti-apoptotic consequence since the removal of estrogen from the cell culture medium is co-incident with the established observation of the onset of apoptotic death in the absence of hormone (Kempkes *et al*, 1995a).
The repression of *bik* mRNA levels in EREB 2-5 was 2.5-fold by 72 hours, in contrast to that for P493-6, where repressed *bik* mRNA levels were 10-fold decreased by just 24 hours after induction of EBNA2. Additionally, the de-repression observed in EREB 2-5 (92-fold) was much greater than that observed in P493-6 (3.4-fold). However, it should be considered that EREB 2-5 cells were starved of estrogen for only 24 hours prior to time 0 as compared to 60 hours in P493-6, and thus differences owing to the physical condition of the cells could be implicated in the differences in the extent of *bik* modulation observed. Significantly, the overall trend is the same. Also, P493-6 cells maintain detectable c-*myc* mRNA protein levels in the presence of tetracycline and may therefore be considered ‘pre-activated’ compared to EREB 2-5 cells, in which EBNA2 activity (and thus also c-Myc) is tightly regulated by hormone

(Schlee *et al*, 2004). It is thus conceivable that a small amount of "leaky" Myc expression leads to an already repressed expression of Bik, and thus the effect we see is not the "full" effect. Accordingly then, even a small level of Myc expression in the cell might hinder the de-repression effect; as would not be the case in EREB 2-5.

As both EBNA2 and endogenous *c-myc* are expressed following activation of functional EBNA2, with *trans*-activation of *c-myc* under the control of EBNA2 (Kaiser *et al*, 1999), it is not possible to conclude from the EREB 2-5 experiments if Bik is a downstream target of c-Myc or is a c-Myc-independent EBNA2 target. Thus, in order to directly ask if c-Myc represses *bik* in the absence of EBNA2, EREB 2.5 cells stably transfected with *c-myc* expression vector was employed. The rationale for this was that P493-6 cells are derived from the same parental EREB 2-5 cells and can be induced to proliferate on a Myc-driven proliferation programme in the absence of the EBV growth programme. Significantly, it was shown that c-Myc can trigger *bik* repression in P493-6 cells in the absence of functional EBNA2. Additionally, repression of exogenous c-*myc* leads to a de-repression of *bik*. Moreover, the steady-state level of *bik* mRNA/Bik protein was considerably lower when cells were cycling due to EBV growth programme when compared to cells proliferating on the *c-myc* programme indicating that the effect of EBNA2 was clearly more potent than c-Myc, and possibly indicating a co-operative role for both EBNA2 and *c-myc* in *bik* repression. This finding is consistent with the hypothesis that the decrease in *bik* is at least in part a consequence of c-Myc activation by EBNA2. Previous studies have identified several other genes as being c-Myc dependent EBNA2 target genes, including eIF5a, PPase, acidic ribosomal protein P0, PA28γ and NM23-H1 (Schlee *et al*, 2004). It was postulated in Chapter 4 that EBNA2-associated repression of *IgM* may mediate the down-regulation of the *bik* gene. EBNA2 down-regulates *sIgM* but the latter is unaffected by c-Myc expression (Pajic *et al*, 2001). Conceivably, then, since EBNA2 modulates both IgM, and c-Myc expression (Jochner *et al*, 1996; Kaiser *et al*, 1999) and since both of these genes can affect *bik* expression (Jiang and Clark, 2001) (Figure 5.2.7) it is possible that EBNA2 mediates the regulation of *bik* in part through both IgM-dependent and c-Myc-dependent pathways. This premise may at least partially explain the considerable differences observed in the levels of *bik* expression when cells are cycling due to EBNA2, (in contrast to cells proliferating due to *c-myc* expression); since EBNA2 would contribute not only to the activation of

c-Myc-mediated *bik* repression (as would be the case in c-Myc-only-driven *bik* repression) but also to the activation of the IgM-associated pathway. Interestingly, EBNA2-mediated regulation of both c-Myc and IgM is only partially CBF1 dependent (Maier *et al*, 2005).

While findings in Chapter 4 (Figure 4.2.5) indicate that the CBF1 interaction may not be required for EBNA2-mediated *bik* repression it should be noted that EBNA2 and the cellular receptor, Notch, have overlapping functions beyond their capacity to interact with CBF1. Significantly, in *trans*-complementation assays carried out in the laboratory (Figure B, Appendix II), *bik* repression was restored in estrogen-starved EREB 2-5 cells following retrovirus transduction with wild-type *EBNA2.* Transduced cells that were selected as expressing high levels of *Notch 1IC*, also showed enhanced transcriptional repression of *bik*. These results further confirmed that *bik* is transcriptionally repressed by the EBNA2-driven growth programme in EBV-immortalised B-cells. Rescue of these cells following retrovirus transduction with either wild-type EBNA2 or Notch 1IC restored *bik* repression. Thus, high levels of Notch can substitute for EBNA2 to repress *bik,* although not to the same extent as EBNA2. EBNA2 may execute notch-like functions to facilitate the regulation of pro-apoptotic *bik.* Although neither the Notch pathway associated protein CBF1 nor the EBNA2-CBF1 interaction are required for EBNA2 repression of *bik* (Chapter 4), it should be considered that CBF1 is not the only common factor connecting Notch and EBNA2 signaling.

The EREB 2-5 cell line, in the presence of EBNA2 is a typical proliferating LCL. However, withdrawal of estrogen results in cessation of cell growth and a significant proportion of the cells die by apoptosis (Kempkes *et al*, 1995a). The gain of expression of this pro-apoptotic EBNA2-regulated gene could contribute substantially to this outcome. Significantly, upon removal of EBNA2 it appears that Bik may be phosphorylated (Figure 5.2.3), an event required for Bik pro-apoptotic activity (Verma *et al*, 2001).

This experiment also adds further substance to the argument that *bik* regulation is not an artefact of estrogen regulation, (Chapter 4). While Bik has been shown to be induced in estrogen-dependent MCF7 breast cancer cells in response to inhibition of estrogen signaling (Hur *et al*, 2004), any correlation between the expression of *bik*

and the presence of estrogen was previously excluded due to the use of the tetracycline-inducible system, DG75-tTA-EBNA2 in Chapter 4. The transduction experiment further confirms this, since the repression of *bik* is due to the transduction of exogenous wild-type EBNA2.

EBV positive tonsillar memory B-cells express a restricted pattern of latent gene transcripts which resembles the pattern of latent gene expression detected in EBV-related tumours (Babcock *et al*, 2000b). One study in which B-cells were LCM-captured from lymphoid follicles from the tonsil, reported elevated *bik* mRNA levels in the proliferating germinal centre (GC) cells, (sites where an activated naïve B-cell, which is responding to foreign antigen during an immune response, undergoes the transition into a long-lived memory B-cell) (MacLennan *et al*, 1988; Liu and Arpin, 1997) relative to non-proliferating naïve cells from the mantle zone (MZ). This finding correlates with an increased sensitivity of GC cells to apoptosis (Shen *et al*, 2004). To assess whether EBNA2 expression was linked to *bik* repression *in vivo* an experiment was undertaken in the laboratory in which PBMC-derived untouched naïve B-cells were infected with EBV. No further decrease in the already low level of *bik* mRNA that was present prior to infection by 96 hours was observed. It may therefore be the case that the EBV growth programme represses the *bik* induction that is seen to occur *in vivo* during the naïve to GC cell transition. This interpretation, however, implies that estrogen-starved EREB 2-5 cells in which *bik* is re-induced concomitant with the onset of apoptosis, do not completely assume the phenotype of a true naïve B-cell. The ability of EBNA2 to down-regulate *bik* may also however be significant in EBV associated malignancies in which EBNA2 is expressed.

Thus, the novel discovery that Bik is repressed in B-cell lines in response to the EBV growth programme has been presented. It is shown the first time that EBV down-regulates expression of the cellular *bik* gene with a key role for the viral transcription factor EBNA2, and possibly the cellular gene c-Myc. This host-virus interaction may be relevant in the prevention of apoptotic death of infected B-cells that are driven to proliferate by EBV.

CHAPTER 6

Ectopic Bik induces Apoptosis in the EBV-infected cell line, IB4.

6.1 Introduction

A fundamental feature of the overall EBV strategy is the modulation of cellular pathways that suppress the cellular apoptotic programme, promoting lymphocyte survival and proliferation (Klein, 1994). The ability of the viral latent proteins to manipulate the balance of *bcl-2* family members has long been recognised, and has been exploited to inhibit cancer in preclinical and clinical settings (Ghobrial *et al*, 2005; Papadopoulos, 2006). Previous work in our laboratory has shown that the anti-apoptotic, *bcl-2* family member, *bfl-1* plays an important role in regulating the survival of EBV-infected B-cells (D'Souza *et al*, 2000) and also has an important role in protecting H/RS cells from apoptosis (Loughran, S.T., manuscript in preparation). Bik is pro-apoptotic with a death-promoting activity (Boyd *et al*, 1995; Tong *et al*, 2001). Bik has been implicated in several cancers (Castells *et al*, 2000; Jiang and Clark, 2001; Hur *et al*, 2004; Sturm *et al*, 2006) and exogenous Bik expression can sensitise tumour cells to apoptosis (Daniel *et al*, 1999; Panaretakis *et al*, 2002) making *bik* a potential therapeutic gene in cancer treatment (Zou *et al*, 2002).

It has already been established in chapters 4 and 5 that *bik* is a transcriptional target of EBNA2 and thus has a potential role to play in EBV regulation of cellular apoptosis. This chapter set out to investigate the effect of ectopic Bik in B-cells in which endogenous Bik expression has been repressed by EBV.

6.2 Effects of ectopic Bik on EBV-infected cell lines.

6.2.1 Bik potently inhibits viability of EBV infected cell lines.

In order to elucidate the significance of the loss of Bik expression to the survival of EBV-infected cells in culture, a strategy was devised to substitute for this loss by ectopic expression of Bik in the EBV-infected cell line, IB4. A paired set of expression plasmids for Bik; pcDNA3-HA-*bik* and pcDNA3-HA-*bik*-ΔBH3 were thus procured for this purpose. The pcDNA3-HA-*bik* construct (Figure 6.2.1A), contains the full length *bik* ORF sub-cloned downstream of a sequence encoding the HA peptide (derived from the human influenza hemagglutinin protein) in the mammalian expression vector pcDNA3-HA (Table 2.1-4). HA is a short epitope tag, commonly used for detection of fusion proteins mediated by highly specific anti-HA antibodies. The partner for this vector pcDNA3-HA-*bik*-ΔBH3 (Figure 6.2.1A) contains a deletion of 5 amino acids within the BH3 domain (Figure 6.2.1B).

In order to verify the expression of HA-Bik and HA-Bik-ΔBH3 protein from these constructs, the EBV positive cell line, AG876 was transiently transfected with pcDNA3-HA-*bik* and pcDNA3-HA-*bik*-ΔBH3, the corresponding empty vector (pcDNA3-HA) and a positive control pcDNA3-HA-*bfl-1* in AG876 (Table 2.1-1) by electroporation (Section 2.2.2.7). AG876 is an EBV-positive BL cell line with a low basal level of *bik* expression (Chapter 3, Figure 3.2.2A). Total protein from these cells was prepared at 48 hours after transfection and the presence of HA-tagged Bik and HA-tagged Bik-ΔBH3 at the expected size was confirmed by Western blotting (Section 2.2.4.3) using an anti-HA antibody (Table 2.1-2) (Figure 6.2.1C).

It can be seen that HA-Bik protein was expressed at the anticipated size (27kDA) with the slightly smaller size observed for Bik-ΔBH3 consistent with the pertinent 5 amino acid deletion (Figure 6.2.1C). Western blot analysis revealed that the level of expression of exogenous Bik from the *bik*-transfected cells appeared lower than cells expressing the BH3 deletion mutant. Because ectopic Bik levels are compared to the total β-Actin protein level in each sample, differences in loading densities between samples can be corrected. However, comparing observed differences in expression levels of protein, does not take into account that the cells transfected with *bik* may

also be less viable, and therefore express a lower level of protein than the protein expressed from the mutant partner. Evaluating differences in expression of potentially toxic protein on a per-transfection basis using Western blot analysis does not take into account these differences and may therefore underestimate the abundance of the protein in a particular cell pool. The potentially toxic effect of Bik relative to BH3-deleted Bik may lead to a distortion in the apparent levels of expression of these ectopic proteins. If ectopic Bik (but not BH3-deleted Bik) has a deleterious effect on the transfected cells in which it is expressed, and assuming a transfection efficiency of X%, then 'normalisation' using total β-actin levels would not sufficiently correct for this. Bik would therefore appear to be under-expressed relative to BH3-deleted Bik.

The nucleotide sequence of the HA-*bik* cDNA and the BH3 deletion (ΔBH3) mutant were verified by sequencing (Section 2.2.1.18) using pcDNA3 vector specific forward and reverse primers (pcDNA3-For and pcDNA3-Rev) (Figure 6.2.1B). Sequencing results, received as linear nucleotide sequences, were aligned with the established nucleotide sequence of the human *bik* gene using the multiple-sequence alignment program MultAlin (Table 2.1-5). The Bik ORF demonstrated 100% homology with the published sequence, as obtained from GenBank, (accession number U34584). There was a deletion of 5 amino acids in the pcDNA3-HA-*bik*-ΔBH3 plasmid, confirming the integrity of the BH3 mutant vector. The HA-Tag sequence was also confirmed.

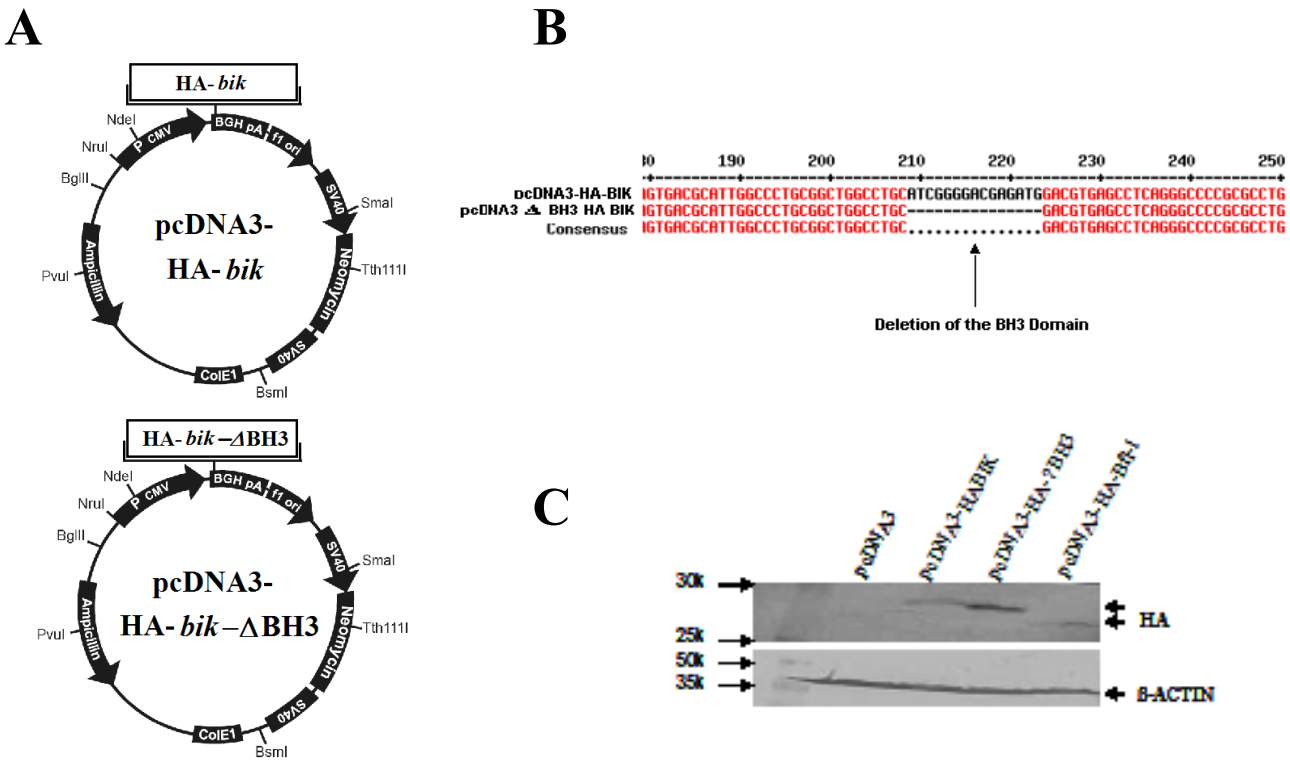


Figure 6.2.1: Detection of ectopic HA-Bik and HA-Bik-ΔBH3 from pcDNA3-HA-*bik* and pcDNA3-HA-*bik*-ΔBH3 plasmids.

(A) Schematic circular maps of the pcDNA3-HA-*bik* expression vector and its mutant partner pcDNA3-HA-*bik*-ΔBH3. The human cytomegalovirus immediate-early promoter (designated as CMV), is suitable for high-level expression in mammalian cells. The plasmids contain the neomycin resistance gene for selection of transfected cell lines (B) Section of Bik ORF sequence highlighting the BH3 domain in pcDNA3-HA-*bik*, and the corresponding deletion of the BH3 domain in pcDNA3-HA-*bik*-ΔBH3. The nucleotide sequence of the HA-tagged *bik* cDNA and the BH3 deletion (ΔBH3) were verified by sequencing using pcDNA3 vector specific forward and reverse primers. Sequence results were aligned with the published *bik* sequence. Sequences highlighted in red demonstrated 100% homology. BH3 domain sequence is highlighted in black. (C) The expression of HA-tagged Bik and HA-tagged Bik-ΔBH3 were confirmed by Western blotting at 48 hours following after transfection using an anti-HA antibody (upper panel) and normalised for β-actin levels (lower panel) using an anti-β-Actin antibody. pcDNA3-HA and pcDNA3-HA-*bfl-1* were used as controls.

6.2.2 Luciferase Expression Assay.

It was next set out to examine the effect of exogenous Bik expression on a variety of EBV-infected cell lines. A cell viability assay, which relies on the expression of a reporter gene in transiently transfected cells as a measure of their viability has previously been reported (Kumar *et al*, 1994; Boyd *et al*, 1995; Hsu *et al*, 1995; Han *et al*, 1996a; Mayo *et al*, 1997; Heussler *et al*, 1999). Thus, a co-transfection luciferase (*luc*)-based assay as an index of cell survival (Mathai *et al*, 2002; Li *et al*, 2003; Lan *et al*, 2007) was adapted here to examine the potential inhibitory effect of HA-*bik* on the viability of LCLs *in vitro*.

In this assay, different EBV-infected cells were transiently co-transfected with 2µg of pCMV-*Luc,* which carries a *luc* reporter gene driven by the cytomegalovirus (CMV) immediate-early promoter and 5µg of pcDNA3-HA-*bik,* pcDNA3-HA-*bik*-ΔBH3 or pcDNA3HA. Forty-eight hours after transfection, cells were harvested, and *luc* activity was measured using the *Luc* assay system (Promega) (Section 2.2.2.13). The relative activities were calculated by setting the *luc* activities obtained from the control plasmid, pcDNA3-HA, at 100%.

It can be seen from Figure 6.2.2A that exogenous Bik was consistently and potently associated with decreased *luc* levels, indicating inhibited cell viability in all three transfected EBV infected cell lines. An examination of the growth-inhibitory effect of Bik on the different cell lines: AG876; a BL type 3 cell line (18.2%), and the LCLs EREB2.5; (22.9%) and IB4; (12.0%) suggests that the delivery of HA-*bik* may alleviate the protection provided by the elevated anti-apoptotic phenotype imposed on the cell due to EBV infection. Expression of HA-Bik was coincident with a large decrease in the levels of co-expressed *luc* and the reduction in *luc* values was seen to be considerably less when the BH3-domain deleted Bik was expressed AG876 (48.3%), EREB2.5 (47.2%) and IB4 (29.1%) and virtually absent when the anti-apoptotic Bfl-1 was substituted for Bik in the same experiment (AG876; 95.0%). These results indicate that Bik may have an anti-survival effect on B-cell lines proliferating due to the EBV growth programme, since co-expression of Bik resulted in a marked reduction in the number of *luc*-expressing cells. In addition the Bik BH3 domain may play a crucial role in this death promoting activity since the reduction in

luc values was seen to be considerably less when the BH3-domain deleted Bik was expressed.

In a second transfection, it was decided to examine if the negative effect on *luc* activity was dependent on the level of co-transfected *bik* expression plasmid. To this end, AG876 cells were co-transfected with 2µg CMV-*Luc* and increasing amounts (0, 0.1, 0.5, 1, 2, 3, 5, or 7µg) of pcDNA3-HA-*bik* or pcDNA3-HA-*bik*-ΔBH3. Forty-Eight hours after transfection, cells were harvested and *luc* activity was assayed as before. The dose dependent response was calculated against the *luc* activity of the data point with the corresponding quantity of pcDNA3-HA, defined as 100% as before.

Overall, it was observed that ectopic *bik* exerted a consistently more toxic effect than the BH3–deleted derivative suggesting that the BH3 domain is crucial to death-promoting activity of *bik* in this cell background, as has previously been observed in other cell backgrounds elsewhere. This finding suggests that these cells maybe dying by apoptosis (Tong *et al*, 2001; Letai *et al*, 2002).
Strikingly at a ratio of one tenth the amount of *luc* plasmid, *bik* expression vector decreased *luc* expression levels to 7.11% of the control values, compared to 77.5% observed with the equivalent quantity of the BH3-deleted mutant (Figure 6.2.2B). Interestingly, the growth inhibitory effect of *bik* appears to increase in the presence of decreasing amounts of the HA-*bik* expression plasmid. *Luc* activity decreased by 14-fold (to 7.11%) with the addition of 0.5µg of pcDNA3-HA-*bik*. Any further increase in the level of this plasmid did not lead to any further reduction in *luc* levels. Indeed at quantities of 1, 2, 3, and 5µg the *luc* activity actually increased the *luc* activity to an average 23.66% of the control.

In general these results indicate that 0.5µg of pcDNA3-HA-*bik* may be the optimal quantity of vector required to achieve maximal potency under the conditions used. Bik expression has an anti-survival effect on B-cell lines proliferating due to the EBV growth programme, and a consistently more toxic effect than pcDNA3-HA-*bik*-ΔBH3 at all quantities suggesting that this mutation alters the death-promoting activity of *bik* in this cell background.

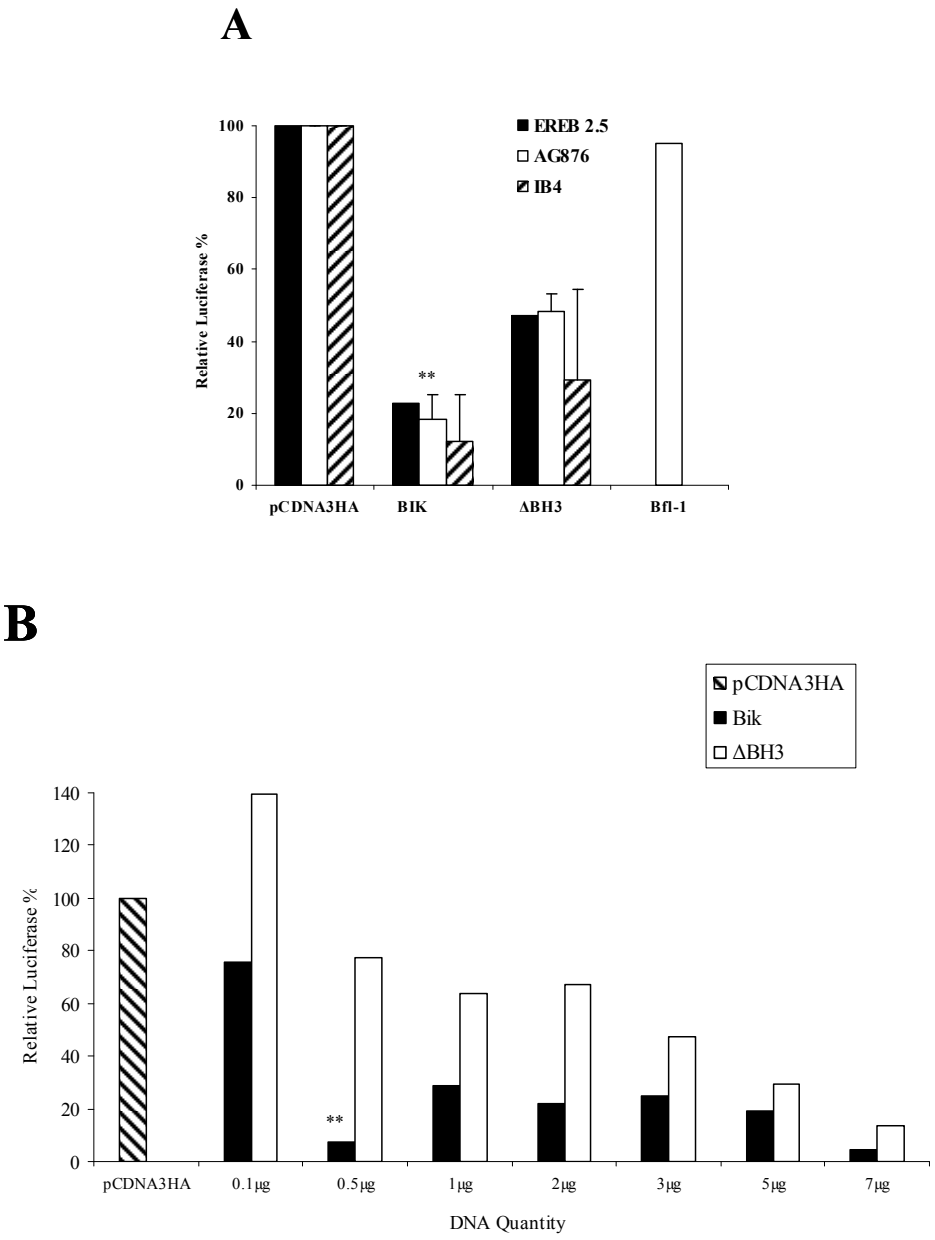


Figure 6.2.2: *Luc* expression assay to measure the effect of ectopic expression of Bik in EBV-infected cell lines.

(A) In order to determine the effect of Bik expression on cell viability different EBV-infected cells were transiently co-transfected with 2µg of pCMV-*Luc,* and 5µg of pcDNA3-HA-*bik* or pcDNA3-HA-*bik* ΔBH3. pcDNA3-HA was used as a control vector. The total amount of DNA transfected was kept constant by adding an appropriate amount of pcDNA3-HA vector. Forty-eight hours after transfection, cells were harvested, and *luc* activity was measured using the *Luc* assay system. The relative activities were calculated by setting the *luc* activities obtained from the control plasmid, pcDNA3-HA, at 100%. (B) To determine the Bik dose effect on cell survival, AG876 was co-transfected with 2µg of CMV-*Luc* and increasing amounts of pcDNA3-HA, pcDNA3-HA-*bik* or pcDNA3-HA-*bik*-ΔBH3 (as indicated on the

graph). The total amount of DNA transfected at each dose was again kept constant by adding an appropriate amount of pcDNA3-HA vector. Cells were harvested 48 hours after transfection, and *luc* activity was measured as before. The relative activities were calculated by setting the Luc activities obtained from transfections with pcDNA3 at 100% (Data are Mean ±SD, ** P<0.01).

6.2.3 The effect of maintenance of Bik expression in EBV-infected cell lines.

pcDNA3-derived vectors can be used to select for transformants by exploiting their encoded geneticin resistance function. To facilitate the generation of stable cell lines, a geneticin drug curve was performed to establish the minimum concentration of drug to cause cell death in this cell line. Geneticin at a concentration of 600μg/ml optimally kills IB4 cells over a twelve day period (data not shown) and was thus selected as a suitable working concentration. In transfection experiments, attempts to develop genetecin-resistant IB4 (Table 2.1-1) cell pools that stably expressed ectopic HA-Bik repeatedly met with failure, whereas the cell pools generated with pcDNA3-HA, pcDNA3-HA-*bik*-ΔBH3 and pcDNA3-HA-*bfl1* did not (not shown). Further attempts to establish a stable cell line expressing HA-tagged *bik* in another cell line L428, met with the same outcome.

6.2.4 Inducible expression of Bik to examine the effect of Bik expression in EBV-infected cell lines.

Given the failure to establish stable cell pools expressing HA-tagged *bik* and taking into account the finding that transient expression of Bik affects cell viability in several EBV-infected cell lines (Section 6.2.1) it was reasoned that a plasmid capable of inducible Bik expression, might be the most amenable to examining the effect of Bik over-expression on a larger cell pool. The tetracycline-regulatable plasmid pRTS-1 (Table 2.1-4), a novel EBV-derived episomally replicating plasmid that carries all the elements for conditional expression of a gene of interest was used for this purpose (Bornkamm *et al*, 2005). In this plasmid, the gene of interest is expressed from the bidirectional promoter P_{tet}bi-1 (Figure 6.2.3) that allows simultaneous expression of two genes, one of which may be used as marker for the expression of the gene of

interest. Tight down regulation is achieved through binding of the silencer tTS_{KRAB} to P_{tet}bi-1 in the absence of tetracycline (or doxycycline). Addition of tetracycline releases repression and via binding of rtTA2s-M2 activates P_{tet}bi-1. The expression cassette contained on the plasmid conferring tetracycline responsiveness is composed of the chicken-β-actin promoter (CAG) flanked by the mouse Ig heavy chain intron enhancer (Eμ) and drives expression of the two genes encoding rtTA2s-M2 and tTS_{KRAB} separated by an internal ribosomal entry site (IRES). The Eμ intron enhancer was included for efficient expression of tetracycline-controlled regulators in B-cells and makes this plasmid especially suited to the study of B-cell lymphomagenesis.

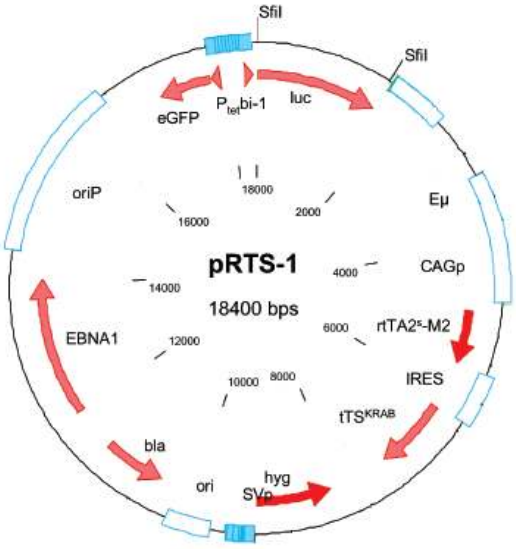


Figure 6.2.3: Schematic map of pRTS-1 vector

Schematic circular map of pRTS-1 vector (From Bornkamm *et al*, 2005). The *luciferase* gene is designated as luc. The bicistronic expression cassette driving expression of rtTAs-M2 and tTSKRAB, separated by an internal ribosomal entry site (IRES), is placed behind the chicken β-actin intron and transcribed from a promoter/enhancer consisting of the mouse heavy chain intron enhancer (Eμ) and the chicken β-actin promoter (CAGp). Ptetbi-1 denotes the bidirectional tetracycline-regulated promoter and oriP the EBV episomal origin of replication. EBNA1, the EBV gene EBNA1; bla, β-lactamase; SVp, the SV40 early promoter; ori, the bacterial origin of replication derived from pMB1; and hyg, the hygromycin phosphotransferase gene.

6.2.4.1 Cloning of pRTS-1-HA-*bik*

In order to generate the tetracycline-responsive HA-tagged Bik and HA-tagged-Bik-ΔBH3 expression plasmids, pRTS-1-HA-*bik* and pRTS-1-HA-*bik*-ΔBH3, a cloning strategy was employed which involved the PCR amplification of the target sequence

and its subsequent sub-cloning into pRTS-1 in place of the *luc* gene. A schematic diagram outlining the construction of the vectors is illustrated in Figure 6.2.4. The steps involved in the generation of pRTS-1-HA-*bik* are illustrated in Figure 6.2.5. The vector was firstly characterised by restriction digestion in order to verify its identity (Section 2.2.1.3). Prior to ligation, the plasmid was linearised by digestion with *Sfi* I to excise the *luc* gene (Figure 6.2.5A), followed by dephosphorylation using CIP to prevent recircularisation of the linearised DNA, or re-cloning of the *luc* ORF (Section 2.2.1.4). The linearised, CIP-treated DNA was then purified by phenol:chloroform extraction followed by ethanol precipitation (Section 2.2.1.2).

pcDNA3-HA-*bik* and pcDNA3-HA-*bik*-ΔBH3 were used as template DNA for PCR in conjunction with a forward primer (HABikForSfiI) with HA tag sequence homology and a reverse primer (BikRevSfiI) specific to the region downstream of the *bik* termination codon (sequences in Table 2.1-5). The primers included *Sfi* I restriction sites for cloning. Following PCR, the products were analysed by agarose gel electrophoresis (Section 2.2.1.15) to check for migration of fragments at the expected size. The PCR products were subsequently purified using the Promega Wizard® DNA purification system (Section 2.2.1.6), digested with *Sfi* I and purified by phenol: chloroform extraction followed by ethanol precipitation (Figure 6.2.5B). The individual digested, purified PCR products were then sub-cloned into the *Sfi* I sites of pRTS-1. Ligations of vector and insert DNA were carried out overnight at 16 ˚C (Section 2.2.1.7).

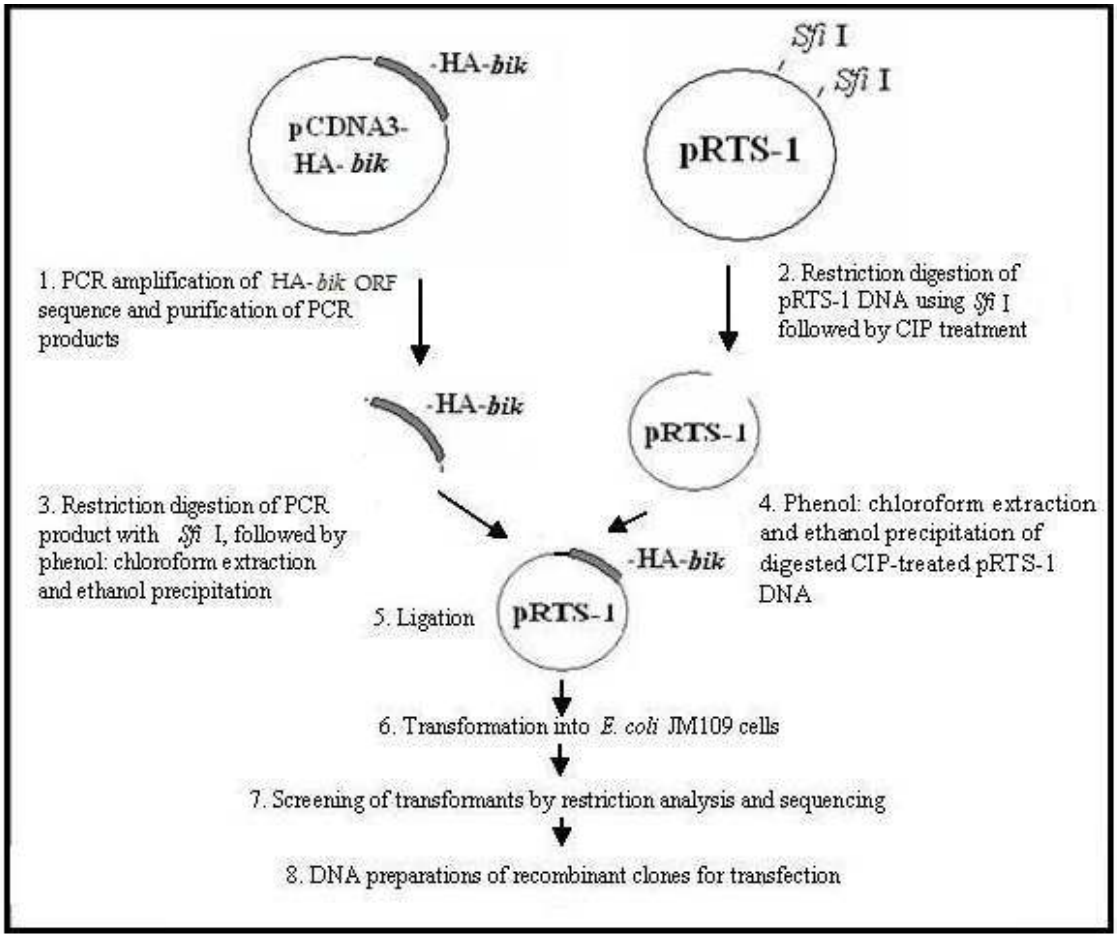


Figure 6.2.4: Cloning strategy

Flow diagram detailing the steps involved in the construction of pRTS-1-HA-*bik* and pRTS-1-HA-*bik*-ΔBH3 constructs. The diagram represents the generation of pRTS-1-HA-*bik* but is reflective of the strategy used in the construction of both pRTS-1-HA-*bik* and pRTS-1-HA-*bik*-ΔBH3. See text for details.

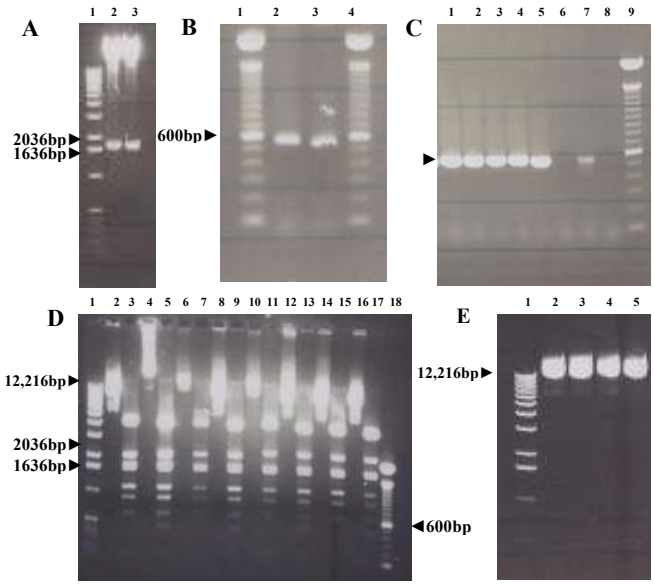


Figure 6.2.5: Construction of the pRTS-1-HA-*bik* and pRTS-1-HA-*bik*-ΔBH3 constructs.

(A) Restriction digestion of pRTS-1 with *Sfi* I excised the *luc* gene (1652bp), as expected in lanes 2 and 3. A 1kb ladder is seen in lane 1. Fragments were analysed using a 0.7% agarose-1X TAE gel and electrophoresis was carried out at 100V for 1 hour in 1X TAE. (B) PCR products amplified from pcDNA3-HA-*bik* and pcDNA3-HA-*bik*-ΔBH3 using forward and reverse primers; HABikForSfiI and BikRevSfiI, were and subsequently digested with *Sfi* I restriction endonuclease, in preparation for ligation, and were then analysed by agarose gel electrophoresis on a 2% agarose-1X TAE gel and electrophoresis was carried out at 100V for 1 hour in 1X TAE. Amplification and subsequent digestion with the *Sfi* I restriction enzyme of the HA-*bik* fragment resulted in a band at 590 bp (lane 2), and a fragment of 575bp in the case of HA-*bik*-ΔBH3 (lane 3). A 100 bp DNA ladder (Invitrogen) is shown in lanes 1 and 4. (C) Recombinant DNA was subject to PCR amplification using the pEBNAseqFor forward primer and the BikRevSfiI reverse primer, to check for orientation. Fragments were analysed by agarose gel electrophoresis on a 2% agarose-1X TAE gel and electrophoresis was carried out at 100V for 1 hour in 1X TAE. A PCR product was generated if the insert DNA had ligated in the forward orientation. Amplification of the pEBNA-HA-*bik* fragment resulted in a band of 597bp (lanes 1-4 correct orientation), while amplification of the pEBNA-HA-*bik*-ΔBH3 resulted in a fragment at 582bp (lanes 5 and 7 correct orientation, lanes 6 and 8 incorrect orientation) (D) Restriction analysis of recombinant pRTS-1-HA-*bik* and pRTS-1-HA-*bik*-ΔBH3 clones to determine correct insert orientation by digestion using *Xma* I enzyme followed by agarose gel electrophoresis on a 1% agarose-1X TAE gel. Correct insert orientation would yield ten fragments of 4236bp, 2220bp, 1729bp, 1641bp 1122bp, 916bp, 639bp, 454bp, 157bp and 59bp while incorrect orientation would produce ten fragments of 4236bp, 2220bp, 1729bp, 1641bp 1122bp, 916bp, 639bp, 157bp, 152bp and 59bp upon digestion. A 1kb ladder is seen in lane 1 and a 100bp ladder is seen in lane 18. Undigested plasmid for each sample analysed is seen in lanes 2, 4, 6, 8, 10, 12, 14 and 16. pRTS-1-HA-*bik* clones (lanes 2-9) harboured the HA-bik fragment in

the correct orientation as discerned by the presence of a digested fragment at 454bp (lane 5 and 9), which was absent from clones with the fragment ligated in the wrong orientation (lanes 3 and 7). While pRTS-1-HA-*bik*-ΔBH3 (lanes 10-17) clones were also discerned by this pattern (lanes 13 and 17 positive clones, lanes 11 and 15 display fragments in the incorrect orientation). (E) The condition and quality of the DNA was assessed by resolving the DNA by agarose gel electrophoresis. In this case 1μg of DNA from the pRTS-1 (lane 2), pRTS-1-HA-*bik* (lane 3) and pRTS-1-HA-*bik*-ΔBH3 (lane 4) and pRTS-1-HA-*bfl-1* (lane 5) expression plasmids are shown after electrophoresis at 100V for 1 hour through a 1% agarose gel using 1X TAE. A 1kb ladder is shown in lane 1.

Following the ligation reaction, competent *E. coli* JM109 cells were transformed with the recombinant plasmid DNA. Single colonies were used to inoculate cultures from which DNA mini-preparations were prepared. Restriction digestion analysis and a PCR assay were performed on the DNA to monitor for the presence of insert DNA. Although both *Sfi* I sites used for cloning were different in base-pair composition it was possible for the insert DNA to ligate into the vector in either the forward or reverse orientation as the overhangs produced are palindromes. To determine whether insert DNA had ligated in the forward orientation, a PCR based strategy was first employed using the pEBNAseqFor forward primer (homologous to region upstream of start codon of pRTS-1) and the BikRevSfiI reverse primer used in PCR amplification of the HA-*bik* /HA-*bik*-ΔBH3 fragments (sequences in Table 2.1-5). A PCR product was generated if the insert DNA had ligated in the forward orientation (Figure 6.2.5C). Correct/incorrect insert orientation was also differentiated by restriction analysis using *Xma* I restriction enzyme (Figure 6.2.5D). Once it was established that both the HA-*bik* and the HA-*bik*-ΔBH3 sequences had ligated into pRTS-1 in the correct orientation, DNA maxi-preparations were carried out using the Qiagen® Midi Kit (Section 2.2.1.12) and the identity of the cloned sequence was verified by DNA sequencing (Section 2.2.1.18) using the pEBNAseqFor primer. Both the pRTS-1-HA-*bik* and the pRTS-1-HA-bik-ΔBH3 derived plasmid DNA demonstrated 100% homology with the predicted sequences as derived from the known HA-tag and *bik* (GenBank accession number U34584) coding sequences (not shown). The condition and quality of the recombinant DNA was subsequently assessed by resolving the DNA by agarose gel electrophoresis at 100V for 1 hour in 1X TAE, and as Figure 6.2.5E reveals DNA used in transfection analysis was in the same topological condition and at similar concentration.

In order to test expression of HA-tagged Bik and HA-tagged Bik-ΔBH3, transient transfection of the pRTS-1-HA-*bik,* pRTS-1-HA-*bik*-ΔBH3, pRTS-1-HA-*bfl1* (Table 2.1-4) and the corresponding empty vector pRTS-1 was carried out in various EBV negative cell lines including DG75, BJAB and AKATA-4E3 cells and in the highly transfectable human embryonic kidney cell line HEK 293 (Table 2.1-1). Cell pools were induced to express their respective proteins by addition of tetracycline (1μg/ml), or not induced by addition of an equal volume of vehicle (100% ethanol) to the culture media. Total protein extracts of induced cell pools were examined for the expression of HA-tagged proteins by Western blotting at 48 hours after addition of tetracycline (Figure 6.2.6). Attempts to detect HA-tagged Bik and HA tagged Bik-ΔBH3 proteins from the harvested cell pools consistently failed however. The expression of HA-tagged Bik and HA-tagged Bik-ΔBH3 were successfully detected from pcDNA3 constructs.

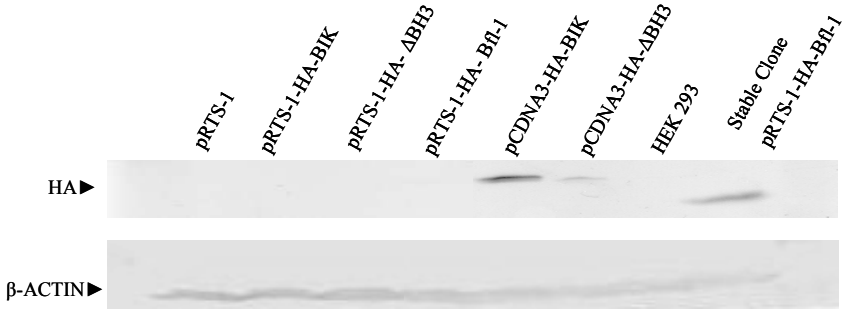


Figure 6.2.6: Western blot analysis of the transiently-transfected cell line HEK-293.

To test the expression of HA-tagged Bik and HA-tagged Bik-ΔBH3 transient transfection of the pRTS-1-HA-*bik,* pRTS-1-HA-*bik*-ΔBH3, pRTS-1-HA-*bfl1* and the corresponding empty vector pRTS-1 was carried out in HEK 293 cells and various EBV-negative cell lines. The pcDNA3 derived constructs, pcDNA3-HA-*bik* and pcDNA3-HA-*bik*-ΔBH3 were also transfected as controls. The expression of the respective HA-tagged proteins was examined by Western blotting at 72 hours after addition of tetracycline using an anti-HA antibody (upper panel) and normalised for β-actin levels (lower panel) using an anti-β-Actin antibody. The Stable clone L428-pRTS-1-HA-*Bfl-1* was used as control for the anti-HA antibody.

In a second series of transfections, the same plasmids were also used to transfect the DG75 cell line (Table 2.1-1) by electroporation (Section 2.2.2.7) followed by selection of positive clones using hygromycin (400μg/ml). DG75 is a readily transfectable, EBV-negative BL cell line, it was once again apparent that cells transfected with pRTS-1-HA-*bik* gave rise to no drug resistant cells as compared to cells transfected with control vector or other constructs. As before, transfected cell pools were induced or non-induced to express their respective proteins. Total protein from these cells was prepared at 48 hours after induction and the extracts were subject to Western blotting (Section 2.2.4.3). Again, the cells transfected with pRTS-1-HA-*bik* cells did not express detectable levels of HA-tagged Bik, in contrast to cells transfected with pRTS-1-HA-*bik*-ΔBH3 and pRTS-1-HA-*bfl1*. It was plausible that a relatively low level of expression of Bik protein from a leaky tet-inducible promoter on the pRTS-1 vector was lethal to the transfected cell population, causing all *bik*-transfected cells to die. In short it seemed likely that these cells could not support Bik expression, providing further, yet indirect evidence that low levels of ectopic Bik was toxic to these cells. Despite several attempts, all efforts to establish stable positive clones of pRTS-1-HA-*bik* in EBV negative (DG75 (Figure 6.2.7A), BJAB (Figure 6.2.7B), AKATA-4E3) and EBV positive cell lines (EREB2.5, IB4, LCL 327) by hygromycin selection were futile. Strikingly however it proved possible to establish both pRTS-1-HA-*Bfl-1* and pRTS-1-HA-*bik*-ΔBH3 stable cell pools expressing HA-tagged proteins (HA-Bfl-1 and HA-ΔBH3) every time. On the basis of this data and also considering that it was possible to establish stable cell pools using pcDNA3-HA-*bik*-ΔBH3 and pcDNA3-HA-*bfl-1* but not pcDNA3-HA-*bik* in IB4 and L428, it was clear that the HA-Bik expressing vectors imposed a powerful negative selection on their host cells and HA-*bik*-transfected cells ultimately died. It should be noted that HA-Bik protein could be detected from the original vector, pcDNA3-HA-*bik* in transient transfection experiments (Figure 6.2.1C) and that the fidelity of the recombinant pRTS-1 cloned vector was confirmed by sequencing analysis (data not shown); all indicative of the production of a functional protein. Additionally, further attempts to express a second tagged Bik protein (MYC-Bik) failed from constitutive (pcDNA3) and inducible (pRTS-1) backgrounds (data not shown).

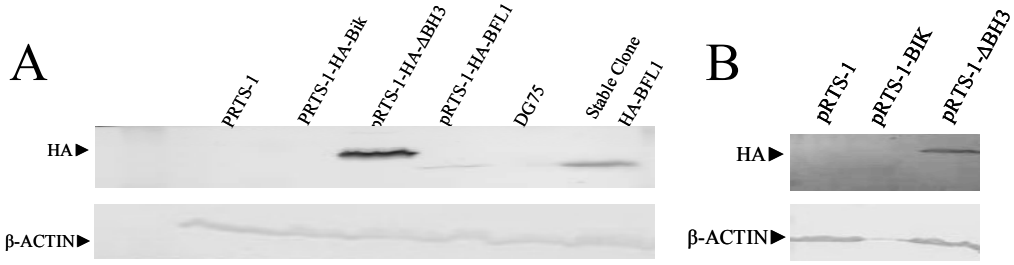


Figure 6.2.7: **Western Blot analysis of the stably-transfected EBV-negative cell lines, DG75 and BJAB**.

(A) To examine the expression of HA-tagged Bik and HA-tagged Bik-ΔBH3 stable transfection of the pRTS-1-HA-*bik,* pRTS-1-HA-*bik*-ΔBH3, pRTS-1-HA-*bfl1* and the corresponding empty vector pRTS-1 was undertaken in the BL DG75 cells by electroporation followed by selection of positive clones using hygromycin (400μg/ml). After a 12 day period of selection DG75-pRTS-1-HA-*bik,* DG75-pRTS-1-HA-*bik*-ΔBH3, DG75-pRTS-1-HA–*bfl-1* and DG75-pRTS-1 clones were induced, by addition of tetracycline (1μg/ml), to express their respective proteins. The expression of HA-tagged Bik and HA-tagged Bik-ΔBH3 were examined by Western blotting at 72 hours after addition of tetracycline using an anti-HA antibody (upper panel) and normalised for β-actin levels (lower panel) using an anti-β-Actin antibody. The Stable clone pRTS-1-HA-*Bfl-1* was used as control for the anti-HA antibody. (B) This experiment was also undertaken in the BL BJAB cell line, as in (A).

Co-expression of GFP from the bi-directional promoters of pRTS-1-HA-*bik*-ΔBH3 and pRTS-1 upon induction with tetracycline was also confirmed using fluorescence microscopy (Section 2.2.2.11) (Figure 6.2.8A).

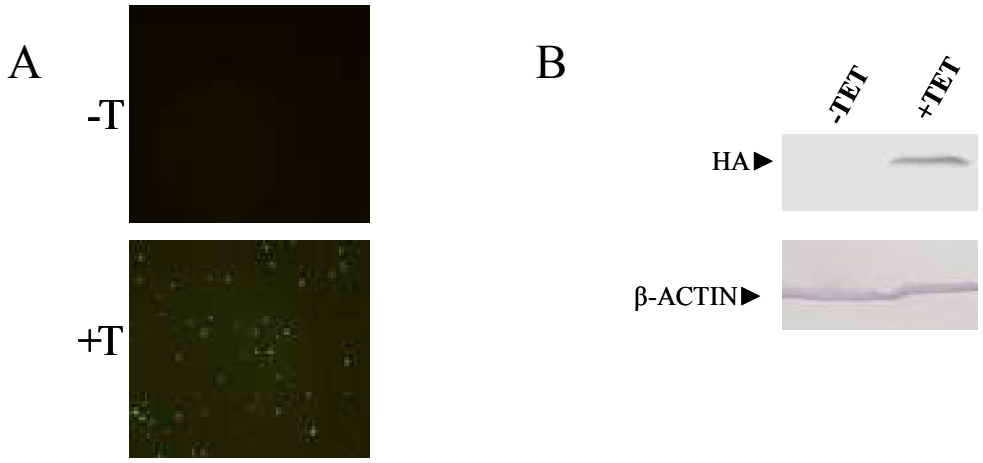


Figure 6.2.8: Ectopic expression of HA-tagged and GFP proteins from DG75 transfected cell pools.

DG75-pRTS-1-HA-*bik*-ΔBH3 cell pools were induced or non-induced by addition of tetracycline to express HA-*bik*-ΔBH3 and GFP proteins. (A) The induction of GFP protein expression was monitored at 48 hours using an Olympus DP-50 fluorescent microscope. (B) Extracts of tetracycline induced/non-induced cell pools were examined for the expression of HA-tagged proteins by Western blotting at 48 hours after addition of tetracycline using an anti-HA antibody (upper panel) and normalised for β-Actin levels (lower panel) using an anti-β-Actin antibody.

These results further confirmed the expression of HA-tagged Bik-ΔBH3 protein from pRTS-1-HA-*bik*-ΔBH3 construct in DG75 cells.

It was next decided to examine the expression of the co-expressed marker, GFP, by fluorescence activated cell-sorting (FACS), to determine if these cells transfected with pRTS-1-HA-*bik* were actually dying due to Bik over-expression. The failure to establish transfected cell pools that could be induced to express Bik, due to its own likely toxicity towards the cell, meant that such cell phenotype assays had to be performed on transiently transfected cells. Initially, the EBV-infected cell lines, EREB2.5 and IB4, were transfected by electroporation (Section 2.2.2.7) with 0.5μg of pRTS-1-HA-*bik,* pRTS-1-HA-*bik*-ΔBH3, pRTS-1-HA-*bfl-1* or the corresponding control construct pRTS-1. Cells were harvested 12 hours after transfection (Section 2.2.2.12). GFP fluorescence was measured on a BD FACSCalibur flow cytometer and CellQuest software was employed for data analysis. No GFP expression was observed upon transfection of IB4 or EREB2.5 cells with any of the pRTS-1

constructs as is represented in Figure 6.2.9, which illustrates the percentage transfection efficiencies achieved in EREB2.5. This experiment was repeated with various quantities of DNA (0.5 – 5μg) but to no avail. Electroporation parameters (voltage setting (200 – 270 V), volume of media in which cells were electroporated (200 – 300μl and supplemented serum/serum-free media), incubation times and duration of transfection were also varied but the cells remained adverse to efficient pRTS-1 uptake by electroporation (data not shown).

In an effort to overcome this obstacle, a range of different EBV negative (DG75, BJAB and L428) and EBV positive cell lines (EREB2.5, IB4, LCL 327) were tested in an attempt to efficiently deliver the pRTS-1 DNA to the cells for the purpose of examining GFP expression. Again, the extent of transfection efficiency was generally too inadequate to permit subsequent phenotypic assays by flow cytometry (usually <1%). A modest transfection efficiency was observed using the IB4 cell line (4.61% pRTS-1). However, the level of transfection efficiency was not sufficient for the purpose intended, since the level of transfection of pRTS-1-HA-*bik* was never more than 0.5% (data not shown). Transfection conditions were again modified as before, and another transfection method was employed (DEAE-Dextran, Section 2.2.2.10) in an attempt to improve efficiency, but no further progress was made.

Next, a modified electroporation technique known as nucleofection (Section 2.2.2.8), which benefited from optimised cell line-specific electroporation parameters together with a cell line-specific buffer, was employed in an attempt to realise a sufficient efficiency of transfection. Satisfactory expression of GFP however was still unattainable. In a parallel experiment, nucleofection with the GFP-reporter plasmid, pmaxGFP revealed a transfection efficiency of 19.36% when transfected IB4 cells were analysed for GFP expression and 51.78% when transfected BJAB cells were examined by FACS analysis (data not shown), indicating the efficacy of transfection seen in this experiment may be specifically due to the properties and quality of pRTS-1 DNA molecules. Recent studies have suggested that the purity of plasmid DNA strongly influences transfection efficiency since contamination with endotoxins may inhibit transfection in various cell lines (Weber *et al*, 1995). In order to improve transfection efficiency, a protocol to isolate endotoxin-free plasmid DNA was

employed (Section 2.2.1.13). However, elimination of pyrogenic endotoxins from the plasmid preparation did not correlate with an improvement in transfection efficiency. In summary, in spite of numerous attempts, all efforts to efficiently transfect pRTS-1 vectors and examine the expression of GFP in several EBV negative (DG75, BJAB, L428) and EBV positive cell lines (EREB2.5, IB4, LCL 327) were ineffective, most likely due to the poor uptake of plasmid DNA during electroporation. It is conceivable that the transfection efficiency was negatively affected by the relatively large size of a the plasmid DNA (18kb) (Kreiss *et al*, 1999). Recognising that an efficient delivery protocol for *bik* was an essential step in this experiment, and that this objective seemed unattainable in the pRTS-1 vector background, it was decided not to proceed with these vectors for these experiments. A co-transfection strategy involving pmaxGFP with pcDNA3-HA-*bik,* pcDNA3-HA-*bik*-ΔBH3 or the corresponding control vector pcDNA3, was therefore adopted in order to realise this goal.

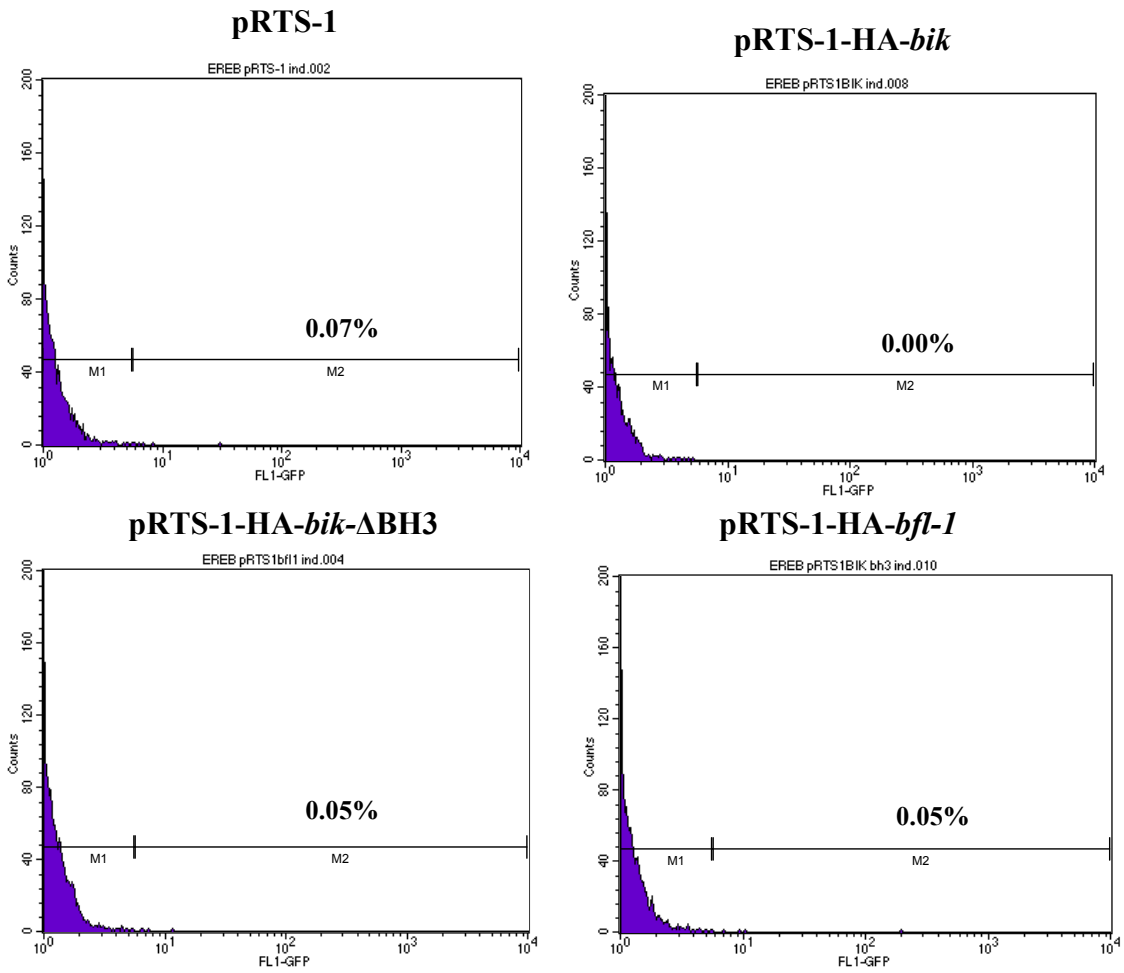


Figure 6.2.9: **No GFP expression was observed upon transfection of EREB2.5 cells with the pRTS-1 constructs.**

EREB2.5 cells, were transfected by electroporation with 0.5µg of pRTS-1-HA-*bik,* pRTS-1-HA-*bik*-ΔBH3, pRTS-1-HA-*bfl-1* or the corresponding control construct pRTS-1. For analysis of GFP fluorescence the cells were washed twice with PBS and re-suspended in PBS before analysis. GFP fluorescence was measured on BD FACSCalibur flow cytometer 12 hours after transfection. CellQuest software was employed for data analysis. The percentage of transfected cells 12 hours after transfection is indicated inside the box.

6.2.5 Green Fluorescent Protein (GFP) Expression Assay

A second cell viability assay was employed in which the expression of intracellular GFP in transfected IB4 cells was used as a measure of cell viability (adapted from Heussler *et al*, 1999). This assay was carried out using the pmaxGFP expression vector, which encodes the green fluorescent protein (GFP) from copepod *Pontellina plumata*. In this experiment, IB4 cells were transiently co-transfected with the pmaxGFP expression plasmid and *bik* or *bik*-ΔBH3-coding plasmids in the pcDNA3 background. The number of cells expressing GFP in the presence of HA-Bik or HA-Bik-ΔBH3 mutant expression was then analysed at 12 hours. Following transfection, cells were washed twice with PBS and re-suspended in PBS at a concentration of 1 X 10^6 cells/ml prior to analysis. GFP fluorescence was measured on BD FACSCalibur flow cytometer. CellQuest software was employed for data analysis and the background level of fluorescence in the IB4 cell line was monitored and used as a template for subsequent analysis Figure 6.2.10A(i).

It is demonstrated in Figure 6.2.10B that when Bik is expressed the number of cells expressing GFP decreases in a dose dependent manner further supporting the hypothesis that Bik expression has a potentially cytotoxic effect on cells. The most obvious observation from the co-transfection experiments was the reduction in the number of GFP-positive cells for the *bik* expression vector compared with empty vector (pcDNA3-HA) or other plasmid constructs (pcDNA3-HA-*bik*-ΔBH3 and pcDNA3-HA-*Bfl-1*), which produced comparable levels of GFP-expressing transfected cells (Figure 6.2.10A(iv)). This observation is representative of at least three independent experiments. The decrease in the number of GFP expressing cells is consistent with the observations made above when a period of drug selection was introduced following transfection (Sections 6.2.3 and 6.2.4) and with the *Luc* assay experiment (Section 6.2.2) and indicates that the biological effects of exogenous Bik expression may require only a relatively short period to take effect. Signifcantly, these *bik*-transfected cells did not express high levels of GFP as compared with those cells transfected with the vector control or other constructs (Figure 6.2.10A(ii) and Figure 6.2.10A(iii)), suggesting that cells that express high GFP levels (and by deduction, high levels of Bik protein), may already be dying or dead. Indeed, microscopic

observations indicated that the majority of the Bik expressing cells were smaller and more condensed as opposed to the morphology of the cells transfected with other constructs (data not shown). This observed effect on IB4 morphology was only seen with the transfection of *bik* and not with the expression of the empty vector or the expression construct carrying the *bfl-1* gene. Furthermore, expression of BH3-deleted Bik cDNA, *bik*-ΔBH3, also failed to show any biological effects upon transfection into IB4 cells, indicating that the *bik*-induced cell death is most likely dependent on the BH3 domain in concordance with previously published findings (Tong *et al*, 2001; Germain *et al*, 2002).

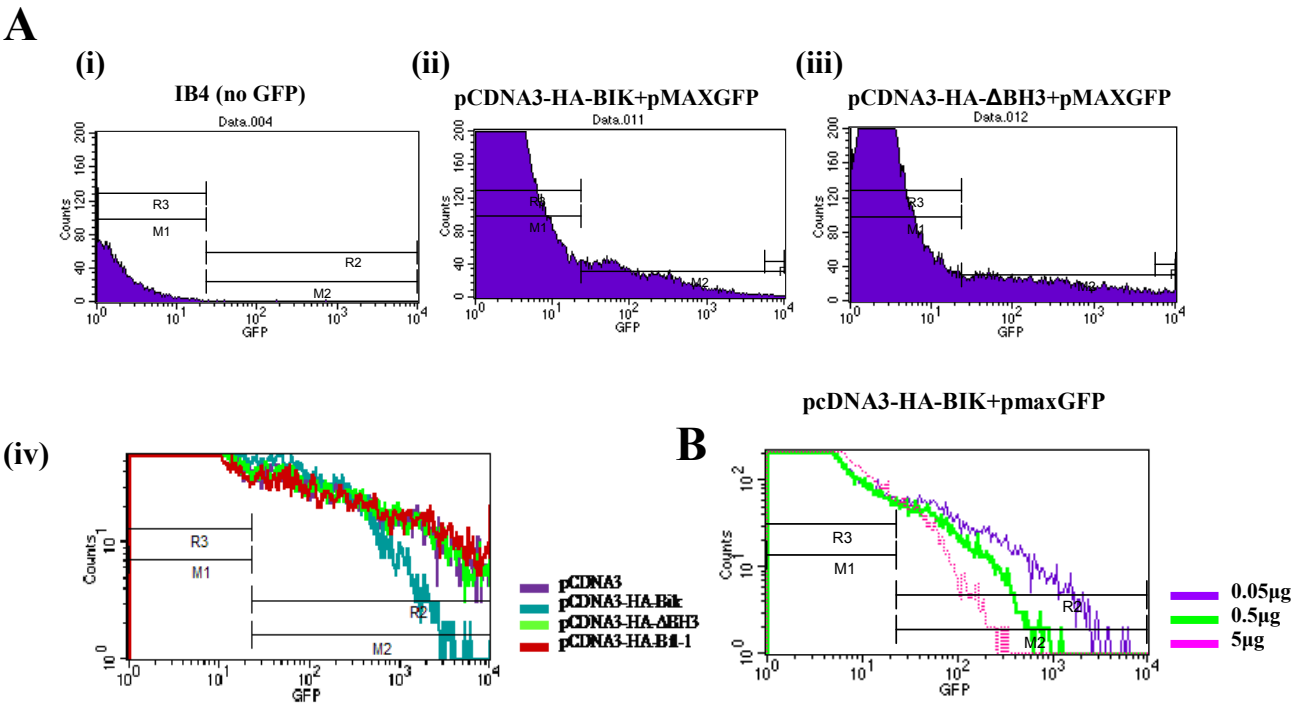


Figure 6.2.10: GFP co-expression in the presence of co-transfected plasmids in IB4.

(A) In order to determine the effect of Bik expression on cell viability, IB4 cells were transiently co-transfected with 2µg of pmaxGFP, and 0.5µg of pcDNA3-HA-*bik* and pcDNA3-HA-*bik* ΔBH3. The vectors pcDNA3 and pcDNA3-HA-*bfl*-1 were used as control vectors. The total amount of DNA transfected was kept constant by adding an appropriate amount of pcDNA3-HA vector. Twelve hours after transfection, cells were harvested, and GFP expression was monitored using FACS analysis. GFP fluorescence was measured on BD FACSCalibur flow cytometer. CellQuest software was employed for data analysis. Figures (A) (i),(ii) and (iii) illustrate the level of GFP expression in mock transfected IB4 cells, and in pcDNA3 and pcDNA3-HA-*bik* transfected cells respectively. Figure (A)(iv) is a comparison of the number of GFP positive cells for *bik,* vector control (pcDNA3-HA), pcDNA3-HA-*bik*-ΔBH3 and

pcDNA3-HA-*Bfl-1*. All observations are representative of at least 3 experiments. (B) To examine the Bik dose effect on cell survival, IB4 was co-transfected with 2µg of pmaxGFP and increasing amounts (0.05, 0.5, 5µg) of pcDNA3, pcDNA3-HA-*bik* and pcDNA3-HA-*bik*-ΔBH3. The total amount of DNA transfected at each dose was kept constant by adding an appropriate amount of pcDNA3 vector. Cells were harvested 12 hours after transfection, and GFP fluorescence was analysed as in (A). These observations are representative of at least 3 experiments.

6.2.6 Ectopic Bik triggers apoptosis in EBV-infected cell lines.

6.2.6.1 MG132 treatment induced apoptosis in the EBV-infected cell line, IB4.

Bik has previously been shown to induce cell death by apoptosis in various cell backgrounds (Han *et al*, 1996a; Tong *et al*, 2001; Germain *et al*, 2002; Hur *et al*, 2004; Oppermann *et al*, 2005). Previous experiments in this chapter have demonstrated that Bik expression is incompatible with IB4 cell viability. Accordingly, it was essential to determine if exogenous Bik could induce death by apoptosis. The premise that ectopic Bik could drive these cells into apoptosis was tested using 7-AAD/Annexin V staining and FACS analysis (Section 2.2.2.16).

A control experiment was initially undertaken to identify the profile of apoptotic cells using 7-AAD/Annexin V staining and FACS. Drastic alterations in membrane structure occur during the apoptotic process (Wyllie *et al*, 1980) principally a progressive loss of membrane permeability that can be evaluated by 7-aminoactinomycin D incorporation (7-AAD) (Schmid *et al*, 1992). This vital dye is excluded from live, healthy cells and early apoptotic cells, but permeates late-stage apoptotic and dead cells that do not have an intact plasma membrane, staining them red, and was detected on the FL3 parameter. Cells were stained with 7-AAD in order to distinguish between cells that were viable (7-AAD-negative) and either dead or in the late stage of apoptosis (7-AAD-positive). Annexin V is a calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine (PS), a membrane component normally localised to the internal face of the cell membrane. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane thus exposing them to the external environment where Annexin V can readily bind to them. The use of Annexin V to monitor the loss of membrane phospholipid asymmetry is thus an indicator of the onset of apoptosis. Annexin V is

detected on the FL2 parameter. The application of Annexin V for PS detection, together with 7-AAD as an indicator of membrane permeability, allows for the easy differentiation of early/late apoptotic, necrotic/nuclear debris and viable cells by FACS analysis. This two-dye strategy allows for identification of these four populations as follows; Non-apoptotic cells (Annexin V^- and 7-AAD^-), early apoptotic cells (Annexin V^+ and 7-AAD^-) late stage apoptotic and dead cells (Annexin V^+ and 7-AAD^+) and mostly cell debris/apoptotic bodies (Annexin V^- and 7-AAD^+).
Abrogation of proteasome activity in EBV-derived cell lines by chemical treatment with the potent proteasome inhibitor Z-Leu-Leu-Leu-al (MG132) was chosen to directly investigate the profile of apoptotic IB4 cells. Proteasome inhibition has been reported to induce apoptosis that is mediated by caspase activation (Wu *et al*, 1999). MG132 is a specific, membrane-permeable inhibitor of proteasome activity. MG132 has been shown to induce apoptosis in a number of different cell types (Giuliano *et al*, 1999; Almond *et al*, 2001; Emanuele *et al*, 2002; Lauricella *et al*, 2003), thus far however, the effect of MG132 in the IB4 cell line has not been reported.

IB4 cells were treated/untreated with 15μM MG132/DMSO and the cell survival profile was analysed at 24 hours after treatment by 7-AAD/Annexin V staining and subsequent flow cytometric analysis (Section 2.2.2.16). Initially measurements were made using forward light scatter (FSC) and side light scatter (SSC). Forward light scatter provides information on the relative size of individual cells, whereas side light scatter provides information on the relative granularity of individual cells. For the purposes of this study these two parameters are combined to identify the light scatter pattern emitted by a healthy population of cells. A population of lymphocytes was gated on region 1 (R1) using forward (FSC) and side scatter (SSC) (data not shown). These cells were subsequently analysed for apoptosis using 7-AAD/Annexin V staining.

MG132-treated IB4 cells were shown to die by apoptosis using an Annexin V/7-AAD dual stain followed by FACS analysis (Figure 6.2.11A). The sets of data are presented as scatter plots. As can be seen in Figure 6.2.11A within the DMSO treated population of cells, 8.0% of cells were non-viable, appearing in the upper right quadrant (Annexin V^+ and 7-AAD^+). Approximately 92% of cells appeared in the upper right quadrant (Annexin V^+ and 7-AAD^+) at 24 hours after treatment with

MG132. These results show clearly the detection of apoptotic cells using 7-AAD/Annexin V staining in response to treatment of IB4 cells with MG132.

6.2.6.2 MG132 treatment increased the population of IB4 cells in the sub-G_1 phase of the cell cycle.

A second method commonly used to assay apoptosis, the sub-G_1 DNA content method was also used here. The enzymatic cleavage of nuclear DNA is a fundamental event in the apoptotic cascade, being the first irreversible step in the process leading to cell death, creating large numbers of small fragments of DNA (Arends *et al*, 1990). The cellular DNA content can be measured following cell fixation which leads to a partial leakage of degraded DNA within apoptotic cells. As a consequence, apoptotic cells contain reduced DNA content and can be recognised, following staining of cellular DNA with Propidium Iodide (PI) (Nicoletti *et al*, 1991; Afanasyev *et al*, 1993). Apoptotic nuclei appear as a broad hypo-diploid DNA peak which is easily discernable from the narrow peak of cells with a normal diploid DNA content in the red fluorescence channels. Further to the previous results, after Annexin V/7-AAD staining, the percentage of cells in G_0/G_1, S phase and G_2/M of the cell cycle was calculated by subjectively applying markers which provide a measurement of the percentage of cells in each phase. The reduced fluorescence of PI in cells undergoing apoptosis resulted in the appearance of a characteristic sub-G_1 fluorescence peak with respect to the G_0/G_1 cell cycle region. In this experiment, cell extracts from the cell pools cultured in MG132 were analysed. The data is displayed in histogram format with the intensity of fluorescence from the propidium iodide stained cells on the X-axis representing the number of cells.

Thirty minutes prior to FACS analysis the cells were stained with PI (Section 2.2.2.19) to detect total DNA within cells. Samples were collected on the FACCalibur flow cytometer as before, and the percentage of cells in each phase of the cell cycle was calculated and analysed using CellQuest software. Cells with increased sub-G_1 DNA content were considered to be apoptotic. The resultant histogram plot of IB4 cells which have been treated with 15μM MG132 for 24 hours is shown in Figure 6.2.11B. The sub-G_1 population was increased from 13.0% of the cells treated with DMSO to 49.6% of the total cell population after treatment with MG132. These

results comply with the apoptosis assay (Figure 6.2.11A), which also indicates a higher proportion of apoptotic cells (92.30%) in the MG132 treated cell pools relative to the DMSO treated cell pool. Table 6-1 indicates that there is a change in the distribution of the cells within in the cell cycle with a reduction in the number of cells in the synthesis phase from 25.80% to 10.63%. Treated cells also failed to accumulate in G_0/G_1, indicated by the percentage of cells in the G_0/G_1 phase of the cell cycle of treated cells (21.0%) as compared to the DMSO control (38.09%). The G_2/M phases in both populations were comparable. Overall this data indicated that compared with DMSO treated cells, the populations of MG132 treated cells displayed a significant decrease in G_0/G_1 phase and S phases, and a dramatic emergence of a sub-G_1 phase. In summary treatment with MG132 caused cell cycle arrest in the IB4 cell line and an accumulation of cells in sub-G_1, indicative of apoptosis.

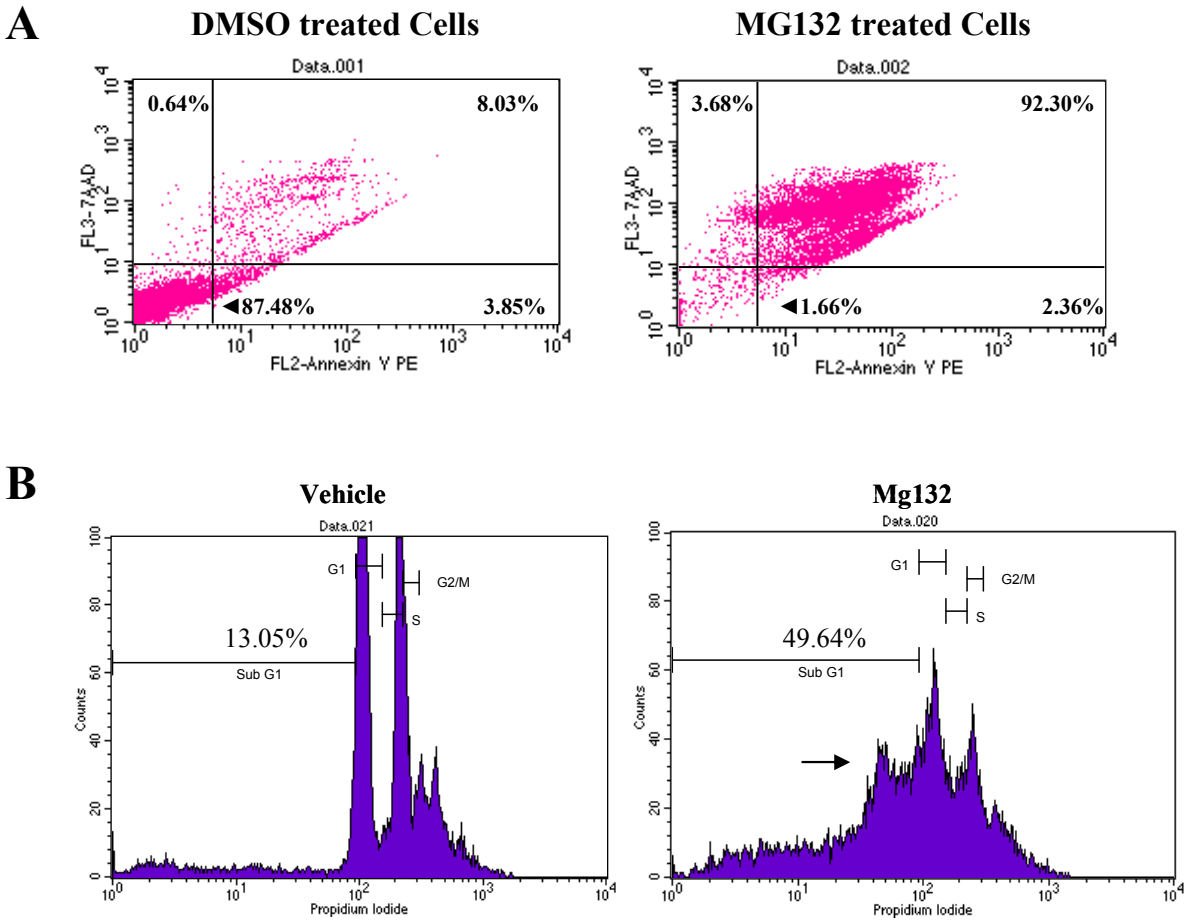


Figure 6.2.11: Treatment of IB4 cells with the proteasome inhibitor, MG132.

(A) IB4 cells were treated with or without 15μM MG132 and the cell survival profile was analysed at 24 hours after treatment by 7-AAD/Annexin V staining and subsequent flow cytometric analysis. Quadrant markers were based on stained/unstained controls and values reflect the percentage of cells in each quadrant. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$/Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$). (B) Apoptosis was assayed using the sub-G_1 DNA content method. Thirty minutes before the assay, cells were stained with PI. Fluorescence-activated cell sorting (FACS) analysis of sub-G_1 was performed by PI staining and flow cytometry. Apoptotic cells determined by sub-G_1 assay in cell samples treated as in (A). The DNA content in the cells was determined 24 hours after treatment. Markers identify cells with hypodiploid DNA content (sub-G_1; indicated by the black arrow), and also those cells in stages G_0/G_1, S, and G_2/M of the cell cycle. The percentage of apoptotic cells 24 hours after treatment is indicated on the left hand side of each box.

Table 6.2-1: Representative flow cytometric profiles of IB4 cells after incubation with MG132.

Treatment	Distribution (% cells)			
	Sub-G_1	G_0/G_1	G_2/M	S
DMSO	13.05	38.09	10.23	25.77
MG132	49.64	21.03	9.54	10.63

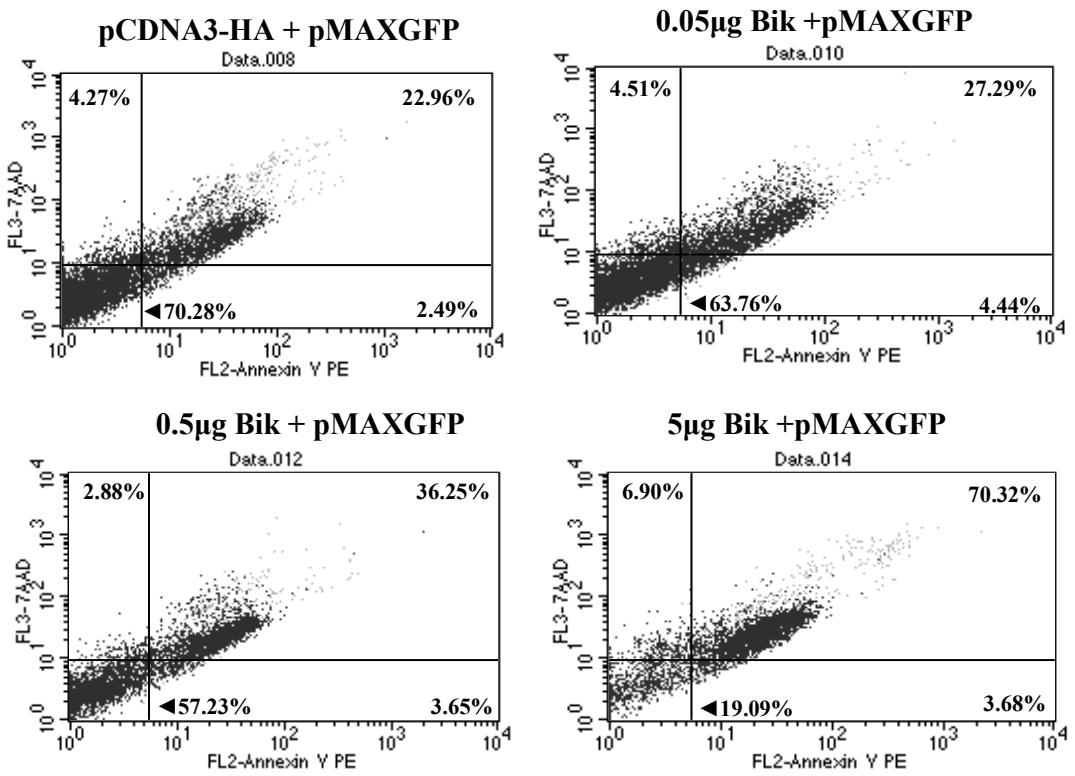
Apoptosis was assayed using the sub-G_1 DNA content. Cells were analysed as in Figure 6.2.11. The percentage of cells present in the different phases of the cell cycle (G_0/G_1, S, G_2/M) are given in the table.

6.2.7 Ectopic Bik induces apoptosis in IB4 cells.

IB4 cells were co-transfected with 2µg pmaxGFP and increasing amounts (0.05, 0.5, or 5µg) of pcDNA3-HA-*bik*, pcDNA3-HA-*bik*-ΔBH3 or the corresponding control plasmid pcDNA3-HA. The total amount of expression plasmid was adjusted in all cases to 7µg with the empty vector pcDNA3-HA. Cell viability was analysed at 6 hours after transfection by double labelling the cells with Annexin V/7-AAD and flow cytometry (Section 2.2.2.16). Using Annexin V/7-AAD staining, FACS analysis for cell surface molecules was performed as described previously (Section 6.2.6.1). A minimum of 4,500 GFP-expressing cells were examined in isolation (by analysing cells in region 2 (R2)).

Co-transfection of IB4 cells with pmaxGFP and together with either *bik* or *bik*-ΔBH3 resulted in death of the Bik-expressing cells only (Figure 6.2.1). A clear dose dependent increase in apoptotic activity occurred in response to addition of increasing quantities of the HA-Bik expression plasmid pcDNA3-HA-*bik* (Figure 6.2.12). Dramatically, cells in late apoptosis accounted for 70.30% of the transfected cell population with the addition of 5µg of pcDNA3-HA-*bik*. The number of cells expressing GFP also decreased with increasing quantities of *bik* plasmid (data not shown). In striking contrast, cells ectopically expressing the same quantites of the BH3-deleteted pcDNA3-HA-*bik*-ΔBH3 did not succumb to apoptosis at all quantities of plasmid used (Figure 6.2.12B). Overall a significant increase in apoptosis occurred in response to the addition of increasing quantities of the HA-Bik expression plasmid. The transfection method may account for the significant numbers of apoptotic cells seen in control transfection (Figure 6.2.12A).

A



B

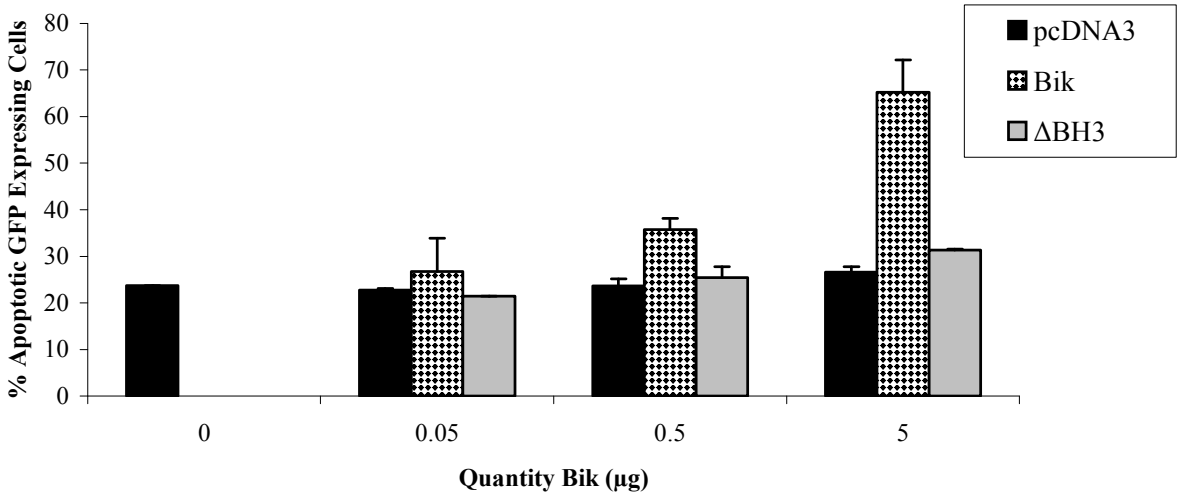


Figure 6.2.12: Increasing quantities of HA-Bik expression augments the apoptotic response at 6 hours.

To examine the Bik dose effect on cell survival, IB4 was co-transfected with 2µg of pmaxGFP and increasing amounts (0.05, 0.5, 5µg) of pcDNA3-HA, pcDNA3-HA-*bik* or pcDNA3-HA-*bik*-ΔBH3. The total amount of DNA transfected at each dose was kept constant at 7µg by adding an appropriate amount of pcDNA3-HA vector. Cells were harvested 6 hours after transfection and analysed by 7-AAD/Annexin V staining

and subsequent flow cytometric analysis. CellQuest software was employed for data analysis. (A) Scatter plots for 0, 0.05, 0.5, and 5µg of pcDNA3-HA-*bik* are shown. Quadrant markers were based on stained/unstained controls and values reflect the percentage of cells in each quadrant. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$ /Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$). (B) The percentage of GFP-expressing dead cells was plotted against the quantity of pcDNA3, pcDNA3-HA-*bik* or pcDNA3-HA-*bik*-ΔBH3 vector (µg) transfected. (Data are Mean ±SD)

6.2.7.1 Apoptosis is a potent and early effect of Bik expression.

IB4 cells were transiently co-transfected with 2µg of pmaxGFP and 0.5µg, of either pcDNA3-HA-*bik*, pcDNA3-HA-*bik*-ΔBH3, pcDNA3-HA-*bfl-1* or the corresponding control plasmid pcDNA3-HA. Cell viability was examined by Annexin V/7-AAD staining and subsequent flow cytometric analysis. Transfected cells were initially examined 3 hours after-transfection, on the basis that the toxic effect of *bik* may require only a relatively short period of time to exert a killing effect on transfected cells (Section 6.2.5). This early analysis was accommodated by the early detectable expression of GFP from the pmaxGFP expression plasmid. Cells were assayed for apoptosis using Annexin V/7-AAD staining, and flow cytomertry as before (Section 2.2.2.16). A minimum of 2,000 GFP-expressing cells were examined in isolation as before.

Examining the effect of Bik expression on viability of IB4-transfected-HA-*bik* cells it can be seen that 33.78% of the transfected population were in the late stages of apoptosis (Figure 6.2.13) (Annexin V$^+$/7-AAD$^+$), and 22.11% of the total GFP population exhibited signs of early apoptosis (Annexin V$^+$/7-AAD$^-$). In contrast, cells expressing HA-Bik-ΔBH3 comprised a 19% late apoptotic and an 18.32% early apoptotic population. Overall, cells expressing HA-Bik were 18.57% more apoptotic than cells harbouring the HA-Bik-ΔBH3. Significantly, only 40.94% of the *bik*-transfected population were viable as compared to pcDNA3-HA (67.94%), pcDNA3-HA-*bik*-ΔBH3 (60.24%) and pcDNA3-HA-*bfl*-1 (61.03%). Thus, Bik-expressing cells showed an average 22.73% reduction in cell viability relative to the controls.

It is obvious from these results that the toxic effect of Bik expression is clear and detectable just 3 hours after transfection. In contrast, cells expressing HA-ΔBH3 were significantly less apoptotic (Figure 6.2.13). This distinction between cells expressing HA-Bik and those that express HA-ΔBH3 indicates that these cells are dying by a mechanism that is dependent on the presence of the BH3 domain, as has been found by others (Tong *et al*, 2001; Germain *et al*, 2002).

Based on these observations, it is clear that Bik can induce cells to die by apoptosis. Transfection with HA-*bik* caused an average 18.8% increase in the number of apoptotic cells relative to the controls (Figure 6.2.13). The observation that some of the cells in the HA-Bik-expressing pool continue to survive may reflect heterogeneity in the levels of transfection or expression between individual transfected cells, some of which may express HA-Bik at very low levels. In addition these results also highlight a key role for the BH3 domain in Bik-mediated apoptosis.

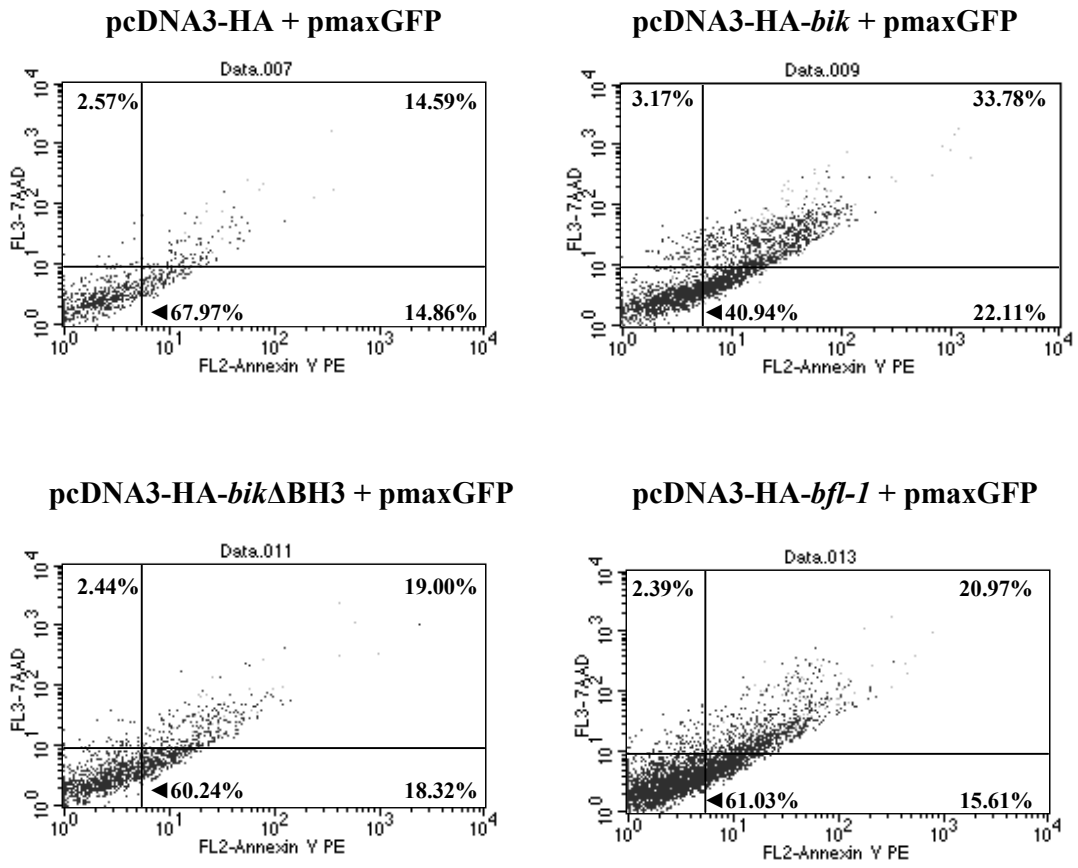


Figure 6.2.13: **Ectopic Bik causes IB4 cells to die by apoptosis after 3 hours**.

In order to determine the effect of Bik expression on cell survival after three hours, IB4 cells were transiently co-transfected with 2µg of pmaxGFP, and 0.5µg of pcDNA3-HA-*bik* and pcDNA3-HA-*bik* ΔBH3. pcDNA3 and pcDNA3-HA-*bfl*-1 were used as control vectors. The total amount of DNA transfected was kept constant by adding an appropriate amount of pcDNA3 vector. Three hours after transfection, cells were harvested, and analysed by 7-AAD/Annexin V staining and subsequent flow cytometric analysis. CellQuest software was employed for data analysis. A minimum of 2,000 GFP-expressing cells were examined in isolation (by analysing cells in R2). Quadrant markers were based on stained/unstained controls and values reflect the percentage of cells in each quadrant. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$ /Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$).

This experiment was also performed at 6, 12, and 24 hour time intervals using 0.5µg of DNA to examine the effect of *bik* expression over time in these cells. The survival of GFP expressing cells was again monitored using Annexin V/7-AAD staining using FACS analysis. Consistent with the earlier time point, at 6 and 12 hours after

transfection over-expression of Bik caused significant apoptosis in the IB4 cell line. However, there is a time-dependent decrease in the level of apoptosis recorded, with cells ectopically expressing HA-Bik at 3, 6 and 12 hours exhibiting 18.57%, 16.12% and 12.51% respectively more apoptotic cells relative to pcDNA3-HA-*bik*-ΔBH3 at the given time points (Figure 6.2.13 and Figure 6.2.14).

Interestingly, in contrast to these results, at 24 hours, the effect of Bik expression had disappeared, and the level of apoptosis in the other transfected cell pools surpassed that of HA-*bik* (data not shown). The use of electroporation-based methods for the delivery of DNA across the cell membrane has a negative effect on cell viability (Canatella *et al*, 2001). It is therefore possible that the cytotoxic effect of nucleofection on the transfected-cell pools led to an ultimate loss of cell viability and committed a substantial number of the transfected cell population to death by apoptosis. The toxic effect of ectopic Bik was most visible in the early stages after-transfection, at 3, 6 and 12 hours (Figure 6.2.14) and the prevalence of cells dying due to apoptosis becomes more evident after 12 hours. Thus, this short-time period of 3-12 hours after-transfection was the most appropriate time to analyse the effect of HA-Bik expression.

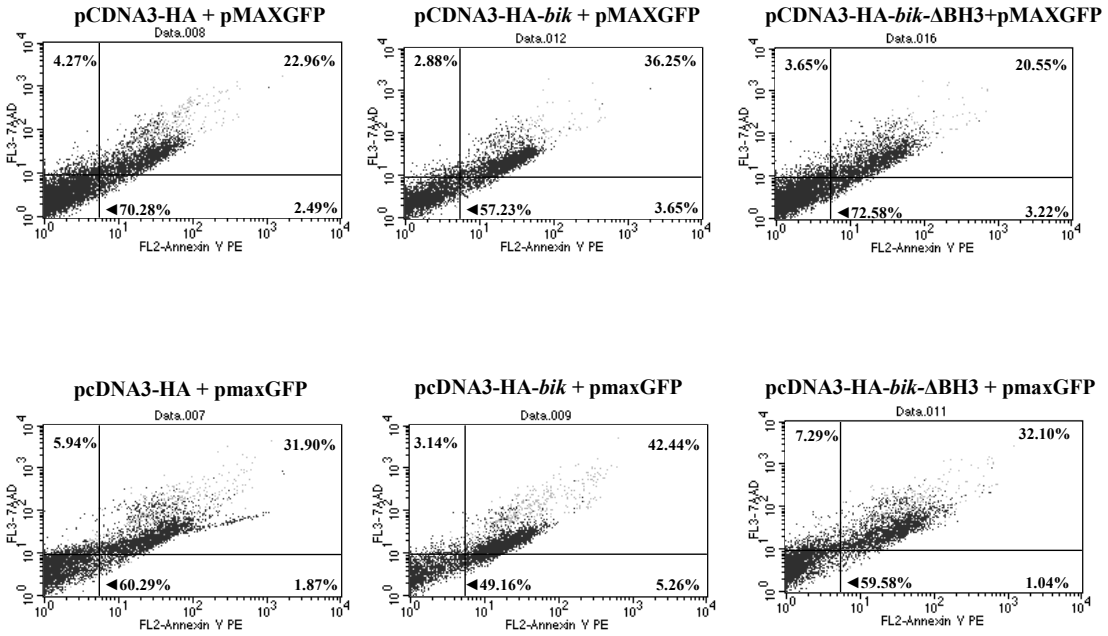


Figure 6.2.14: **Ectopic HA-*bik* causes significant apoptosis in the IB4 cell line at 6 and 12 hours**.

In order to determine the effect of Bik expression on cell survival over the course of 24 hours, IB4 cells were transiently co-transfected with 2µg of pmaxGFP, and 0.5µg of pcDNA3-HA-*bik* and pcDNA3-HA-*bik* ΔBH3. pcDNA3 and pcDNA3-HA-*bfl*-1 were used as control vectors. The total amount of DNA transfected was kept constant by adding an appropriate amount of pcDNA3 vector. At 6 and 12 after transfection, cells were harvested, and analysed by 7-AAD/Annexin V staining and subsequent flow cytometric analysis. A minimum of 6,000 GFP-expressing cells were examined in isolation (by analysing cells in R2). CellQuest software was employed for data analysis. Quadrant markers were based on stained/unstained controls and values reflect the percentage of cells in each quadrant. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$/Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$).

6.2.7.2 Sub-G1 analysis of *bik*-transfected cells.

The cell cycle status of cells expressing ectopic Bik was next examined by analysing their DNA content of cells as determined by PI staining. However, as illustrated in Figure 6.2.15, since only a small minority of the cells actually take up and express the genes available in the transient transfection, the overall result of the experiment is shrouded by the non-GFP expressing population many of which are undoubtedly apoptotic due to the nucleofection procedure. It can be seen from the results, that the percentage of cells in the sub-G_1 phase of the cell cycle in the pcDNA3-HA, HA-*bik* and ΔBH3 cell pools was 53.29%, 52.06% and 63.21% respectively (Figure 6.2.15). This data illustrates that a protocol to identify and segregate the transfected cell population is required here. This is generally accomplished by including a plasmid that expresses a marker gene in the transfection cocktail, as with pmaxGFP (Section 6.2.5). However the simultaneous detection of GFP and DNA content using propidium iodide (PI) by flow cytometry is made difficult by virtue of the unique nature of these two fluorogenic reagents. In order for PI to enter cells efficiently and to stain DNA quantitatively, the cells must first be permeabilised; and ethanol treatment is a routine method to achieve this. However, this permeabilisation step causes GFP, which is normally found in the cytoplasm, to leach out of the cells thus abrogating the possibility of examining the GFP population. In order to circumvent the problem of simultaneous detection of transfected cells and PI-based cell cycle analysis, a protocol (adapted from Lamm *et al*, 1997) was employed, using paraformaldehyde-based fixatives which allow GFP to be maintained in cells and retain its fluorescence even after ethanol permeabilisation. The use of fixatives, however, resulted in poor staining of the DNA with PI and provided data of insufficient resolution for quantitative cell cycle analysis (data not shown).

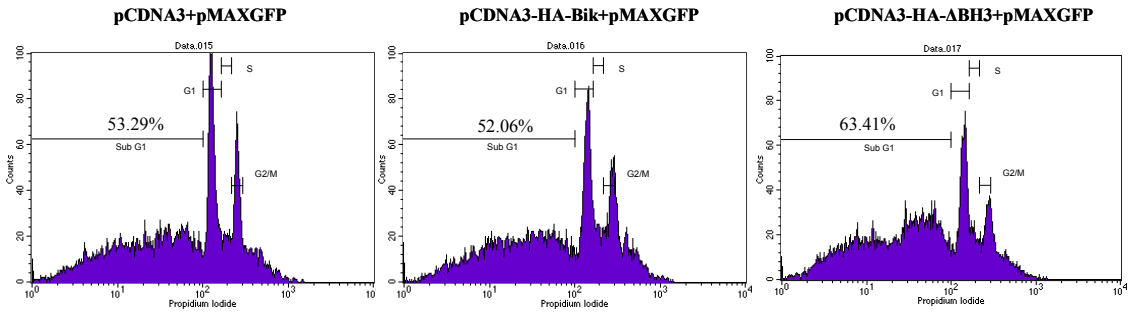


Figure 6.2.15: **Sub-G_1 analysis of Bik-transfected cells**.

Apoptotic cells determined by sub-G_1assay in cell samples treated as in Figure 6.2.14 above after 12 hours. Apoptosis was assayed using the sub-G_1 DNA content. Thirty minutes before the assay, cells were stained with PI. FACS analysis of sub-G_1 was performed by PI staining and flowcytometry. The DNA content in the cells was determined 24 hours after treatment. Markers identify cells with hypodiploid DNA content (sub-G_1), and also those cells in stages G_0/G_1, S, and G_2/M of the cell cycle. The percentage of apoptotic cells is indicated on the left hand side of each box.

6.2.8 The effect of the pan-caspase inhibitor, zVAD-fmk, on Bik-mediated apoptosis.

The caspase protease family plays a central role in the execution of apoptosis (reviewed in Cryns and Yuan, 1998) and the Bcl-2 family members are known to regulate caspase activation (Youle and Strasser, 2008). Bik is known to elicit pro-apoptotic signals which lead to cell death by pathways which involve the activation of caspases (Germain *et al*, 2002; Gillissen *et al*, 2003; Mathai *et al*, 2005; Zhao *et al*, 2007). It is widely published that caspase dependent Bik-induced cell death can be inhibited by the broad spectrum caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD-fmk) (Mathai *et al*, 2002; Naumann *et al*, 2003; Hur *et al*, 2004). zVAD-fmk is a tripeptide-based inhibitor (VAD) linked to a chemical moiety; fluoromethyl ketone (fmk) which covalently modifies the enzyme, thereby inactivating its catalytic function. zVAD-fmk is an irreversible inhibitor of several caspases, including caspases 1, 3, 5, 7, 8 and 9. Since IB4 cells transfected with HA-*bik* show the classical changes characteristic of the apoptotic phenotype (Figure 6.2.13), it was next important to investigate if the mechanism of apoptosis was caspase dependent, by analysing the affect of the caspase inhibitor zVAD-fmk on HA-*bik* and HA-*bik*-ΔBH3-transfected cells.

Initially, the efficacy of zVAD-fmk inhibitor in the IB4 cell line was confirmed in a control experiment. Since proteasome inhibition has been reported to induce apoptosis that is mediated by caspase activation (Wu *et al*, 1999), extracts derived from IB4 cells treated with the proteasome inhibitor MG132 were monitored for cell viability in the absence or presence of the caspase inhibitor zVAD-fmk. Cytotoxicity was assessed at 12 hours by Annexin V/7-AAD staining and FACS analysis. It can be seen that, the abrogation of proteasome activity by MG132 was significantly reversed in the presence of zVAD-fmk (44%) confirming that this inhibitor was biochemically effective in this model system (Figure 6.2.16). Furthermore, the inhibition of apoptosis is specific to the zVAD-fmk reagent and not due to the DMSO solvent. These results demonstrate that MG132-induced activation of apoptosis is significantly inhibited by hindering cysteine protease activity in IB4 cells.

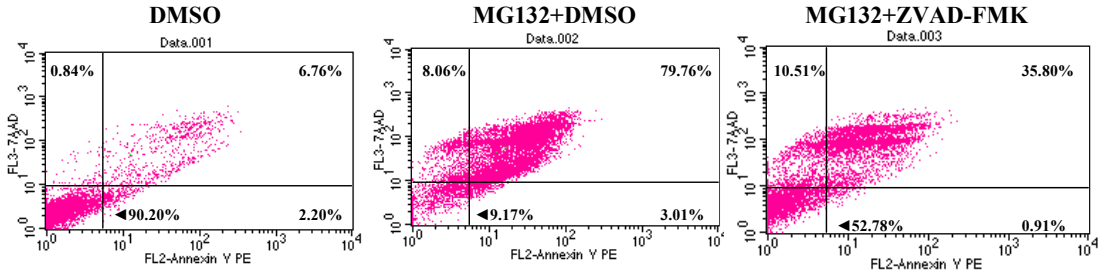


Figure 6.2.16: **MG132-Induced Apoptosis is significantly inhibited by the pan-caspase inhibitor zVAD-fmk.**

IB4 cells were treated with 15µM MG132 in the presence or absence of 50µM zVAD-fmk. Cytotoxicity was evaluated at 12 hours after treatment by 7-AAD/Annexin V staining and subsequent flow cytometric analysis. 10,000 cells were analysed per scatter plot. Quadrant markers were based on stained/unstained controls and values reflect the percentage of cells in each quadrant. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$/Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$).

6.2.8.1 Pre-treatment of cells does not increase the inhibitory effect of zVAD-fmk on MG132 mediated apoptosis.

It has previously shown that a period of pretreatment with zVADfmk yields increased protection from apoptosis compared to just treatment alone (Clayton *et al*, 1997). Since the observed inhibition of MG132 mediated apoptosis by zVAD-FMK (Figure

6.2.16) did not completely rescue the cells, it was decided to pre-treat the cells for 1 hour prior to the addition of 15µM MG132, in an effort to enhance the protective efficacy of the caspase inhibitor. Extracts were monitored for cell viability in the absence or presence of the caspase inhibitor zVAD-fmk as before at 12 hours by Annexin V/7-AAD staining and FACS analysis. As the result in Figure 6.2.17 indicates, pre-treatment with zVAD-fmk had no significant effect relative to treatment alone (Figure 6.16). In fact inhibition of MG132-induced cytotoxicity by zVAD-fmk accounted for a 43% increase in cell viability in this instance as compared to 44% without pretreatment (Figure 6.2.16 and Figure 6.2.17).

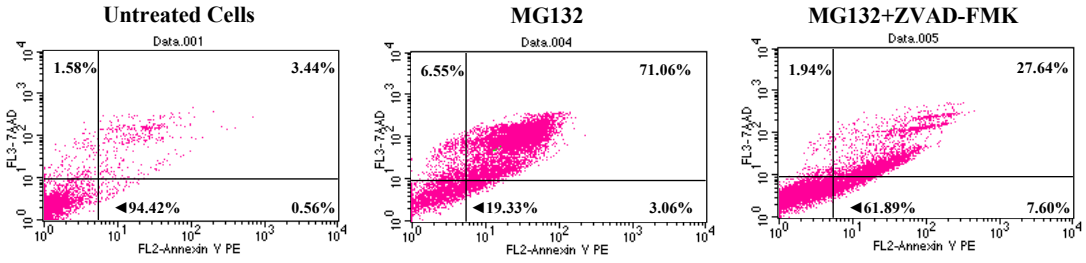


Figure 6.2.17: **Pre-treatment of cells does not increase the inhibitory effect of zVAD-fmk on MG132 mediated apoptosis**.

IB4 cells were pretreated with 50µM zVAD-fmk 1 hour prior to the addition of 15µM MG132. Cell viability was evaluated at 12 hours after treatment by 7-AAD/Annexin V staining and subsequent flow cytometric analysis. 10,000 cells were analysed per scatter plot. Quadrant markers were based on stained/unstained controls and values reflect the percentage of cells in each quadrant. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$/Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$).

6.2.8.2 The pan-caspase inhibitor zVAD-fmk blocks the activation of caspases and loss of cell viability resulting from exposure of IB4 cells to exogenous Bik expression.

The effect of zVAD-fmk treatment on IB4 Bik-transfected cell pools was examined by FACS analysis. In this experiment, IB4 cells were transiently co-transfected with the 2µg pmaxGFP expression plasmid and 5µg *bik* or *bik* ΔBH3 as before.

Transfected cell pools were immediately divided and subsequently treated, or untreated with 50μM zVAD-fmk. The population of cells expressing GFP in the presence of Bik or Bik-ΔBH3 expression was assessed for apoptosis by Annexin V/7-AAD staining. GFP fluorescence of 10,000 events for each sample was measured on the BD FACSCalibur flow cytometer. CellQuest software was employed for data analysis (BD).

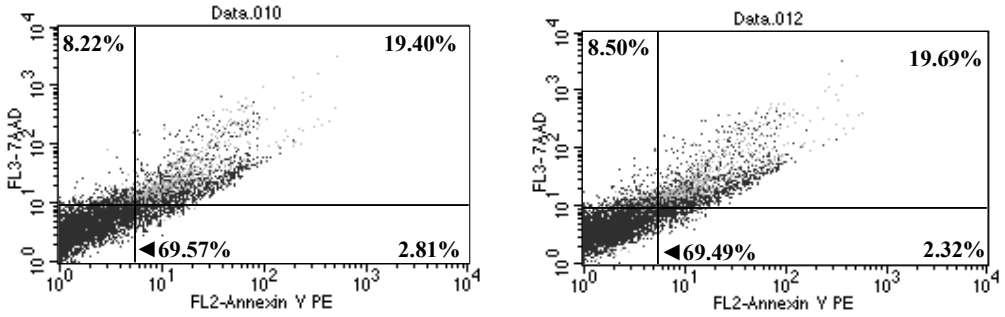
Significantly, zVAD-fmk treatment of IB4-pcDNA3-HA-*bik* cells caused a 25% decrease in the number of apoptotic cells relative to IB4-pcDNA3-HA and a 22% decrease relative to IB4-pcDNA3-HA-*bik*-Δ-BH3 after 3 hours (Figure 6.2.18). Bik-expressing cells were 25% more viable in the presence of zVAD-fmk, compared with -0.17% for pcDNA3-HA and 3.2% for pcDNA3-HA-*bik*-Δ-BH3. Thus, induction of cell death by Bik was strongly inhibited by the caspase inhibitor zVAD-fmk, indicating the requirement for caspases in this context. Cell death in the presence of HA-Bik showed the largest reversal of fate in the presence of the caspase inhibitor. From these results it is clear that zVAD-fmk effectively blocks the activation of caspases and loss of cell viability (Figure 6.2.18) that can result from exposure of these cells to exogenous Bik.

In summary, Bik induces apoptosis through a caspase-dependent pathway in the IB4 LCL. During caspase inhibition, Bik-induced apoptosis decreased substantially. This result further corroborates the involvement of apoptosis in Bik-induced inhibition of cell survival, clearly demonstrating that the zVAD-fmk cysteine protease inhibitor can block Bik-triggered cell death in IB4. In conclusion, this data identifies that Bik initiates a pathway, upstream of the caspases stimulating the induction of apoptosis in IB4. This pathway is likely to be dependent on zVAD-fmk-sensitive caspases and is dependent on a functional BH3 domain.

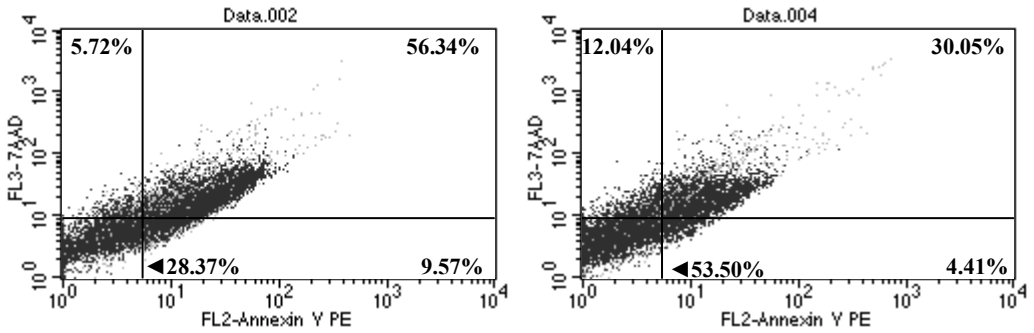
A

Vehicle **zVAD-fmk**

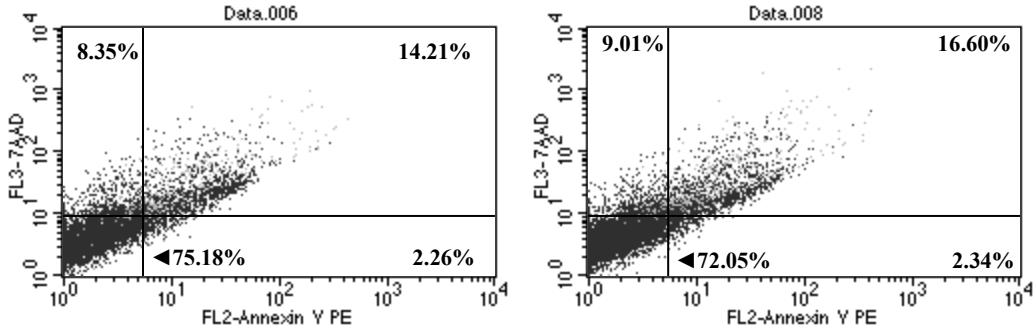
pCDNA3-HA + pMAXGFP



pCDNA3-HA-Bik+ pMAXGFP



pCDNA3-HA-BikΔBH3 + pMAXGFP



B

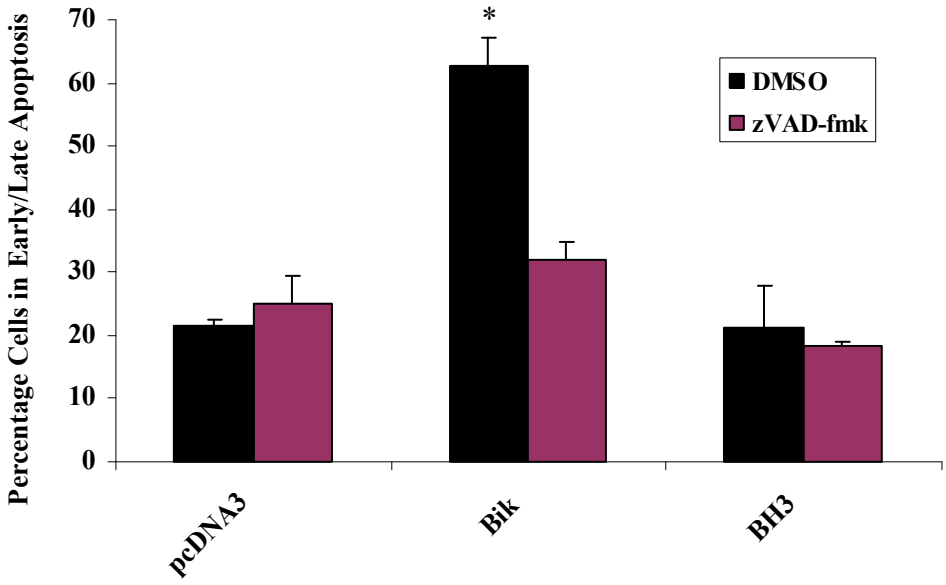


Figure 6.2.18: **Ectopic Bik induces apoptosis through a caspase-dependent pathway in IB4 cells**.

(A) IB4 cells were firstly transiently co-transfected with 2μg of pmaxGFP, and 5μg of pcDNA3-HA-*bik* and pcDNA3-HA-*bik* ΔBH3. pcDNA3 and pcDNA3-HA-*bfl*-1 were used as control vectors. The total amount of DNA transfected was kept constant at 7μg by adding an appropriate amount of pcDNA3 vector. Transfected cells were treated or untreated with of 50μM zVAD-fmk immediately after transfection. Three hours after transfection, cells were harvested, and cell viability was analysed by 7-AAD/Annexin V staining and subsequent flow cytometric analysis. CellQuest software was employed for data analysis and representative scatter plots are presented above. GFP fluorescence (10,000 events for each sample) was measured on the BD FACSCalibur flow cytometer. Quadrant markers were based on stained/unstained controls and values reflect the percentage of cells in each quadrant. Cells in the lower left quadrant are viable (7-AAD$^-$/Annexin V$^-$), cells in the lower right quadrant are early apoptotic (7-AAD$^-$/Annexin V$^+$), cells in the upper right quadrant are late apoptotic (7-AAD$^+$/Annexin V$^+$) and cells in the upper left quadrant are necrotic/mostly nuclear debris (7-AAD$^+$/Annexin V$^-$). (B) The percentage of GFP-expressing cells in late apoptosis over the course of three experiments was plotted (Data are Mean ±SD, *P<0.5).

6.3 Discussion

EBV has been shown to protect B-cells from apoptosis via the expression of select EBV proteins and up-regulation of Bcl-2 or its homologues (Henderson *et al*, 1991; Wang *et al*, 1996; Pegman *et al*, 2006). The virus has specifically been thought to counter apoptosis during latency, principally through LMP1-mediated activation of NF-κB-regulated genes (Fries *et al*, 1996; Cahir-McFarland *et al*, 2000). More recently EBNA2 mimicry of Notch activity has been identified as having the ability to protect B-cells against various apoptotic agents, including Nur-77 mediated cell death (Lee *et al*, 2002; Lee *et al*, 2004b). In recent months, LMP2A has also been implicated with a role in averting apoptosis through the activation of NF-κB-regulated genes (Guasparri *et al*, 2008).

Previous work in our laboratory has shown that the anti-apoptotic, *bfl-1* gene, a CBF1-dependent transcriptional target of EBNA2 (Pegman *et al*, 2006), plays an important role in regulating the survival of EBV-infected B-cells (D'Souza *et al*, 2000). Thus, the findings presented here demonstrating that the EBNA2-mediated suppression of *Bik* contributes to the protection of infected cells from apoptosis, is an indication that this viral protein contributes to cell survival, by up-regulating the anti-apoptotic *bfl-1* and down-regulating the pro-apoptotic *bik*.

Aberrations in the expression of many of the pro-apoptotic Bcl-2 family members has previously been shown to be involved in protecting malignant cells from apoptosis. Bax is mutated in human colon cancer as well as in some leukaemias (Rampino *et al*, 1997; Meijerink *et al*, 1998) and loss of Bax increases tumourigenicity and reduces apoptosis (McCurrach *et al*, 1997; Yin *et al*, 1997; Ionov *et al*, 2000). Bak was found to be mutated in some human gastric and colorectal cancers (Rampino *et al*, 1997; Kondo *et al*, 2000). Furthermore, the absence of both Bax and Bak can enhance transformation, to a greater extent than loss of either protein alone (Zong *et al*, 2001). Preliminary evidence also suggests that the absence of *bim* accelerates *Myc*-induced lymphomagenesis (Cory and Adams, 2002) and there is also indication that the p53 targets, Noxa and Puma, might mediate p53 apoptotic function, and that Bmf might inhibit metastasis (Puthalakath *et al*, 2001). Importantly loss of Bik expression has

been implicated in renal cell carcinoma, and in human breast and colorectal cancers (Castells *et al*, 2000; Sturm *et al*, 2006; Zantl *et al*, 2007).

In this chapter the effect of *bik* expression in EBV-infected cell lines was examined. Evidence is presented that exogenous Bik can induce apoptosis in EBV-infected B-cells by a mechanism that is dependent on its BH3 domain. The evidence presented thus far indicates that down-regulation of *bik* promotes B-cell survival during the Epstein-Barr virus growth programme. Other results in this thesis suggest that EBNA2 plays a key role in repressing *bik* and it is now clear that this host-virus interaction contributes to the survival of EBV-infected B-cells and may therefore play a role in pathogenesis of EBV.

The results presented here indicate that the death-promoting activity of Bik is dependent on a functional BH3 domain in this context. The BH3 domain is of central importance in mediating apoptosis in the pro-apoptotic members of the Bcl-2 family. It has been reported that mutations within the BH3 domain of Bik abrogate its ability to induce cell death and to complex with anti-apoptotic proteins (Chittenden *et al*, 1995). This result is consistent with the hypothesis that Bik may promote cell death by complexing with and antagonising the function of endogenous cellular anti-apoptosis proteins such as Bcl-2 and Bcl-$_{XL}$.

It has been postulated that Bik may induce apoptosis by preferentially sequestering Bcl-2-like apoptosis inhibitors (Boyd *et al*, 1995). The fact that the growth inhibitory effect of Bik appeared to increase in the presence of decreasing amounts of its effector plasmid HA-*bik* in the *Luc* assay experiment, (seen in Figure 6.2.2B) could possibly indicate that a relatively low level of Bik protein is required to reach the saturation point of inhibition, by which time all the binding sites of the pro-survival molecule in question may be engaged by Bik. In that case, Bcl-2 would be the effector that is inhibited by interaction with the BH3-containing Bik protein. In support of this theory, Bik has an affinity for Bcl-2, Bcl-$_{XL}$ and Mcl-1 (Boyd *et al*, 1995; Chen *et al*, 2005) and the Bcl-2 homolog encoded by EBV; BHRF1 (Boyd *et al*, 1995). Thus, the ratio of Bik to its anti-apoptotic homologues may influence cell survival. Any further small increases in the expression of Bik may amplify signals from any of several anti-apoptotic molecules, thus relieving some of the death effect, although this effect is not

seen in the later FACs experiments. Of interest, at 7ug, the level of *luc* activity decreases again to an absolute minimum of 4.28%. However, it is conceivable that this effect is due to non-specific effects of high levels of protein over-expression.

Earlier studies have shown that Bik-induced apoptosis is dependant on caspase activation in a variety of mammalian cell lines, and can be inhibited by the pan-caspase inhibitor zVAD-fmk (Orth and Dixit, 1997; Mathai *et al*, 2002; Naumann *et al*, 2003; Hur *et al*, 2004) (Chapter 1). While apoptosis mediated by Bcl-2 family members predominantly leads to caspase activation (Youle and Strasser, 2008) the ability of these proteins to mediate caspase-independent cell death has also been established (Xiang *et al*, 1996), and indeed the caspase dependency of Bik is thought to be cell type specific (Orth and Dixit, 1997; Mathai *et al*, 2002; Naumann *et al*, 2003; Hur *et al*, 2004; Oppermann *et al*, 2005). It was therefore important to determine if Bik-induced cell death triggers the activation of caspases in this context. The data presented here demonstrates that Bik induces apoptosis by a caspase dependent pathway in the EBV-infected cell line, IB4.

Thus, it has been established that the EBNA2 mediated suppression of *bik* has a role to play in the EBV regulation of cellular apoptosis, with the down-regulation of Bik contributing to the protection of infected cells from apoptosis. Conceivably then the apoptosis deficiency of EBV-associated malignancies may be overcome by enforced expression of pro-apoptotic Bcl-2 proteins such as Bik.

CHAPTER 7

Regulation of *bik* by EBV

7.1 Introduction

The regulation of gene expression in eukaryotic cells is the consequence of a series of intricate mechanisms that are instigated in the nucleus by transcription and completed by translation in the cytoplasm (Lorkowski and Cullen, 2003). Evolution has led to the development of multiple different BH3-only proteins, and several mechanisms have been put in place to keep these pro-apoptotic proteins under control (Huang and Strasser, 2000). The existence of many, otherwise distinct proteins, that share a common functional domain, is indicative of a sophisticated and intricately structured intracellular regulatory system (Kelekar and Thompson, 1998).

The pro-apoptotic activity of BH3-only molecules may be kept in check by either transcriptional control or post-translational modification (as reviewed in Huang and Strasser, 2000; Puthalakath and Strasser, 2002). Since the steady state level of a particular mRNA species in a cell is dependent on both the rates of transcription and decay a co-ordinated balance between transcriptional and post-transcriptional events is required. It is clear that external and internal stimuli lead to regulation of *bik* at several different levels, including tissue-specific expression, transcriptional and translational control and intracellular localisation. This study attempts to define the transcriptional and post-translational mechanisms that regulate *bik* during EBV-infection.

It has been established in previous chapters that EBNA2 is capable of directly regulating *bik* expression at a transcriptional level (Chapters 4 and 5). Indeed, in all of the nine Lat III cell lines analysed (disregarding DAUDI and BL419A which both harbour deletions spanning the EBNA2 coding sequence), only low or undetectable levels of *bik* mRNA were detected by QPCR. Also, significantly, the induction of EBNA2 correlated with a substantial decrease in the level of *bik* mRNA in both BL-derived cell lines and LCLs.

7.2 Regulation of the *bik* Promoter

Having established that EBNA2 alone can regulate *bik* in B-cell lines, it was thus of interest to determine if this down-regulation was due to a decrease in the rate of transcription initiation from the *bik* promoter

7.2.1 Bik promoter construct, p-1710/+203-*bik*-Luc.

A suitable *bik* promoter-reporter construct was first generated. Two of the most commonly used reporter genes in promoter reporter constructs encode the enzymes chloramphenicol acetyl transferase (CAT) and luciferase (Luc). In recent years, the greater sensitivity and rapidity of luciferase assays has resulted in the popular usage of Luc-based rather than CAT-based promoter-reporter constructs for evaluating transcriptional activity in transfected cells. It was therefore decided to investigate the regulation of *bik* promoter activity by EBNA2 using a luciferase based system. For this purpose the pGL2-Basic Luciferase reporter vector (Table: 2.1-4) was employed to monitor transcriptional activity of the *bik* promoter in transfected BL-derived cells. A schematic circular map of the pGL2-basic vector can be seen in Figure 7.2.1.

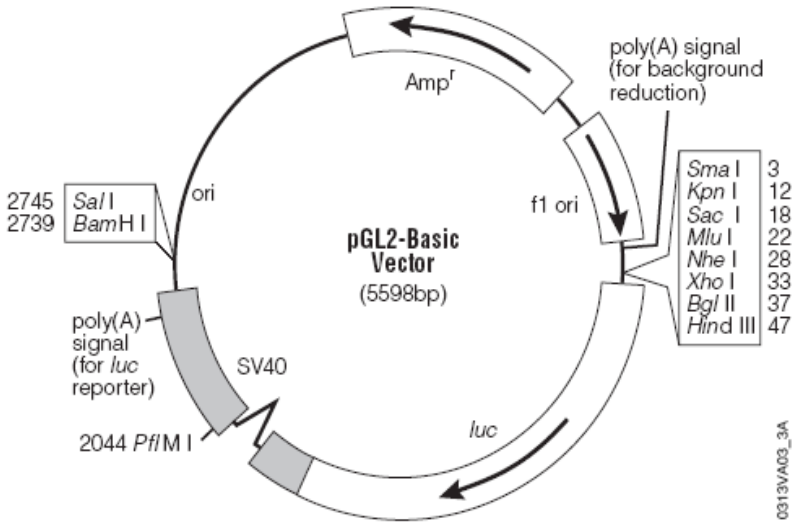


Figure 7.2.1: Schematic circular map of pGL2-basic vector (Promega).

The pGL2-Basic Vector is a reporter construct lacking eukaryotic promoter and enhancer sequences. The vector carries the coding region for firefly (*Photinus pyralis*) luciferase which is designated 'luc' on the map. The multiple cloning site, which contains recognition sites for *Nhe* I and *Bgl* II sites amoung others is also indicated.

A 1.9kb DNA sequence from the 5' flanking region of the *bik* gene (position -1710/+203) was obtained as a fragment cloned into the promoterless pBLCAT-2 ΔTK (in which the *tk* promoter has been deleted) (Verma *et al*, 2000). This plasmid, designated p-1710/+203BikCAT-2 (Table: 2.1-4) was firstly characterised by restriction digestion in order to confirm its identity (data not shown). In order to change the reporter context of the p-1710/+203BikCAT-2 construct to a luciferase-based system, an approach based on sub-cloning the -1710/+203Bik fragment from the p-1710/+203Bik-CAT-2 construct into the pGL2-basic vector was employed. Initially, since PCR was the method of choice, a cloning strategy which involved the PCR amplification of the -1710/+203-*bik* promoter fragment, and subsequent sub-cloning into the pGL2-basic plasmid upstream of the lucifearse gene was used.

The -1710/+203-*bik* promoter fragment was used as template DNA and PCR was undertaken using primers specific to the *bik* promoter region (BikPromFor and BikPromRev, Table: 2.1-5). The primers included *Bam* HI restriction sites in order to facilitate directional cloning. Following PCR, the products were analysed by agarose gel electrophoresis (Section 2.2.2.15) to check for migration of fragments at the expected size, however, no PCR product was observed. This experiment was repeated

with various dilutions of template DNA (1/10–1/500) and primers (25pmole-100pmole) but to no benefit. Further PCR parameters including annealing and denaturation temperatures, $MgCl_2$ concentration (increased in 0.5mM increments from 15mM), concentration and commercial source of Taq DNA polymerase (REDTaq™, AmpliTaq Gold® and SYBR® Green PCR Master Mix), extension times and dNTP concentration were also varied but amplification of PCR product was not obtained (data not shown). Although, in some instances non-specific bands were observed, no product of the correct size was amplified. Conventional PCR conditions were modified to include a 'Hot-Start' step (95^{o}C, 15mins) at the beginning of the reaction, in a further attempt to reduce non-specific artifacts and increase potential PCR product yield, however once again no amplification was achieved. Another modified PCR method, 'Touchdown PCR' was also employed in an additional attempt to reduce any non-specific amplification. This technique involved the use of an annealing temperature greater than the target optimum in the early PCR cycles. The annealing temperature was then decreased by 1°C every cycle until the specified or 'touchdown' annealing temperature was reached. The 'touchdown' temperature was then used for the remaining number of cycles, allowing for the enrichment of the correct product over any non-specific product. Once again however no progress was made. In a final attempt to optimise the reaction, a separate new set of primers (lower self-annealing/self-hybridisation potential Table: 2.1-5) were designed, however no amplification was achieved and consequently a new strategy which involved the excision of the -1710/+203Bik fragment from the p-1710/+203BikCAT-2 construct using restriction digestion, and its insertion into pGL2-basic, was successfully adopted. A schematic diagram outlining the construction of the vector is illustrated in Figure 7.2.2. The steps involved in the generation of -1710/+203 *bik* Luc can be seen in Figure 7.2.3.

pGL2-basic was first characterised by restriction digestion in order to confirm its identity using *Rsa* I and *Hind* III (Figure 7.2.3A). Prior to ligation, pGL2-Basic was linearised by digestion with *Nhe* I and *Bgl* II, followed by dephosphorylation using CIP to prevent recircularisation of the linearised DNA (Section 2.2.1.4). The linearised, CIP-treated DNA was then purified by phenol: chloroform extraction followed by ethanol precipitation (Section 2.2.1.2).

The *bik* promoter region (-1710/+203) had previously been inserted by directional cloning into the *Xba* I and *Bam* HI sites of pBLCAT-2 ΔTK to create the p-1710/+203BikCAT-2 plasmid (Verma *et al*, 2000). Thus, in order to construct the p-1710/+203 *bik* Luc plasmid, the 1.9kb *Xba* I - *BamH* I DNA fragment (containing the *bik* promoter region) was excised from p-1710/+203Bik-CAT by digestion with these enzymes. The digested product was analysed by agarose gel electrophoresis (Section 2.2.1.15) to check for migration of fragments at the expected size (Figure 7.2.3B) and the integrity of the *Xba* I and *Bam* HI digested *bik* promoter fragment was tested by restriction digestion using *Kpn* I, *Rsa* I and *Hind* III (Figure 7.2.3C). The *Xba* I-*Bam* HI fragment was subsequently purified using the Promega Wizard® DNA purification system (Section 2.2.1.6) and the digested, purified product was then sub-cloned into the *Nhe* I and *Bgl* II sites of pGL2-basic. Ligations of vector and insert DNA were carried out overnight at 16 ˚C (Section 2.2.1.7).

Following the ligation reaction, competent *E. coli* JM109 cells were transformed with the recombinant plasmid DNA. Single colonies were used to inoculate cultures from which DNA mini-preparations were prepared. Restriction digestion analysis was performed on the DNA to monitor for the presence of insert DNA using *Kpn* I, *Hind* III and *Eco* RI restriction enzymes (Figure 7.2.3D). Once it was established that the reporter plasmid contained the –1710/+203 region of the human *bik* promoter cloned just upstream of the luciferase gene in pGL2-basic, DNA maxi-preparations were carried out using the Qiagen® Midi Kit (Section 2.2.1.12) and the fidelity of the cloned sequence was verified by sequencing (Section 2.2.1.18) using pGL2-Basic specific primers (Table 2.1-5). The pGL2-1710/+203 *bik* Luc derived plasmid DNA, demonstrated 100% homology with the predicted sequences as derived from the known *bik* promoter sequence (GenBank accession number U34584) (data not shown).

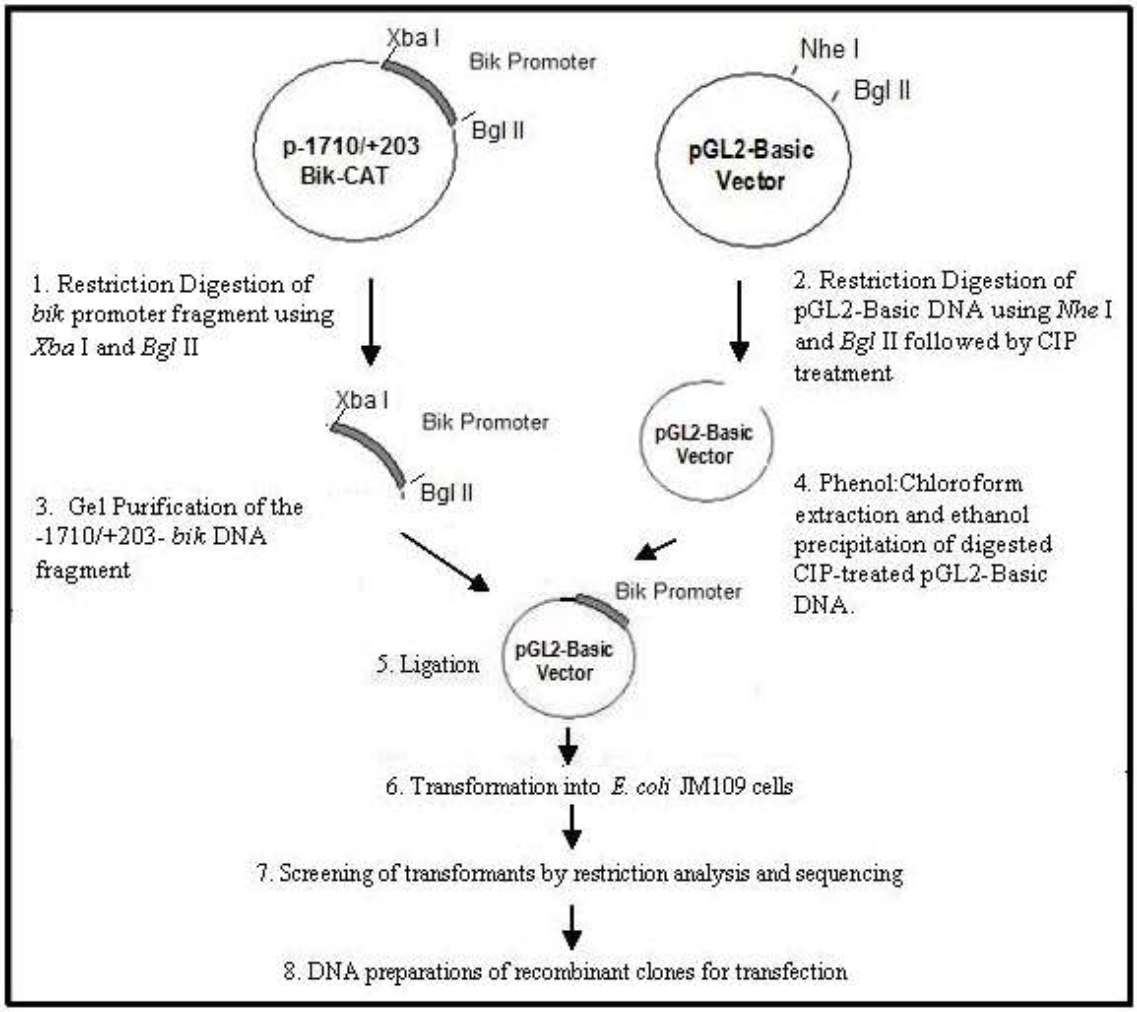


Figure 7.2.2: Cloning Strategy.

Flow diagram detailing the steps involved in the construction of the pGL2-1710/+203 *bik* Luc construct. See text for details.

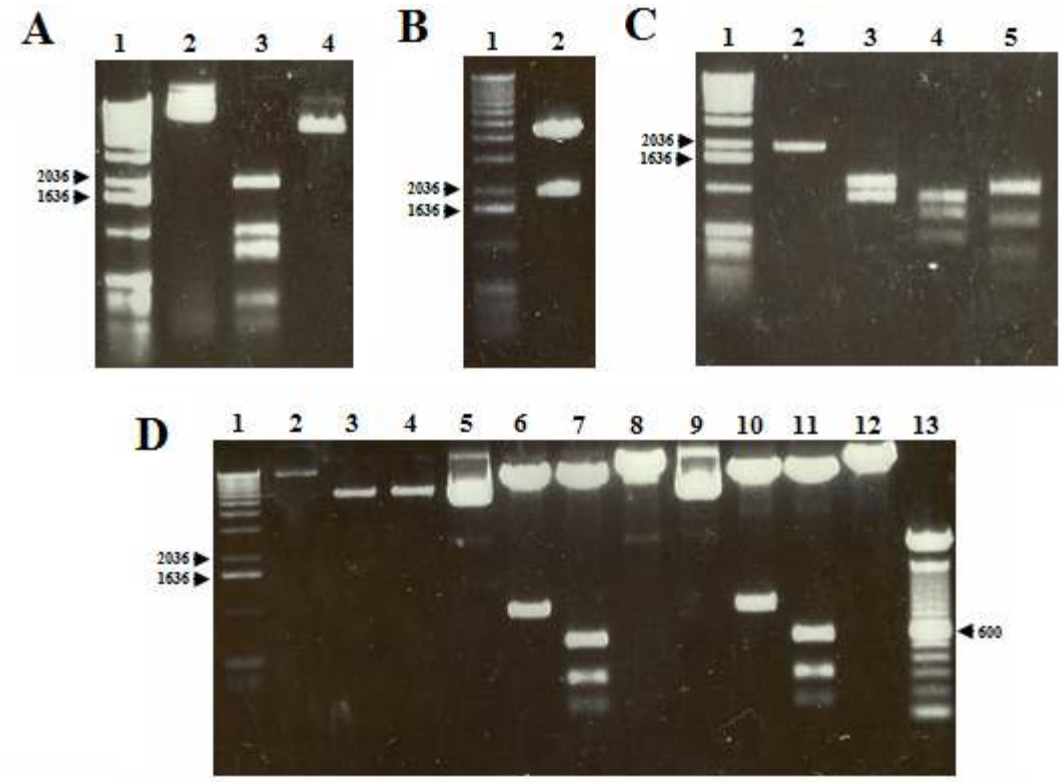


Figure 7.2.3: Construction of the p-1710/+203-*bik* Luc construct.

(A) Restriction digestion of the 5,598bp pGL2-basic with *Rsa* I and *Hind* III. Fragments were analysed using a 1% agarose 1X TAE gel and electophoresis was carried out at 100V for 1 hour in 1X TAE. Undigested pGL2-basic plasmid is shown in lane 2. Digestion with the *Rsa* I restriction enzyme resulted in 12 bands at 1932bp, 1000bp, 795bp, 702bp, 336bp, 318bp 209bp, 169bp, 53bp, 52bp, 21bp and 10bp (lane 3, bands at 53bp, 52bp, 21bp and 10bp to faint to discern), and digestion with *Hind* III (lane 4) linearised the 5,598bp fragment as expected. A 1Kb DNA ladder is shown in lane 1. (B) Restriction digestion of the p-1710/+203BikCAT-2 construct with *Xba* I and *Bam* HI excised the promoter fragment (1913bp), as expected in lane 2. Fragments were analysed using a 0.7% agarose-1X TAE gel and electrophoresis was carried out at 100V for 1 hour in 1X TAE. A 1Kb ladder is shown in lane 1. (C) The *Xba* I-*Bam* HI digested *bik* promoter fragment was subject to restriction digestion using *Kpn* I, *Rsa* I and *Hind* III to check integrity and was then analysed by agarose gel electrophoresis on a 1.2% agarose-1X TAE gel and electrophoresis was carried out at 100V for 1 hour in 1X TAE. Digested, purified *bik* promoter fragment is shown in lane 2 (1913bp). Lanes 3, 4 and 5 show *Kpn* I, *Rsa* I and *Hind* III digestions respectively. Digestion with the *Kpn* I restriction enzyme resulted in 2 bands at 1058bp and 855bp (lane 3), restriction analysis using *Rsa* I yielded 3 fragments at 853bp, 656bp and 404bp (lane 4) while *Hind* III digestion resulted in 4 fragments at 945bp, 558bp, 268bp and 142bp (lane 5). A 1Kb DNA ladder is shown in lane 1. (D) Restriction analysis of recombinant p-1710/+203-*bik* Luc clones by digestion using *Kpn* I, *Hind* III and *Eco* RI enzymes followed by agarose gel electrophoresis on a 1% agarose-1X TAE gel at 100V for 1 hour. Undigested pGL2-Basic Vector is shown in lane 2. Restriction digestion of pGL2-basic with *Kpn* I (lane 3) and *Hind* III (lane 4) linearised the plasmid. Lanes 5-12 illustrate analysis of selected positive clones. Lanes 5-8 show DNA from a single recombinant clone, while lanes 9-12 show DNA

from a second recombinant clone. Undigested recombinant plasmid is shown in lanes 5 and 9. Restriction analysis of p-1710/+203 *bik* Luc with *Kpn* I yielded fragments at 6613bp and 869bp (lanes 6 and 10). Digestion of recombinant DNA with *Hind* III resulted in 4 bands at 6523bp, 558bp, 277bp and 142bp (lanes 7 and 11) while digestion with *Eco* RI linearised the DNA (7511bp; lanes 8 and 12). A 1Kb DNA ladder is shown in lane 1 and a 100bp ladder in lane 13.

7.2.2 Effect of EBNA2 on *bik* promoter activity in the DG75 cell line.

A strategy involving co-transfection experiments with either of two EBNA2 expression plasmids, pSG5EBNA2 and pSG5EBNA2ww323sr (Table 2.1-4), together with the -1710/+203 construct p-1710/+203 *bik* Luc was employed. pSG5EBNA2 expresses wild-type EBNA2. pSG5EBNA2ww323sr on the other hand expresses a non-CBF1 binding EBNA2 mutant in which two tryptophan residues at positions 323 and 324 in the CBF1 binding domain were replaced with a serine and arginine residue respectively, thus abrogating the ability of EBNA2 to bind CBF1 (Ling *et al*, 1993a). The down-regulation of *bik* mRNA by EBNA2 has been observed in the context of the EBV BL cell lines, DG75, BL41 and BJAB (Chapter 4). Thus, to examine if the sub-cloned *bik* promoter fragment could be *trans*-activated by EBNA2 in BL cell lines, co-transfection experiments were undertaken in DG75, BL41K3 and BJAB.

The EBNA2 expression vector and the CBF1-regulated luciferase reporter construct pGa981-6 (Table 2.1-4) were co-transfected as a control in each transfection experiment to monitor EBNA2/CBF1 *trans*-activation. pGa981-6 contains a 50bp oligonucleotide harbouring both CBF1 binding sites of the EBV *TP1* promoter sub-cloned as a hexamer into the plasmid pGa50-7 (Minoguchi *et al*, 1997) . EBNA2 is a well established *trans*-activator of this CBF1-regulated reporter construct, and a consistent *trans*-activation of the CBF1-responsive artificial promoter was detected in every assay.

In a second control experiment to validate the promoter-reporter assay, co-transfections were carried out using a known EBNA2-responsive reporter construct, -1374/+81 Bfl-1 Luc. Accordingly DG75 cells were transiently co-transfected with 7µg of pSG5EBNA2 or pSG5ww323sr with 1µg of the *bfl-1* promoter-reporter construct (-1374/+81 Bfl-1 Luc). The total quantity of transfected plasmid was adjusted in all cases to 10µg using empty vector (pSG5). *Bfl-1* promoter activity was

then analysed 48 hours after transfection using the luciferase assay (Section 2.2.2.13). *Trans*-activation rates were determined by standardising the luciferase values obtained with luciferase values recorded from transfections with 1374/+81 Bfl-1 Luc alone. A plasmid carrying the Lac Z (pCMVLac Z) gene was also included in each transfection and efficiency was accounted for by measuring beta-galactosidase (β-gal) activity (Section 2.2.2.14). As can be seen in Figure 7.2.4A an increase in *bfl-1* promoter activity (approximately 6-fold) is apparent in response to the addition of the EBNA2 expression plasmid, pSG5EBNA2. The non-CBF1 binding mutant pSG5ww323sr, however, failed to *trans*-activate the promoter (Figure 7.2.4A) thus confirming the significance of the EBNA2-CBF1 interaction in this context as previously established (Pegman *et al*, 2006), and also verifying the reliability of the promoter-reporter assay. In the same experiment EBNA2 *trans*-activated the pGa981-6 reporter construct approximately 1,600-fold (Figure 7.2.4B).

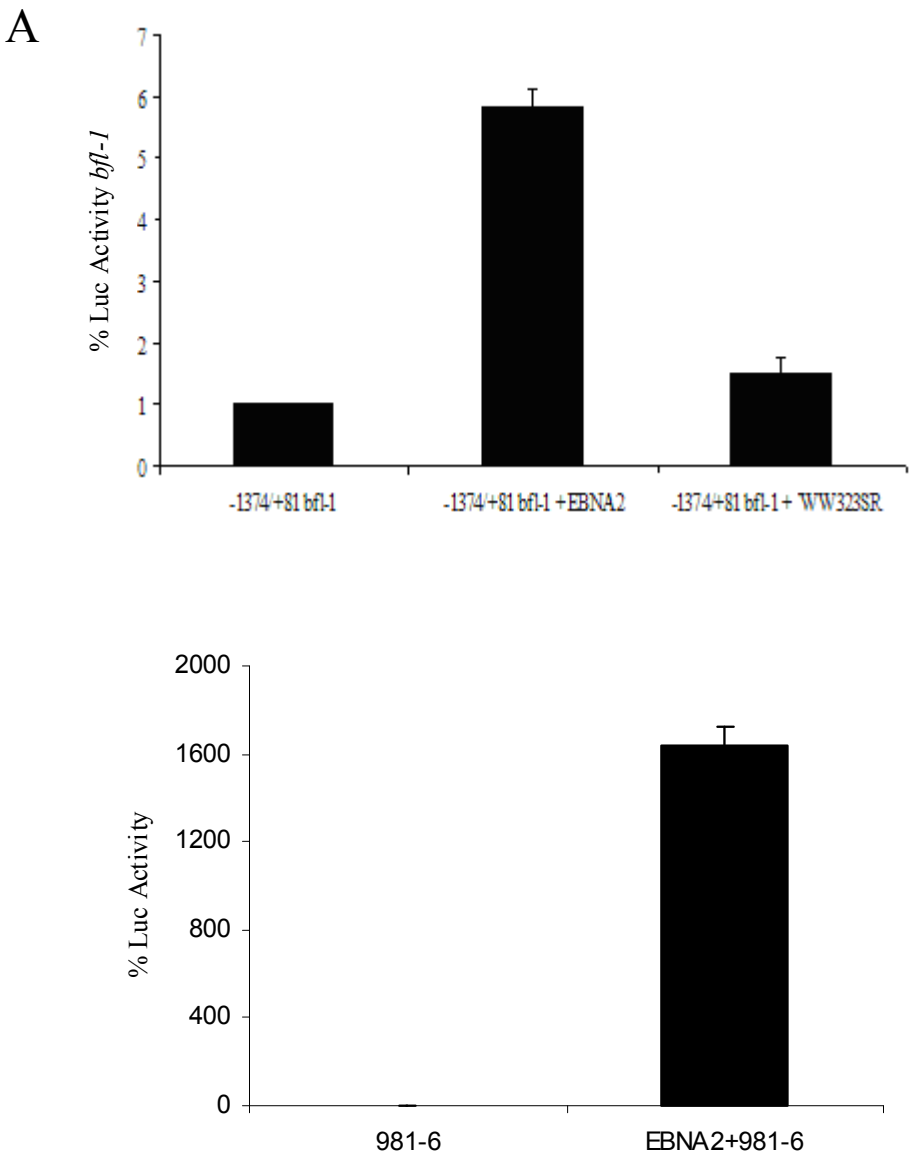


Figure 7.2.4: EBNA2 is a well established *trans*-activator of CBF1-regulated promoters.

(A) DG75 cells were co-transfected with wild type *bfl-1* promoter (–1374/+81-Luc) reporter construct, pCMVLacZ and either the EBNA2 expression vector pSG5EBNA2 or pSG5EBNA2ww323sr. The total quantity of transfected plasmid was adjusted to 10µg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained the –1374/+81-Luc reporter construct when co-transfected with control vector pSG5. The absolute luciferase values obtained for pSG5 and –1374/+81-Luc after transfection was set at 1. (B) DG75 cells were co-transfected with the pGa981-6 artificial reporter construct, pCMVLacZ and EBNA2. The total quantity of transfected plasmid was adjusted to 10µg with the empty vector pSG5. Normalised luciferase values were expressed relative to the corresponding value obtained for the pGa981-6 reporter construct when

co-transfected with control vector pSG5. The absolute luciferase values obtained for pSG5 and 981-6 after transfection was set at 1. (Data are Mean ±SD).

Having established an appropriate assay for analysis of promoter activity in response to EBNA2 expression, DG75 cells were co-transfected with 7µg of pSG5EBNA2 or pSG5EBNA2ww323sr with 1µg of the *bik* promoter-reporter construct -1710/+203-*bik*-Luc and pCMVLac Z. *Bik* promoter activity was then analysed using the luciferase assay as before. Normalised luciferase values were expressed relative to the corresponding value obtained for each reporter construct when co-transfected with control vector pSG5. As can be seen from Figure 7.2.5 a clear reduction in *bik* promoter activity (approximately 40%) was observed in response to EBNA2. Moreover, a decrease in promoter activity was also observed in response to pSG5EBNA2ww323sr, at levels equivalent to that seen for pSG5EBNA2. This result indicates that EBNA2 regulates the *bik* promoter via a mechanism not requiring the cellular DNA binding protein CBF1. EBNA2 *trans*-activated the pGa981-6 reporter construct approximately 1,400-fold (data not shown).

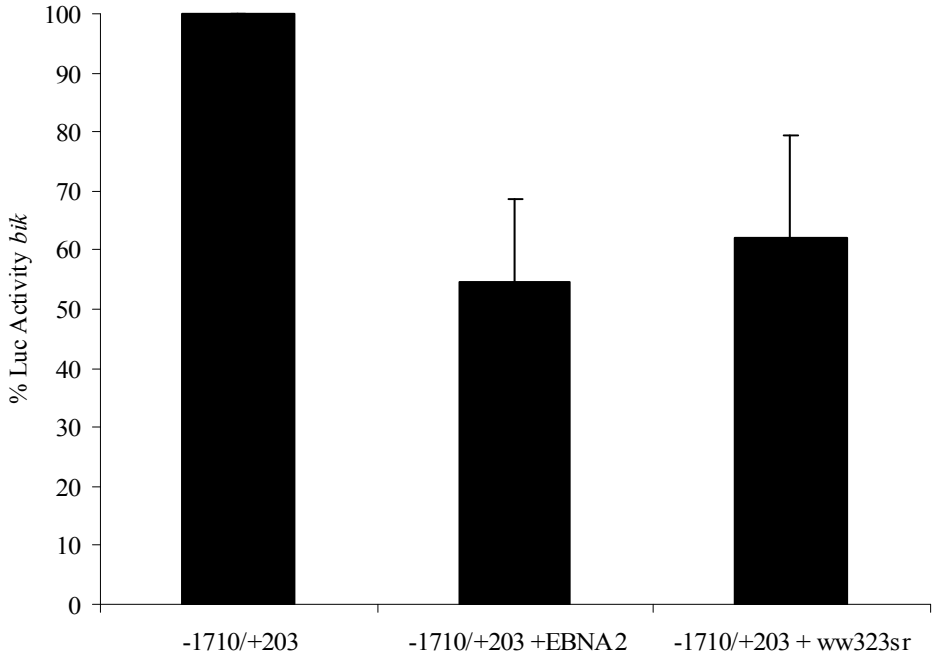


Figure 7.2.5: EBNA2 represses the *bik* promoter; repression does not require the EBNA2-CBF1 interaction

DG75 cells were co-transfected with *bik* promoter (–1710/+203-Luc) reporter construct, pCMVLacZ and the EBNA2 expression vector pSG5EBNA2 or

pSG5EBNA2ww323sr. The total quantity of transfected plasmid was adjusted to 10μg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct when co-transfected with control vector pSG5. The absolute luciferase values obtained for pSG5 and -1710/+203-Luc-*bik* after transfection was set at 100. (Data are Mean ±SD).

7.2.3 EBNA2 mediated repression of the *bik* promoter is not potentiated by EBNA-LP in DG75.

The EBNA Leader Protein (EBNA-LP), together with EBNA2 is the first viral gene product to be expressed during EBV-induced B-cell immortalisation (Alfieri *et al*, 1991). EBNA-LP is known principally as a transcriptional co-activator of EBNA2. It has previously been shown that EBNA-LP co-operates with EBNA2 in the up-regulation of LMP1 in B-cells (Harada and Kieff, 1997; Nitsche *et al*, 1997) and that EBNA-LP and EBNA2 co-operate to stimulate expression of cyclin D2 in resting B-cells in addition to mediating G_0 to G_1 transition during immortalisation (Sinclair *et al*, 1994). Furthermore, co-transfection of EBNA-LP also considerably potentiates EBNA2 *trans*-activation of the *bfl-1* promoter (Pegman,P.M. unpublished data). Thus, considering EBNA-LP as a co-activator of EBNA2, it was decided to determine if there was a co-operative role for EBNA2 and EBNA-LP in the repression of the *bik* promoter. For this purpose an EBNA-LP expression plasmid, pJT125, (Table 2.1-4) and the corresponding non-functional mutant, pRSP83 (Table 2.1-4) were employed. The construction of these plasmids has been published elsewhere (Harada and Kieff, 1997; Peng *et al*, 2000).

Co-transfection experiments were performed using DG75 with 5μg of the EBNA-LP expression plasmids together with 7μg of EBNA2 and 1μg of the *bik*-promoter construct. The total quantity of transfected plasmid was adjusted in all cases to 12μg using empty vector (pSG5). Luciferase activities which were normalised for transfection efficiency (based on β-galactosidase activity). Normalised luciferase values were expressed relative to the corresponding value obtained for the reporter construct when co-transfected with control vector pSG5. The results are shown in Figure 7.2.6. In this experiment, EBNA2 *trans*-activated the pGa981-6 reporter

construct approximately 1,500-fold (data not shown). Co-transfection with pSG5EBNA2 resulted in 50% decrease in promoter activity, while co-transfection of EBNA2 with both the EBNA-LP expression plasmid, pJT125, and the non-functional mutant partner pRSP83, caused a similar decrease in *bik* promoter activity. Thus, co-transfection of EBNA2 with EBNA-LP did not result in any further decrease in promoter activity than was the case with EBNA2 alone. Thus, EBNA-LP does not appear to potentiate EBNA2 repression of the *bik* promoter in this context.

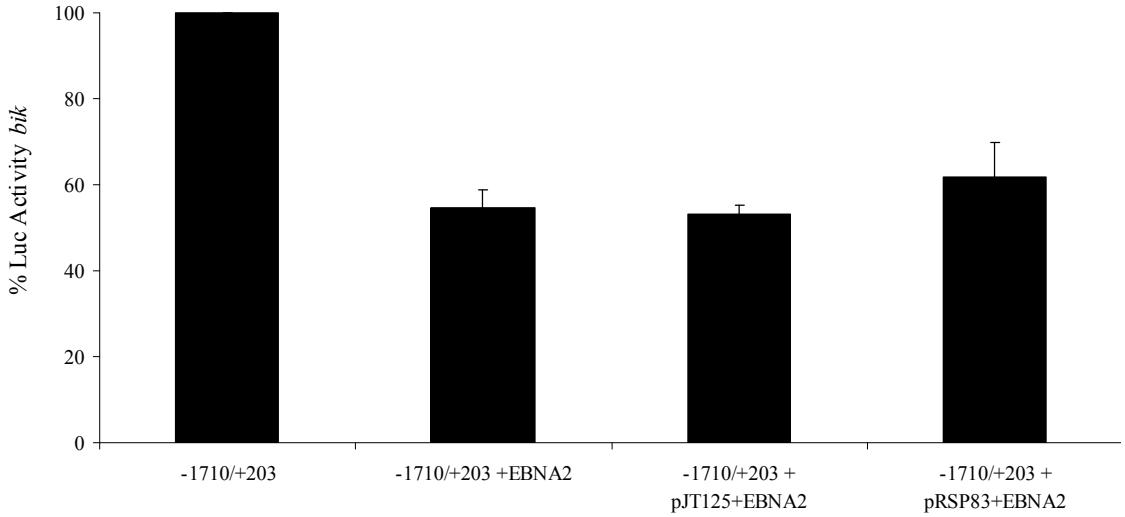


Figure 7.2.6: EBNA2 mediated repression of the *bik* promoter is not potentiated by EBNA-LP in DG75.

DG75 cells were co-transfected with *bik* promoter (–1710/+203-Luc) reporter construct, pCMV LacZ and the EBNA2 expression vector pSG5EBNA2, and either the EBNA-LP expression plasmid pJT125, or its non-functional mutant partner pRSP83. The total quantity of transfected plasmid was adjusted to 12μg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct when co-transfected with control vector pSG5. The absolute luciferase values obtained for pSG5 and -1710/+203-Luc-*bik* after transfection was set at 100. (Data are Mean ±SD).

Of note, co-transfection experiments with the EBNA2 expression vector failed to consistently show repression of the *bik* promoter, whereas consistent responses from the *bfl-1* and *981-6* promoters were readily detectable in every experiment. These experiments were repeated on different occasions; however, as can be seen from the

summary of the results given in Table 7.2-1, no consistency between different experiments could be achieved. While 8 out of 11 (approximately 72%) transfections indicate some degree of down-regulation nonetheless it cannot be concluded that EBNA2 down-regulated transcription initiated from the 1.9kb *bik* promoter fragment in these experiments.

Table 7.2-1: Summary of *bik* promoter Transfections in DG75

Transfection	Luc Activity of in response to EBNA2
1	200%
2	200%
3	100%
4	90%
5	90%
6	50%
7	78%
8	50%
9	58%
10	50%
11	76%

Accordingly, it was thus important to establish if this inconsistency was specific to DG75. For this reason, similar experiments were carried out in another well-studied BL cell line, namely BL41K3.

7.2.4 Effect of EBNA2 on *bik* promoter activity in the BL41K3 cell line.

BL41K3 cells were transfected by electroporation (Section 2.2.4.1). In all cases, cells were harvested 48 hours after transfection and activity of the *bik* promoter was then assessed using the luciferase assay. Luciferase activities which were normalised for transfection efficiency (based on β-galactosidase activity).

Initially, in order to validate the promoter-reporter assay in another cellular context, pSG5EBNA2 and pSG5EBNA2ww323sr were used in a co-transfection experiment with *bfl-1* promoter-reporter construct -1374/+81 Bfl-1 Luc. As before an increase in *bfl-1* promoter activity occurred in response to addition of the EBNA2 expression

plasmid, while the CBF1-mutant pSG5EBNA2ww323sr failed to *trans*-activate the promoter (Figure 7.2.7A).

A similar strategy to that just described for DG75 was then employed to investigate the effect of EBNA2 on *bik* promoter activity in the BL41K3 cell line. Again, EBNA2 *trans*-activated the artificial CBF-1 responsive promoter in pGa981-6 reporter as reflected in an approximately 40-fold increase in luciferase activity (data not shown). As illustrated in Figure 7.2.7B, an obvious reduction in *bik* promoter activity was observed in response to addition of the EBNA2 expression plasmid. Furthermore, a decrease in promoter activity was also observed in response to the CBF1 non-binding EBNA2 mutant vector, comparable to that seen using pSG5EBNA2. Thus, the ability of EBNA2 to bind CBF1 does not appear essential for EBNA2-mediated repression of the *bik* promoter in BL41K3.

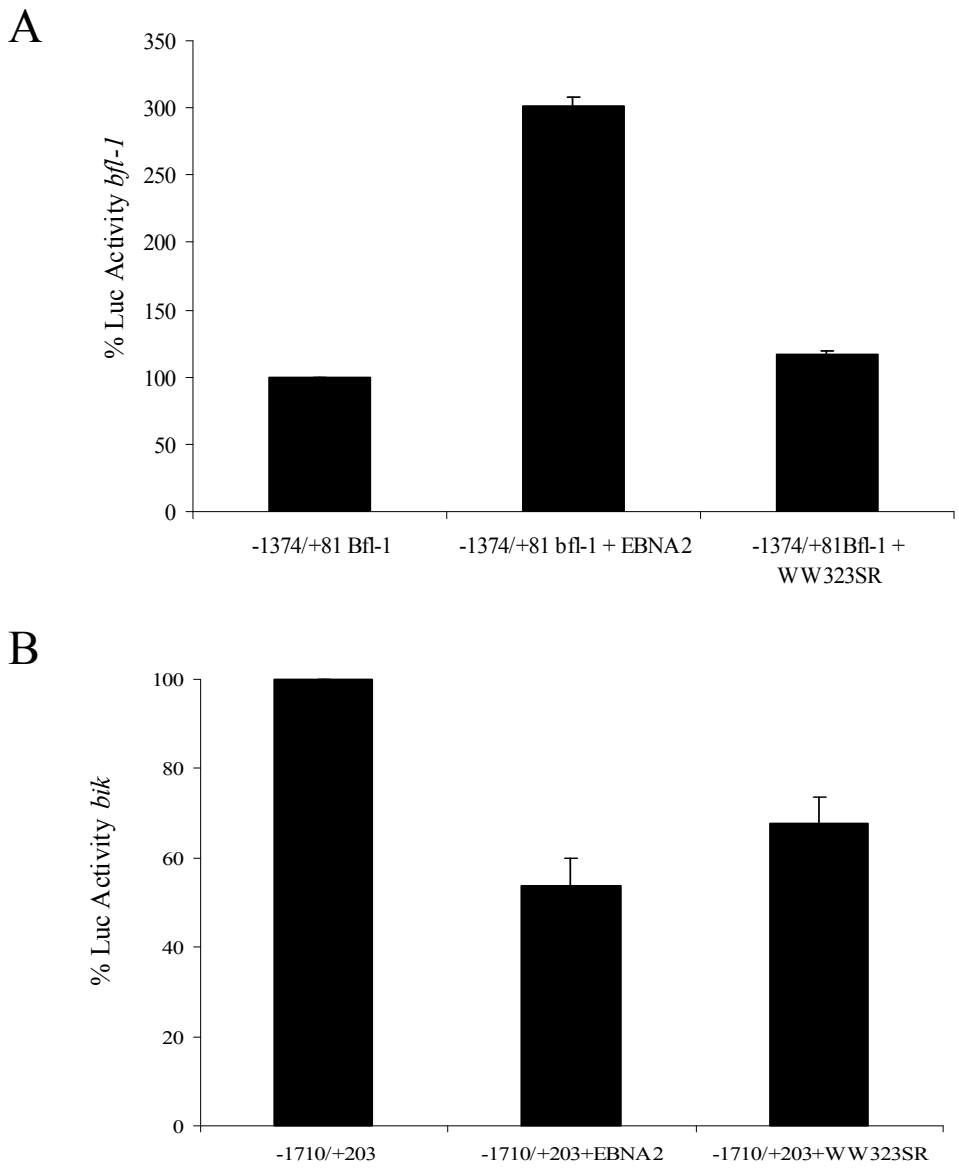


Figure 7.2.7: EBNA2 represses the *bik* promoter; repression does not require the EBNA2-CBF1 interaction.

(A) BL41K3 cells were co-transfected by electroporation with wild type *bfl-1* promoter (–1374/+81-Luc) reporter construct, pCMVLacZ and either the EBNA2 expression vector pSG5EBNA2 or pSG5EBNA2ww323sr. The total quantity of transfected plasmid was adjusted to 10μg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the –1374/+81-Luc reporter construct when co-transfected with control vector pSG5. The absolute luciferase values obtained for pSG5 and –1374/+81-Luc after transfection was set at 100. (B) BL41K3 cells were co-transfected by electroporation with *bik* promoter (–1710/+203-Luc) reporter construct, pCMVLacZ and the EBNA2 expression vector pSG5EBNA2 or pSG5EBNA2ww323sr. The total quantity of transfected plasmid was adjusted to 10μg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were

performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct when co-transfected with control vector pSG5. The absolute luciferase values obtained for pSG5 and -1710/+203-Luc-*bik* after transfection was set at 100. (Data are Mean ±SD).

The influence of EBNA2 expression on *bik* promoter activity was also examined in BL41K3, which although EBV negative, does expresses an inactive ER/EBNA2. BL41K3 cells were transiently transfected with the –1710/+203-*bik*-Luc as before. After transfection the cells were cultured in the presence of 1μM estrogen, and cells were harvested after 48 hours. A control transfection, in which the EBNA2 expression vector was co-transfected with the *bik* promoter and cells were incubated in the absence of estrogen was also performed. As can be seen in Figure 7.2.8, transient co-transfection with pSG5EBNA2 led to approximately 70% reduction *bik* promoter activity. Significantly less promoter repression was observed due to the effect of ER/EBNA2, where *bik* promoter activity was reduced by approximately 30% (Figure 7.2.8) upon addition of estrogen to the medium. This might reflect the limited quantity of ER/EBNA2 molecules available in comparison to the quantity of EBNA2 expressed from co-transfected pSG5EBNA2. These results are evidence of a role for EBNA2 in repression on the *bik* promoter in the BL41 background.

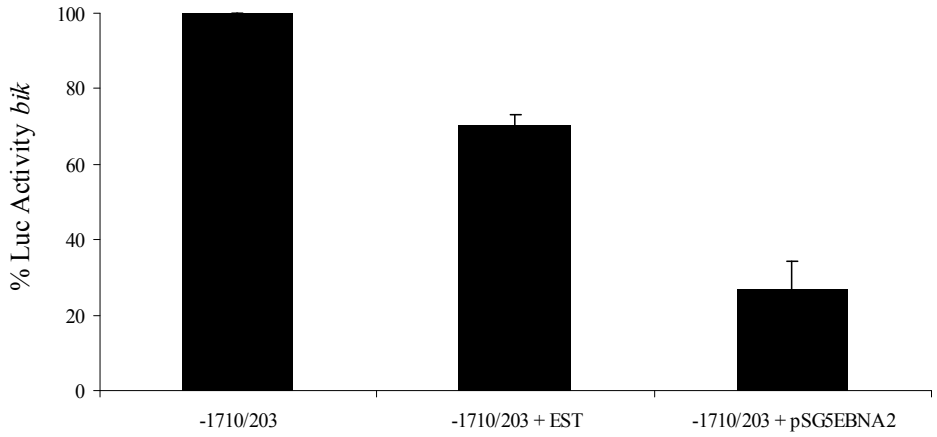


Figure 7.2.8: **Repression of the *bik* promoter in response to EBNA2 activation by estrogen in BL41K3.**

BL41K3 cells were transiently co-transfected by electroporation with the wild type *bik* promoter (–1710/+203-Luc) reporter construct and pCMVLacZ. A co-transfection with EBNA2 expression vector pSG5EBNA2 and the *bik* promoter was also undertaken. The total quantity of transfected plasmid was adjusted to 10μg with the empty vector pSG5. Cells were induced to express ER/EBNA2 by the addition of

1μM estrogen to the cell culture medium and harvested 48 hours after transfection, luciferase and β-gal assays were performed as before. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct when cultivated in the absence of estrogen. The absolute luciferase values obtained for -1710/+203-Luc-*bik* after transfection was set at 100. (Data are Mean ±SD).

The results obtained using the BL41K3 cell line were more consistent than those obtained in DG75, and undoubtedly EBNA2 down-regulates the *bik* promoter in this cell context. For this reason, DG75 cells were not used in any further EBNA2-related promoter co-transfection experiments in this study. However, it should be noted that the level of repression observed in BL41K3 varied considerably, ranging from 88%-18% reduction in promoter activity (Table 7.2-2).

Table 7.2-2: Summary of *bik* promoter transfections in BL41K3

Transfection	Luc Activity in response to EBNA2
1	82%
2	67%
3	61.5%
4	54%
5	50%
6	30.4%
7	23%
8	20%
9	12%

The summation of these results is clear, while there is the observation that the level of repression varies considerably, nevertheless, EBNA2 consistently represses the activity of the *bik* reporter construct in transfections using BL41K3.

7.2.5 Generation of promoter deletion constructs -855/+203 *bik* Luc and -191/+203 *bik* Luc.

In order to delineate the DNA sequence elements that mediate down-regulation by EBNA2 it was initially important to examine the sequence of the *bik* promoter for putative transcription factor binding sites that may be relevant to this study.

7.2.5.1 Putative CBF1 and Ets-family transcription factor binding sites exist on the *bik* promoter sequence.

To identify possible regions of regulatory significance on the *bik* promoter, the upstream sequences were analysed for putative transcription factor binding sites using the MatInspector search tool on the transfac database (http://www.genomatix.de/) in combination with Transcription Element Search Software (TESS) available on the BCM Search Launcher website (http://dot.imgen.bcm.tmc.edu/) (Table 2.1-6). Examination of the –1710 to +203 region of the promoter region revealed putative transcription factor binding sites for a number of transcription factors including transcription factors such as GATA-1, Oct-1, Sp1 and NF-kB as has been previously reported (Verma *et al*, 2000). As discussed in Chapter 1 EBNA2 has no inherent DNA-binding activity, but it can associate with EBNA2-responsive promoters through contact with multiple cellular factors including CBF1, PU.1 and CBP (Zimber-Strobl *et al*, 1993; Henkel *et al*, 1994; Laux *et al*, 1994a; Ling *et al*, 1994; Waltzer *et al*, 1994; Johannsen *et al*, 1995; Wang *et al*, 2000; Pegman *et al*, 2006).

Of interest to this study, analysis of the sequence indicated that the promoter contained the sequence motif gaggTGGGaagaaca at position –871 to –895 (Figure 7.2.9). This sequence has previously been found in many EBNA2-responsive promoters that have been characterised to date, and is part of the consensus binding sequence for CBF1 (CGTGGGAA; (Tun *et al*, 1994)). Further analysis of the promoter sequence, revealed additional '5-GGAA-3' sites, which is the core sequence motif recognised as fundamental in binding of the Ets family of transcription factors (Karim *et al*, 1990). This sequence was identified in two separate regions; between –968 and –984, and also between -1017 and -1033 on the promoter (Figure 7.2.9). Nucleotides adjacent to this core sequence serve to determine the binding specificity of the individual members of this family to the DNA and in this way the two sites were additionally designated putative Elk-1 (-1017 and -1033) and PU.1 (–968 and –984) binding sites. Another potential EBNA2-related regulatory transcription factor binding site, CREB, was found in six places on the promoter, and there was also a consensus ATF/CRE site between -314 and -294. There were 14 potential Myc-related binding sites spread throughout the 1.9kb sequence (Figure 7.2.9), although

this is not surprising since Myc is a central transcriptional hub in growth and proliferation control. Indeed, it is thought that Myc could regulate up to 10–15% of all genes, occupying 4,296 genomic loci in B-cells (Zeller *et al*, 2006)

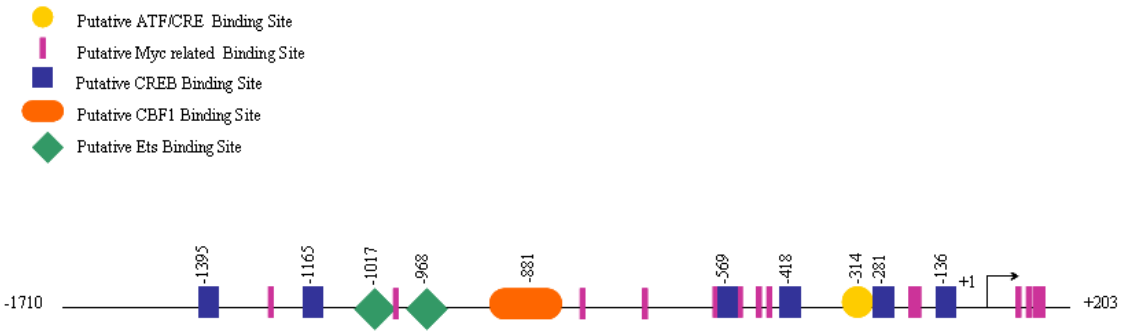


Figure 7.2.9: Schematic representation of the *bik* promoter fragment under study indicating possible transcription factor binding sites.

The region of the *bik*-Luc promoter from position -1710 to +203 relative to the transcription start site is illustrated on the schematic diagram. Transcription Element Search Software from the Transfac database and from the BCM Search Launcher website, revealed putative transcription factor binding sites for a number of relevant transcription factors including potential binding sites for various transcription factors such ATF/CRE (-314 and -294), CBF1/RBPJκ (–871 to –895), Ets (–968 and –984, and also between -1017 and 1033) and CREB (-1395 and -1375, -1165 and -1145, -569 and -549, -418 and -398, -281 and -261 and between -136 and -116). The locations of putative RBP-Jκ/CBF1 (orange), Ets (green) transcription factor binding sites and others are indicated in the legend. There were 14 potential Myc- related binding sites spread throughout the promoter.

7.2.5.2 Cloning of the 5' progressive deletion fragments -855/+203-*bik* Luc and -191/+203-*bik* Luc.

Hence, two further *bik*-luciferase constructs containing progressive deletions from the 5' end of the promoter were developed for use in transient transfection assays. Two distinct regions were nominated for examination, (1) the region upstream of –855 eliminating the putative CBF1 binding site, two potential CREB sites and both potential Ets binding sites, and (2) the area upstream of –191, thus abolishing the potential for binding to most of the relevant transcription factor sites, including

ATF/CRE, CBF1, and Ets. The area downstream of -191 harbours one consensus CREB site and several possible Myc binding sites.

Since the -1710/+203-*bik* Luc reporter construct had already been generated (Section 7.2.1) it was decided to generate the series of *bik* promoter-luciferase reporter constructs to compliment this –1710/+203 promoter. The –855/+203 and -191/+203 regions of the *bik* promoter fused to the luciferase gene were thus derived from the -1710/+203-*bik*-Luc construct using a similiar approach to that employed in the derivation of the p-1710/+203-*bik*-Luc construct (Figure 7.2.2).

The individual promoter deletion fragments were generated by restriction digestion targeted to specific regions of the promoter. Briefly, the 2 truncated promoter regions were excised from -1710/+203-*bik*-Luc by double digestion using *Kpn* I and *Bgl* II in the case of p-855/+203 *bik* Luc and *Sac* I and *Bgl* II in the case of pGL2-191/+203 *bik* Luc. Prior to ligation, the pGL2-Basic plasmid was linearised by digestion with *Kpn* I and *Bgl* II (for ligation to the *Kpn* I-*Bgl* II *bik*-promoter fragment), and *Sac I* and *Bgl* II (for ligation to the *Sac* I- *Bgl* II *bik*-promoter fragment), followed by dephosphorylation using CIP to prevent recircularisation of the linearised DNA (Section 2.2.1.4). The linearised, CIP-treated DNA was then purified by phenol: chloroform extraction followed by ethanol precipitation (Section 2.2.1.2). The *Kpn* I-*Bgl* II and *Sac* I- *Bgl* II fragments were subsequently purified using the Promega Wizard® DNA purification system (Section 2.2.1.6) and the digested, purified product was then sub-cloned into the *Kpn* I and *Bgl* II sites and *Sac* I and *Bgl* II sites of pGL2-basic respectively. Ligations of vector and insert DNA were carried out overnight at 16 ˚C (Section 2.2.1.7).

Following the ligation reaction, competent *E. coli* JM109 cells were transformed with the recombinant plasmid DNA (Section 2.2.1.9). Single colonies were used to inoculate cultures from which DNA mini-preparations were prepared. Restriction digestion analysis was performed on the DNA to monitor for the presence of insert DNA using *Hind* III and *Sma* I restriction enzymes (Figure 7.2.10). Once it was established that the two reporter plasmids contained the –855/+203 region and –191/+203 region of the human *bik* promoter cloned just upstream of the luciferase gene in pGL2-basic, DNA maxi-preparations were carried out using the Qiagen® Midi

Kit (Section 2.2.1.12) and these constructs were then used in transient co-transfection assays of BL-derived cell lines. A schematic representation of the luciferase reporter gene constructs driven by various regions of the *bik* promoter is shown in Figure 7.2.11A. Agarose gel electrophoresis confirmed the quality of the *bik*-promoter-luciferase constructs used in transfection studies (Figure 7.2.11B).

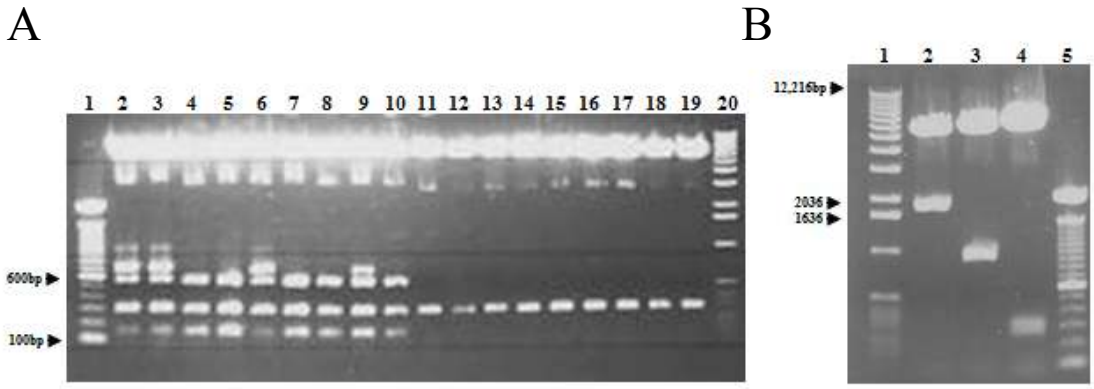


Figure 7.2.10: **Generation of the *bik-luc* Promoter Reporter Constructs, -855/+203 *bik* Luc, and -191/+203 *bik* Luc**.

(A) Restriction analysis of recombinant p-855/+203-*bik*-Luc and p-191/+203-*bik*-Luc clones by digestion using *Hind* III enzyme followed by agarose gel electrophoresis on a 1% agarose-1X TAE gel at 100V for 1 hour. Lanes 2-10 illustrate analysis of potential -855/+203 *bik* Luc recombinant clones. Lanes 11-19 show DNA from putative p-191/+203-*bik*-Luc recombinant clones. A 100bp ladder and a 1Kb ladder are shown in lanes 1 and 20 respectively. Digestion of positive p-855/+203-*bik*-Luc clones with *Hind* III resulted in 5 bands at 5563bp, 558bp, 277bp, 142bp and 91bp (lanes 4,5,7,8 and 10, band at 91bp to faint to discern). Restriction digestion analysis of positive p-191/+203-*bik*-Luc yielded 2 fragments at 5969bp and 277bp (lanes 11-19). (B) Restriction digestion analysis comparing recombinant p-1710/+203-*bik*-Luc, p-855/+203-*bik*-Luc and p-191/+203-*bik*-Luc clones using *Sma* I. Restriction analysis of the promoter p-1710/+203-*bik*-Luc using *Sma* I yielded 5 fragments at 5601bp, **1802bp**, 51bp, 27bp and 17bp (lane 2, bands at 51bp, 27bp and 17bp to faint to discern) while *Sma* I digestion of -855/+203-*bik*-Luc resulted in 5 fragments at 5601bp, **924bp**, 51bp, 27bp and 17bp (lane 3, again bands at 51bp, 27bp and 17bp to faint to discern). *Sma* I digestion of the shortest promoter fragment p-191/+203-*bik*-Luc resulted in 5 fragments, 5601bp, **260bp**, 51bp, 27bp and 17bp (lane 4, bands at 51bp, 27bp and 17bp to faint to discern). Agarose gel electrophoresis on a 1% agarose-1X TAE gel at 100V was carried out for 1 hour. A 1Kb DNA ladder is shown in lane 1 and a 100bp ladder is shown in lane 5.

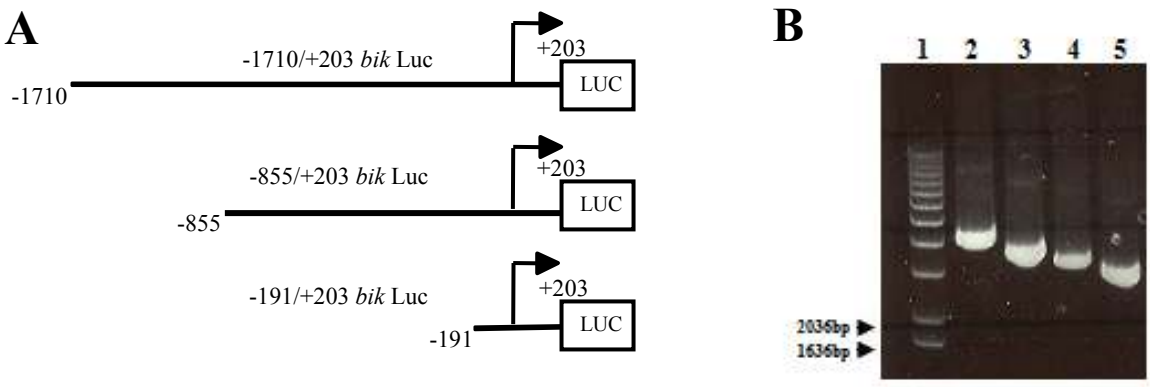


Figure 7.2.11: 5' progressive deletion *bik*-Luc promoter-reporter constructs.

(A) Schematic representation of the -1710/+203 region wild type *bik* promoter and the truncated promoter-reporter constructs. These constructs share a common 3' terminus 203bp downstream from the transcription initiation site (designated by a bent arrow), at which point they are joined to the luciferase gene (Luc). (B) The condition and quality of the DNA was assessed by resolving the DNA by agarose gel electrophoresis. In this case 1µg of DNA from the pGL2-1710/+203-*bik* Luc (lane 2), pGL2-855/+203-*bik* Luc (lane 3), pGL2-191/+203-*bik* Luc (lane 4) and pGL2-basic (lane 5) reporter plasmids are shown after electrophoresis at 100V for 1 hour through a 1% agarose gel using 1X TAE. A 1kb ladder is shown in lane 1.

7.2.6 Transfection experiments using the 5'progressive deletion *bik* promoter-reporter constructs.

DG75 cells were transiently co-transfected with the *bik*-promoter-luciferase constructs and luciferase activity was measured 48 hours after transfection. Importantly, an equivalent number of reporter molecules of each of the individual constructs was transfected in each case, rather than the same concentration of DNA, in order to account for the size differences of the individual constructs. The total quantity of plasmid was adjusted in all cases to 2µg with the empty vector pSG5. As seen in Figure 7.2.12, the level of luciferase activity fell as the promoter fragment was progressively deleted from the 5' end. The difference between the activity of the longest promoter sequence (-1710/+203-*bik*-Luc) and the shorter fragments suggests that several sequence elements along the length of the promoter contribute to positively affect its activity.

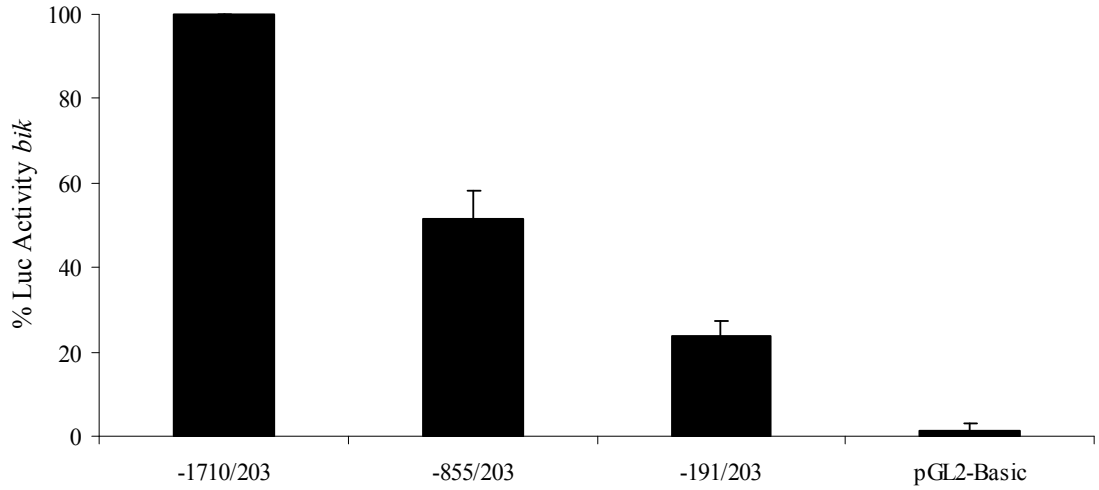


Figure 7.2.12: Analysis of 5' progressive deletion promoter- constructs in DG75

DG75 cells were transiently co-transfected by electroporation with *bik* promoter (–1710/+203-Luc) reporter construct or the promoter deletion constructs (-855/+203-Luc /-191/+203-Luc) and pCMVLacZ. The total quantity of transfected plasmid was adjusted to 2µg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct. The absolute luciferase value obtained for -1710/+203-Luc-*bik* after transfection was set at 100. (Data are Mean ±SD)

In EBV-infected cell lines EBNA2 is expressed and only low levels of *bik* mRNA are detectable (Chapter 3), and so low levels of *bik* promoter activity are to be expected. Thus, in order to examine the activity of the *bik* promoter in this background, AG876 and IB4 were transiently co-transfected by electroporation with the *bik*-promoter-luciferase constructs and luciferase activity was measured 48 hours after transfection. Again, an equivalent number of reporter molecules of each of the individual constructs was transfected in each case. The total quantity of transfected plasmid was adjusted in all cases to 2µg with the empty vector pSG5. As seen in Figure 7.2.13A and B, similar to what was seen in DG75, luciferase activity fell as the promoter fragment was progressively deleted from the 5' end. However, the level of basal activity observed with the –855/+203-Luc-*bik* fragment was considerably different in both cell lines. The −191/+203-Luc-*bik* fragment was consistently lower than the levels observed with the longer promoter construct, −1710/+203.

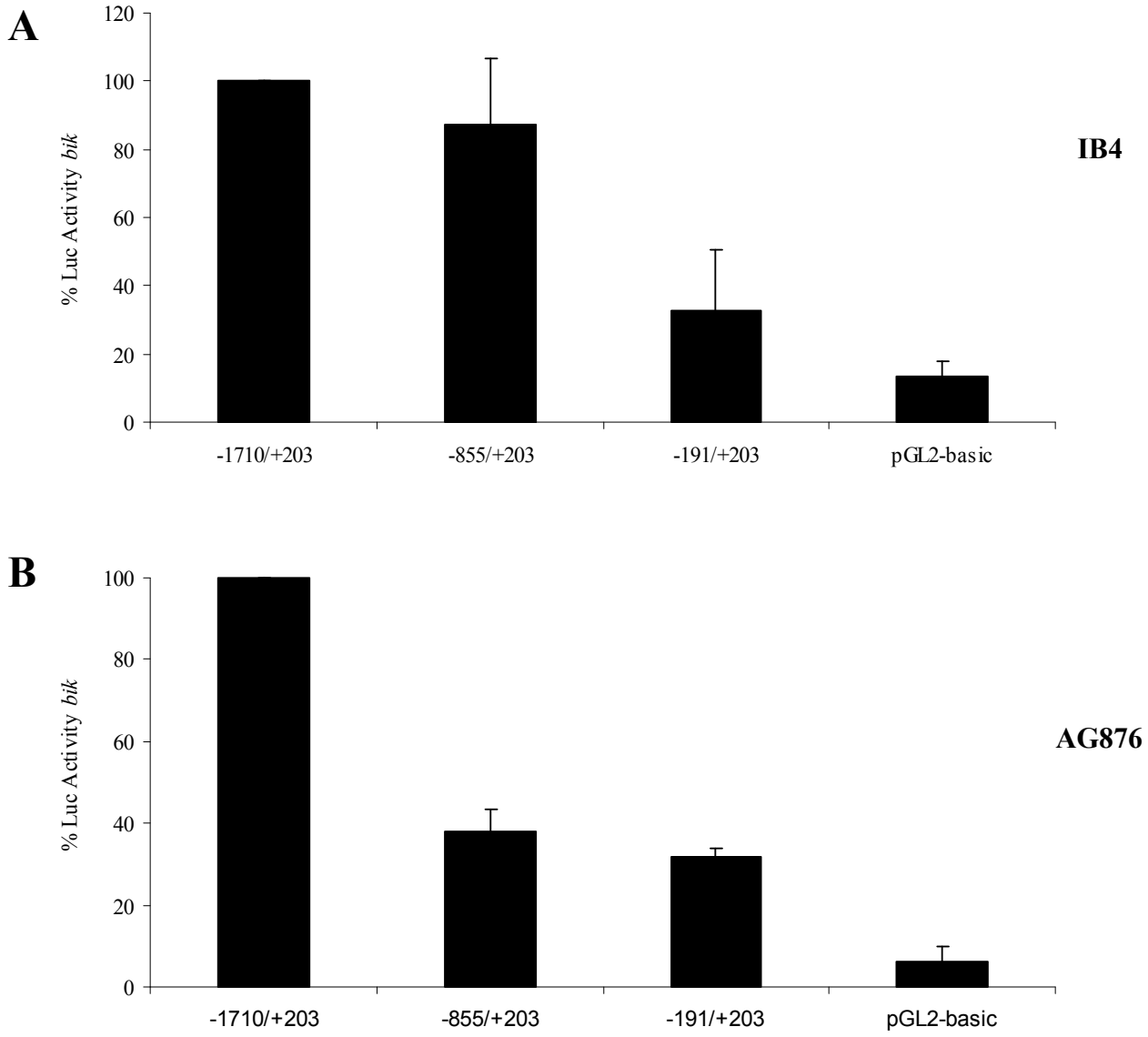


Figure 7.2.13: Analysis of 5' progressive deletion constructs in AG876 and IB4

IB4 (A) and AG876 (B) were transiently co-transfected by electroporation with *bik* promoter (–1710/+203-Luc) reporter construct or the promoter deletion constructs (-855/+203-Luc /-191/+203-Luc) and pCMVLacZ. The total quantity of transfected plasmid was adjusted to 2μg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct. The absolute luciferase value obtained for -1710/+203-Luc-*bik* after transfection was set at 100. (Data are Mean ±SD).

In an attempt to directly investigate the effect of expression of EBNA2 on the transcriptional activity of the *bik* promoter fragments, a number of transient co-transfection experiments were carried out in the BL41K3 and BJAB cell lines. As

shown in Figure 7.2.14A, EBNA2 expression in BL41K3 cells mediated a 30% reduction in transcription from the –1710/+203 region of the *bik* promoter, and analysis of 5' deletions of the promoter again revealed a gradual loss of activity, indicating the loss of elements that are necessary to promote gene transcription in this context. The repression effect by EBNA2 was lost upon deletion to -855, indicating the possible deletion of elements conferring EBNA2 responsiveness within this region of the promoter. Thus, these results indicate that sequence elements in the -1710 to -855 region of the *bik* promoter may make a considerable contribution to promoter activity in this context.

In contrast to this a different trend was observed when this experiment was performed in the BJAB cell line. The longest construct illustrated a 65% reduction in transcription from the *bik* promoter as a consequence of EBNA2 expression (Figure 7.2.14B). Again the basal level of the *bik* promoter was greatly decreased by the removal of elements downstream of this region, but the suppressive effect of EBNA2 on the *bik* promoter was still effective down to -855/+203. Therefore, the deletion of potential CBF1 and Ets binding sites from the promoter did not prevent EBNA2-mediated activation in this experiment. EBNA2 expression however, failed to repress the shorter promoter fragment -191/+203 (Figure 7.2.14B). This result indicated that the region -855 to -191 may harbour a transcription factor binding site essential for EBNA2 associated *bik* repression and is inconsistent to what was found in BL41K3.

Deletion analysis of the *bik* promoter revealed that removal of sequences containing several consensus DNA binding sequences did not affect EBNA2-mediated repression of the *bik* promoter in the same way between different cell lines. When comparing the constructs themselves in all three cell lines, irrespective of EBNA2 expression; it is clear that there is a gradual loss of promoter activity upon deletion down to –191, as was seen in IB4, AG876 previously, seemingly indicating the loss of elements that are important for promoter activity. Overall these results were inconclusive, and did not indicate the significance of any specific region within the *bik* promoter that be important in EBNA-associated *bik* repression.

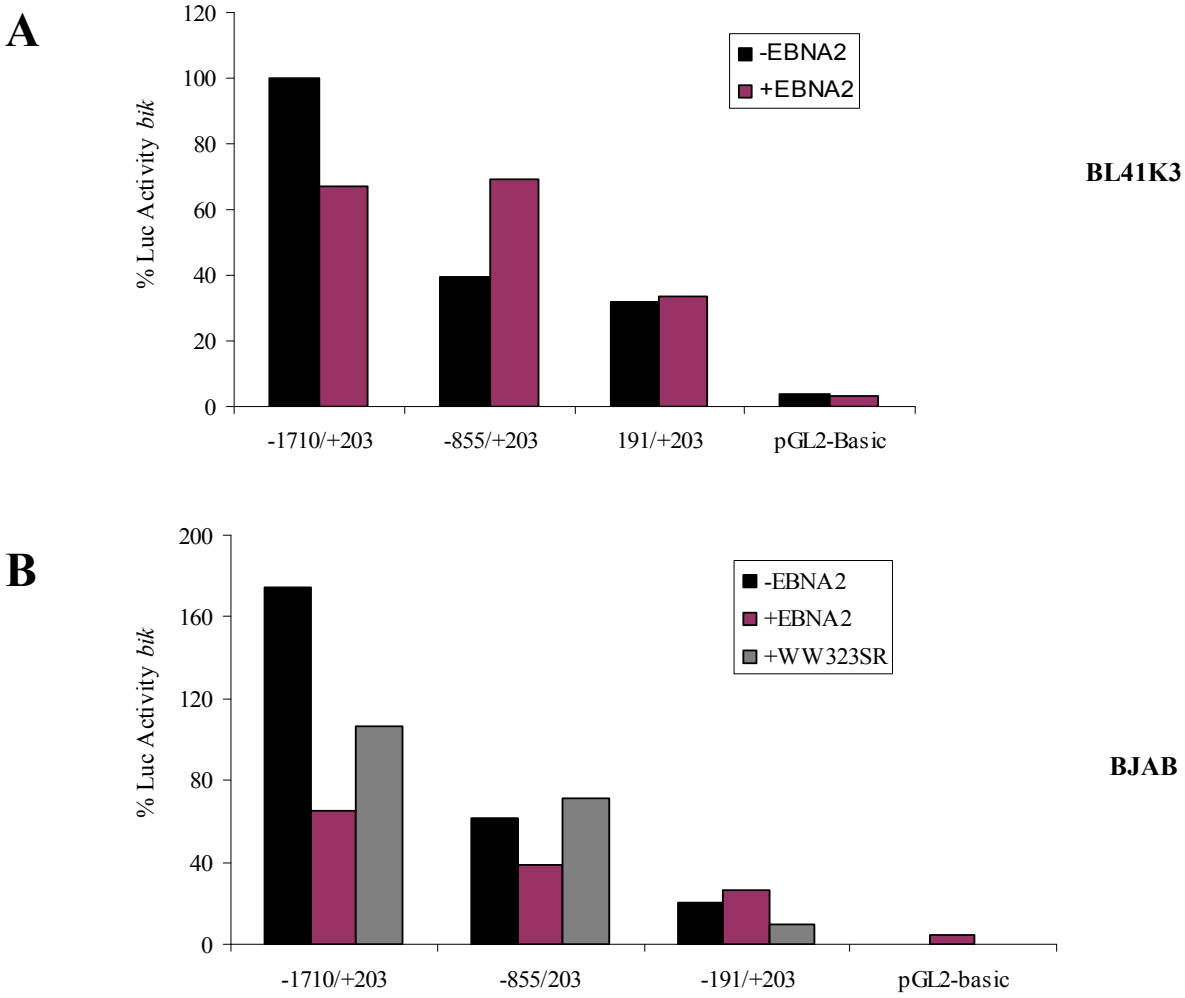


Figure 7.2.14 : Analysis of promoter deletion constructs BL41K3 and BJAB

BL41K3 (A) BJAB (B) were transiently co-transfected by electroporation with *bik* promoter (–1710/+203-Luc) reporter construct, the promoter deletion constructs (-855/+203-Luc/-191/+203-Luc) or pGL2-basic (empty vector), pCMV LacZ and either the EBNA2 expression plasmid pSG5EBNA2 or the CBFI-deleted pSG5EBNA2ww323sr. The total quantity of transfected plasmid was adjusted to 10µg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct when co-tranfected with the control vector pSG5. The absolute luciferase value obtained for -1710/+203-Luc-*bik* after transfection was set at 100.

7.2.7 Effect of c-MYC on *bik* promoter activity.

Having established that c-Myc alone can down-regulate *bik* mRNA and protein levels in the P493-6 LCL, (Chapter 5), it was decided to determine if this down-regulation in the levels of *bik* mRNA was due to a decrease in the rate of transcription from the promoter of the *bik* gene in response to c-Myc and so the effect of expression of this cellular gene on the promoter activity of *bik* was also analysed. For this reason, the effect of the c-Myc expression on the *bik*-promoter constructs was examined in the P493-6 cell line, where c-Myc function was induced by the removal of tetracycline from the cell culture medium (Section 2.2.2.3). P493-6 cells, which had been seeded in tetracycline supplemented medium (- c-Myc) for the previous 24 hours were transiently transfected with the –1710/+203-*bik*-Luc construct by electroporation. After transfection the cells were cultured in tetracycline-free medium. At 48 hours after transfection the cells were harvested and cell extracts were assayed for luciferase activity and β-gal as before. As seen in Figure 7.2.15, progressive deletion of the –1710/+203 region of the *bik* promoter to position –191 revealed a gradual loss of the basal activity of the promoter. This data indicates that *cis*-elements located between -1710 and -855 are important in *bik* promoter activity in these cells which are driven into proliferation due to the *c-myc* gene (Pajic *et al*, 2001).

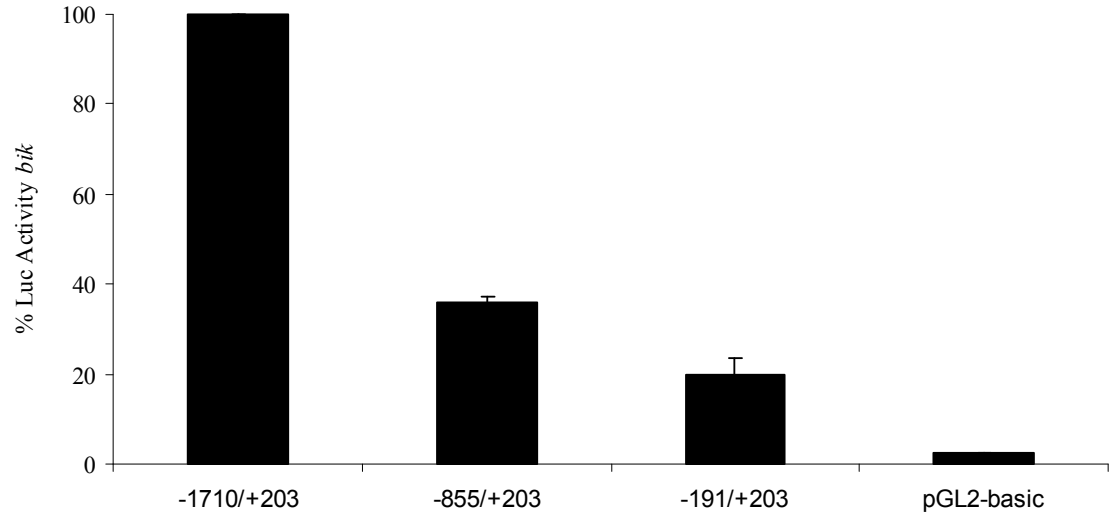


Figure 7.2.15: P493-6 Myc transfections

P493-6 cells were transiently co-transfected by electroporation with *bik* promoter (–1710/+203-Luc) reporter construct, the promoter deletion constructs (-855/+203-Luc/-191/+203-Luc) or pGL2-basic (empty vector) and pCMV LacZ. Cells were induced to express c-Myc by removal of tetracycline from the cell culture medium. The total quantity of transfected plasmid was adjusted to 2µg with the empty vector pSG5. Cells were harvested 48 hours after transfection and luciferase and β-gal assays were performed. Normalised luciferase values were expressed relative to the corresponding value obtained for the -1710/+203-Luc-*bik* reporter construct. The absolute luciferase value obtained for -1710/+203-Luc-*bik* after transfection was set at 100. (Data are Mean ±SD).

Thus, in an attempt to directly investigate the effect of expression of c-Myc expression on the activity of the *bik* promoter a number co-transfection experiments were carried out using the *bik* promoter (–1710/+203-Luc) reporter construct, the promoter deletion constructs (-855/+203-Luc/-191/+203-Luc) or pGL2-basic (empty vector), pCMV LacZ and various quantities (0µg, 3µg, 5µg and 7µg) of the c-Myc expression plasmid pcDNA3-HA-Myc (Table 2.1-4) in the BL41K3, BJAB and DG75 cell lines. *Bik* promoter activity was then analysed using the luciferase assay as before. However, results obtained from these experiments were highly inconsistent; c-Myc failed to consistently show direct *trans*-repression by c-Myc in any cell line (data not shown). These results indicate that under these conditions, c-Myc may have no role in the regulation of transcription initiated from the *bik* promoter in question and that transcriptional regulation of *bik* by c-Myc is potentially mediated by far upstream or downstream/intronic regulatory elements, or by changes in RNA stability.

A number of co-transfection experiments involving both EBNA2 and c-Myc together with the –1710/+203-Luc *bik* promoter reporter construct were undertaken in the BL41K3 cell line in order to determine if there was a synergistic effect on *bik* promoter activity when both proteins were expressed together. However the expression of these proteins did not consistently repress the *bik* promoter, and the results obtained were also inconclusive (data not shown).

7.3 RNA Stability

mRNA stability plays a major role in gene expression, affecting the rates at which mRNAs disappear following transcriptional repression and at which they accumulate following transcriptional induction. The abundance of a particular mRNA can fluctuate several fold following a change in the mRNA half-life (Ross, 1995). Investigations into the mechanism of up-regulation of *bfl-1* mRNA levels by LMP-1 in the laboratory revealed the importance of a role for both mRNA stabilisation and increased promoter activity (D'Souza *et al*, 2000; D'Souza *et al*, 2004). The observed decrease in the levels of *bik* mRNA upon activation of EBNA2 could also be attributable to the altered stability of previously transcribed mRNA. It was therefore of interest to investigate if mRNA stability was a factor in regulating *bik* expression in B-cells in response to EBNA2 expression.

7.3.1 The effect of Actinomycin D treatment on the levels of *bik* mRNA expression in response to EBNA2 in BL41K3.

Accordingly then, BL41K3 was treated with an inhibitor of RNA polymerase II, actinomycin D, to block transcription and monitor the rate of decay of *bik* transcript in the presence and absence of EBNA2. It was initially important to establish an appropriate interval to ensure the activation of functional EBNA2, prior to the inhibition of transcription. Thus, in order to monitor EBNA2 induction following addition of estrogen, a time course was undertaken with the BL41K3 cell line cultivated in the presence of 1μM estrogen. Protein extracts were prepared at various time points over a 24 hour period, and the levels of EBNA2 expression were monitored by Western blotting. Changes in EBNA2 expression over time are shown in Figure 7.3.1.

Western blot analysis confirmed that the activation of functional ER/EBNA2 (indicated by a molecular weight shift) in BL41K3 cells had occurred as early as 2 hours after the addition of estrogen to the cell culture medium. Hence actinomycin D was added in subsequent experiments two hours after the addition of estrogen.

Figure 7.3.1: Western Blot analysis of induction of EBNA2 protein expression in the BL-derived cell line BL41K3.

ER/EBNA2 was activated in BL41K3 cells by the addition of estrogen to the cell culture medium. Total protein was prepared at the indicated time points after induction, and was used in Western blot analysis. Binding of primary antibodies was detected using an anti-mouse conjugated alkaline phosphatase secondary antibody.

In order to monitor the rate of decay of *bik* mRNA a time course experiment was performed in which BL41K3 cells were first seeded in the presence and absence of estrogen. Cells were washed twice with PBS and divided into medium supplemented with either 1μM estrogen or and equal volume of vehicle (ETOH) (Kempkes *et al*, 1995a). Estrogen treated samples were run in parallel with a vehicle-treated control. After a two hour time interval, cells were then treated with 5μg/ml actinomycin D or an equal volume of second vehicle (DMSO). A control experiment in which BL41K3 cells were cultivated in the presence of estrogen only was also performed. Total RNA was prepared at various time points over a 24 hour time period. RT reactions were carried out to generate cDNA, which was subsequently used in a QPCR assay to measure *bik* mRNA levels as before. mRNA levels of the endogenous control gene *gapdh* were also monitored, and the C_T values for *gapdh* transcripts in the treated and untreated RNA samples were used as a control, and results were normalised to *gapdh*. Importantly, *gapdh* C_T values indicated that the quantity of mRNA reverse transcribed and potentially amplifiable by PCR was similar in estrogen-treated and estrogen-untreated samples.

Changes in *bik* mRNA levels due to EBNA2 and in the presence of actinomycin D over the 24 hour time course are shown in Figure 7.3.2. In the control experiment *bik* mRNA levels were seen to diminish over time in response to increasing levels of EBNA2 expression as previously established (Checkered boxes Figure 7.3.2; see also Chapter 4). In contrast to this, a significant increase in *bik* mRNA levels is seen in the

actinomycin D treated cells after 12 hours, particularly in the presence of EBNA2 (approximately 3-fold -EBNA2, 7-fold +EBNA2). Cells induced to express EBNA2 showed an approximately 11-fold increase in *bik* transcript by 24 hours after treatment with actinomycin D, and in contrast to this *bik* mRNA levels had returned to a near basal level in the uninduced, actinomycin D treated cell pool by this time.

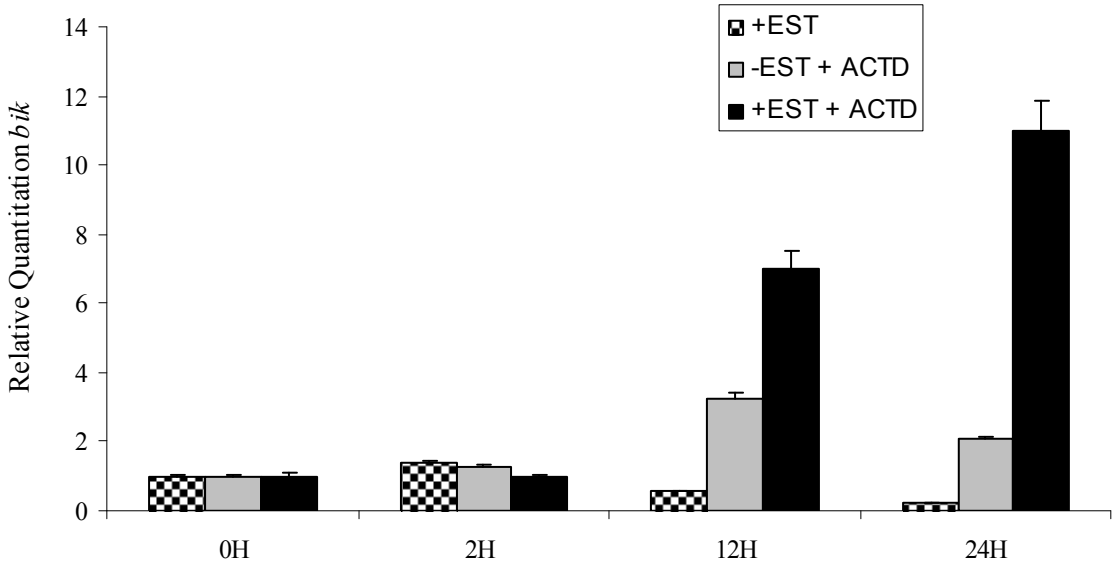


Figure 7.3.2: **Investigation of *bik* RNA stability in response to induction of EBNA2 in BL41K3 cells.**

The Effect of EBNA on the stability of *bik* mRNA. Exponentially proliferating cells were exposed to β-estradiol (1μM), or vehicle for 2 hours. After this time, total cellular transcriptional activity was suppressed by Actinomycin D (5μg/ml). Total cellular RNA was then extracted at the indicated time points up to 24 hours. Amounts of the *bik* mRNA transcripts were determined by Taqman QPCR assay as before. (Data are Mean ±SD).

In general, it is clear from these results that the degradation pathway for *bik* mRNA is different in the presence of EBNA2.

7.4 Investigation into the Epigenetic Regulation of Bik in EBV-infected cell lines.

Epigenetic alterations, chages that affect gene expression but not the gene sequence itself, have been widely implicated in the inactivation of tumour suppressor genes in human cancers (Jones and Laird, 1999). Epigenetic gene regulation involves methylation of cytosine residues in CpG islands and post-translational covalent modifications of the N-terminal residues of histones within the nucleosome. A tightly regulated relationship between DNA methylation and histone modifications guarantees the stability and integrity of the epigenetic programming. Aberrant expression of histone-modifying enzymes or DNA methyltransferases can result in deregulated gene expression, leading to the establishment of pathologic epigenetic alterations in human malignancies (Jones and Baylin, 2002; Zelent *et al*, 2005).

Acetylation and deacetylation of histone proteins, DNA-binding transcription factors, nuclear hormone receptors and signal-transduction proteins, have critical roles in regulating gene expression. Aberrant protein acetylation stemming from misregulation of either histone acetyltransferases (HATs) or histone deacetylases **(**HDACs) has been linked to cancer and various neurological diseases (Di Prospero and Fischbeck, 2005; Drummond *et al*, 2005; Langley *et al*, 2005). HDAC inhibitors (HDACi) may revert silent heterochromatin to an active chromatin conformation and restore the normal function of genes that are silenced in these diseases (Di Prospero and Fischbeck, 2005).

Thus, histone modifications including acetylation and methylation can affect gene transcription forming interacting epigenetic networks that regulate gene expression (Fuks, 2005). The inhibition of histone-modifying enzymes and DNA methyltransferases might restore the expression profile of several genes that are implicated in tumourogenesis. Accordingly, two main groups of drugs have been developed for this purpose. The first group is comprised of substances that target the action of DNA methyltransferases (DNMTs), through either competing with cytosine or with S-adenosylmethionine (SAM, AdoMet) or acting over the DNMTs themselves, (including 5-azacytidine, (5-azaC), Doxorubicin and Zebularine). The second group involves compounds that inhibit subunits of the repressor complexes,

such as HDACs (including Sodium Butyrate (NaB), Trichostatin A (TSA), Apicidin, APHA Compound 8, (-)-Depudecin and suberoylanilide hydroxamic acid (SAHA)).

It has been established in Chapter 3 that loss of Bik protein expression is a general feature of BL-derived cell lines exhibiting type III latency and in LCLs. In order to assess the possibility that epigenetic change might contribute to silencing of the *bik* gene during EBV infection the sequence of the *bik* promoter was initially examined to investigate the presence of CpG islands in order to give an indication of a potential role for methylation.

7.4.1 A CpG islands is present on the *bik* promoter sequence.

The identification of a CpG island on the *bik* promoter might indicate a potential role for methylation in *bik* promoter silencing in EBV-infected cells. To investigate the methylation status of the *bik* 5' region by identifing possible CpG rich regions on the *bik* promoter, the upstream sequences were analysed for C+G content using 'CpG island searcher' software (http://cpgislands.usc.edu/cpg.aspx) (Table 2.1-6). A CpG island is generally accepted as a 200bp stretch of DNA with a C+G content of 50% and an observed CpG/expected CpG (Obs_{CpG}/Exp_{CpG}) ratio in excess of 0.6 (Gardiner-Garden and Frommer, 1987). Examination of the –1710 to +203 region of the promoter region, revealed a CpG island from position –590bp to +203bp (52% GC, Obs_{CpG}/Exp_{CpG} 1.051, 793bp) of the *bik* promoter (Figure 7.4.1). Thus, the *bik* 5' region is associated with CpG island as indicated by an elevated GC content in this region.

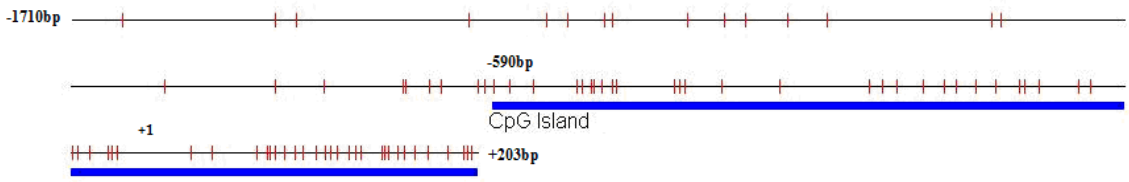


Figure 7.4.1: Schematic representation of the *bik* promoter indicating a CpG island around the transcriptional start site.

The region of the *bik* promoter from position -1710 to +203 relative to the transcription start site is illustrated on the schematic diagram. CpG island searcher,

revealed a CpG island from postition -590bp to +203bp (52% GC) on the promoter. The location of the CpG island is indicated by a blue line.

This analysis indicates that *bik* contains a CpG island around its transcriptional start site, thus rendering it susceptible to control by methylation.

7.4.2 Treatment of EBV-infected cell lines IB4 and AG876 with the DNMT Inhibitor, 5-azacytidine. does not lead to de-repression of *bik.*

Initially, the pyrimidine analog 5-Azacytidine (5-azaC), a potent effector of decreased DNA methylation in mammalian cells, was employed. Cells are known to incorporate 5-5-azaC into their DNA, forming covalent adducts with cellular DNMT1, thereby depleting the cells from enzyme activity and causing demethylation of genomic DNA as a secondary consequence (Christman, 2002).

IB4 (EBV-positive LCL) and AG876 (EBV-positive Lat III) cells were treated with 5-azaC at a range of concentrations (0.5μM to 1.5μM as determined from literature) or an equal volume of vehicle (acetic acid). Protein extracts were prepared at various time points after treatment and Bik protein expression was monitored by Western blotting (Section 2.2.4.3). Levels of the endogenous control, β-Actin, were also examined and confirmed equal loading in each case (Figure 7.4.2). A representation of the results is given in Figure 7.4.2. As can be seen from the results illustrated in Figure 7.4.2A and B, no significant increase in the level Bik protein could be detected upon treatment with this compound over the course of the experiment.

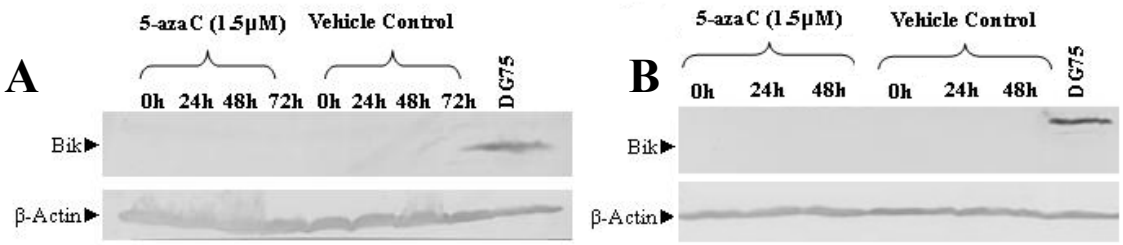


Figure 7.4.2: Western Blot analysis of Bik protein expression in response to induction of treatment with 5azaC in AG876 (A) and IB4 (B).

(A) AG876 and (B) IB4 were treated with 1.5μM 5azaC or an equal volume of vehicle (acetic acid) and total protein was prepared at various time following treatment and used in Western blot analysis. The level of Bik protein expression was detected using a mouse anti-Bik antibody (BD Biosciences 557040). The upper panel illustrates the analysis of Bik protein expression in response to 5azaC treatment. The lower panel shows the same blot probed for β-Actin. Binding of primary antibodies was detected using an anti-mouse conjugated alkaline phosphatase secondary antibody in each case.

Both cell lines examined showed no evidence of *bik* promoter methylation after treatment. These results may suggest that promoter methylation is not a mechanism of *bik* down-regulation during the EBV growth programme.

7.4.3 Treatment of EBV-infected cell lines with the HDAC Inhibitiors, Sodium Butyrate (NaB) and Trichostatin A (TSA).

Induction of apoptosis by HDACi has been shown to induce changes in several of the pro-apoptotic and anti-apoptotic members of the Bcl-2 family, including Bcl-2, Bcl-$_{XL,}$ Mcl-1, Bim, Bax, Bak, Bmf and Noxa (Mandal and Kumar, 1996; Hague *et al*, 1997; Cao *et al*, 2001; Zhang *et al*, 2004; Zhao *et al*, 2005; Zhang *et al*, 2006; Inoue *et al*, 2007), suggesting that HDACi may induce apoptosis by altering the balance between pro-apoptotic and anti-apoptotic proteins in the cell.

In an attempt to assess whether histone modifications are involved in Bik silencing in EBV infected cell lines, two well-established HDACi were employed to examine the effects of histone deactylation on Bik expression in the EBV-infected cell lines AG876 and IB4. The first of these compounds was the naturally occurring fatty acid

Sodium Butyrate (Boffa *et al*, 1978; Candido *et al*, 1978; Sealy and Chalkley, 1978; Vidali *et al*, 1978) and the second, Trichostatin A (TSA) is a well-established inhibitor which leads to loss of structure of the heterochromatin compartment (Yoshida *et al*, 1990; Taddei *et al*, 2001).

IB4 and AG876 cells were treated with a range of concentrations of these chemical inhibitors; either NaB (0.5mM to 1.5mM) or TSA (200ng/ml to 400ng/ml) (concentrations as determined from literature) or an equal volume of vehicle (cell culture medium/ETOH respectively). Protein extracts were prepared at various time points after treatment and Bik protein expression was monitored by Western blot as before (Section 2.2.4.3). Levels of the endogenous control, β-Actin, were also examined and confirmed equal loading of protein in each lane (Figure 7.4.3). A representation of the results is illustrated in Figure 7.4.3A and B and it can be seen that no significant increase in the level Bik protein could be detected with either compound over the course of the experiment.

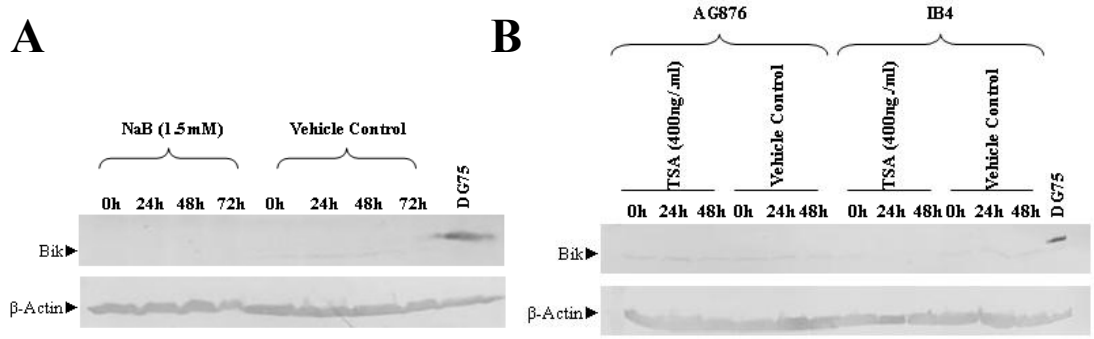


Figure 7.4.3: **Western Blot analysis of Bik protein expression in response to induction of treatment with the HDACi Sodium Butyrate and Trichostatin A in AG876 (A) and IB4 (B).**

(A) AG876 cells were treated with 1.5mM NaB or an equal volume of vehicle (cell culture medium) and total protein was prepared at various time points after treatment and used in Western blot analysis. (B) AG876 and IB4 cells were treated with 400ng/ml TSA or an equal volume of vehicle (ETOH) and total protein was prepared at the indicated time points and used in Western blot analysis. The level of Bik protein expression was detected using a mouse anti-Bik antibody (BD Biosciences 557040). The upper panel (A and B) illustrates the analysis of Bik protein expression in response to NaB treatment. The lower panel shows the same blot probed for β-Actin.

Binding of primary antibodies was detected using an anti-mouse conjugated alkaline phosphatase secondary antibody in each case.

Thus, neither of the HDACi tested affected the level of Bik protein expression in either of the cell lines examined. Although, the effects of the HDACi were assessed at a range of concentrations; there was no apparent correlation between HDAC inhibition and the activity of *bik*. It may be that the acetylation status and chromatin structure of the *bik* gene are unlikely to be dominant regulators of transcription in this context.

It is clear from the results presented here, that neither of the HDACi investigated, nor the DNMT inhibitor, had any effect on the level of *bik* expression in this context.

7.4.4 The HDAC inhibitor, Trichostatin A inhibits ER/EBNA2 activation and the expression of plasmid encoded EBNA2.

In order to investigate if TSA was capable of directly regulating the expression of ER/EBNA2-mediated Bik repression the levels of Bik protein in ER/EBNA2-induced BJABK3 cells were examined after treatment TSA. ER/EBNA2 was induced over an 84 hour time period in the BJABK3 cell line, by the addition of estrogen to the cell culture medium. Treated cells were immediately divided, into medium supplemented with either 200ng/ml TSA, or an equal volume of vehicle (ETOH). Total protein was prepared from cells harvested at various time-points after ER/EBNA2 induction, (Figure 7.4.4) and subsequently used in Western blot analyses for ER-EBNA2, Bik and c-Myc (Figure 7.4.4). Levels of the endogenous control, β-Actin, were also examined and confirmed equal loading in each case (Figure 7.4.4). In the control experiment, (in which estrogen-induced cells were incubated in the presence of vehicle), the activation of functional ER/EBNA2 (indicated by a molecular weight shift) was confirmed, and can be seen at 24 hours after induction (Figure 7.4.4). ER/EBNA2 remained detectable for up to 84 hours (Figure 7.4.4). A significant decrease in the level of Bik protein can be seen to coincide with the induction of ER/EBNA2 activation over the course of the experiment, confirming Bik down-regulation in response to ER/EBNA2 induction as previously established. c-Myc expression was transiently up-regulated at 24 hours in response to ER/EBNA2

expression after which time, the levels of c-Myc protein returned to the pre-induction level of expression.

However, TSA inhibited the activation of ER/EBNA2 (Figure 7.4.4). ER/EBNA2 is a fusion protein of an apparent molecular weight of 120kDa in contrast to the 86kDa protein of wild type EBNA2 (Kempkes *et al*, 1995a). Addition of estrogen activates the function of ER/EBNA2, and affects the electrophoretic mobility of the ER/EBNA2 fusion protein. While an initial activation of ER/EBNA2 is apparent at 24 hours, the levels induced are low relative to the control experiment and by 48 hours, only non-functional EBNA2 is detectable. Thus, TSA does not inhibit the basal level of ER/EBNA2, rather it appears to interfere with its ability to retain bound estrogen, the loss of which is reflected in the inability of ER/EBNA2 to positively regulate its own promoter. Accordingly, ER/EBNA2-mediated Bik repression is inhibited, and Bik levels remain unchanged over the course of the experiment. c-Myc protein levels are also unaffected and remain constant over the 84 hour time course.

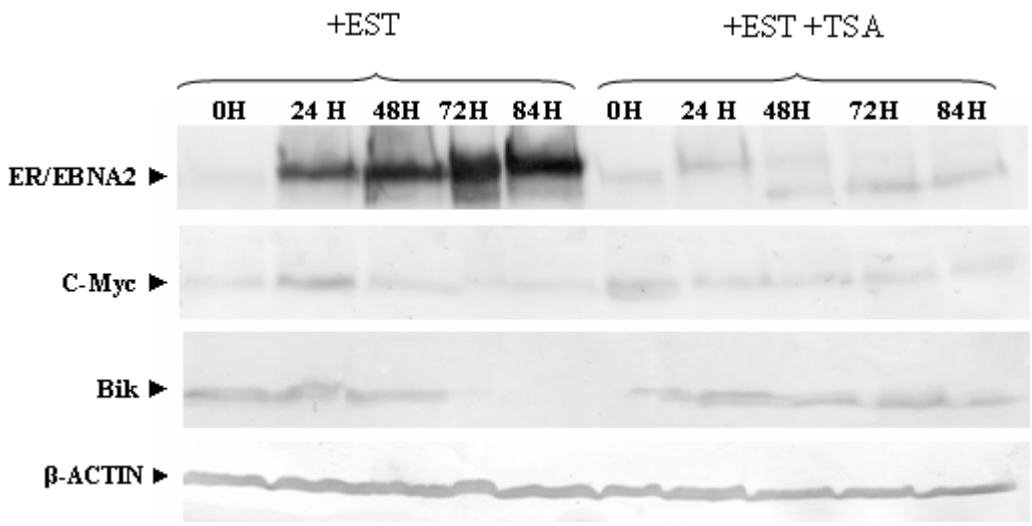


Figure 7.4.4: **Western Blot analysis of protein expression in response to treatment with the HDAC inhibitor TSA in BJABK3.**

BJABK3 cells were induced to express ER/EBNA2 by addition of estrogen to the cell culture medium and cells were immediately treated with the HDAC inhibitor TSA (200ng/ml). Total protein was prepared at the indicated time points after induction and used in Western blot analysis. The level of Bik protein expression was detected using a goat anti-Bik antibody (Santa Cruz, sc-1710). Analysis of ER/EBNA2 induction is shown in the upper panel. c-Myc protein expression is illustrated in the second panel. Western blot analysis of EBNA2-mediated Bik protein repression in the presence of TSA treatment is illustrated in the third panel. The lower panel shows the same blot probed for β-Actin. Binding of primary antibodies was detected using an anti-mouse conjugated alkaline phosphatase secondary antibody in the case of EBNA2, c-Myc and β-Actin and an anti-goat conjugated horseradish peroxidase secondary in the case of Bik.

Having established that the HDAC inhibitor, TSA, inhibits the activity of ER/EBNA2 in the BJABK3 cell line, and in an attempt to establish a suitable context to investigate the effect of the methylation inhibitor on EBNA2-associated *bik* repression, it was next decided to do a similar experiment using the DG75-tTA-EBNA2 cell line, in which EBNA2 expression is not under EBNA2 control, rather its expression is driven by a heterologous promoter.

Accordingly, EBNA2 was induced over a 72 hour time period in the DG75-tTA-EBNA2 cell line, by the removal of tetracycline from the cell culture medium as before (Chapter 4). Cells were immediately divided, either into medium supplemented

with 200ng/ml TSA, or an equal volume of vehicle (ETOH). Total protein was prepared from cells harvested at various time-points after EBNA2 induction, (Figure 7.4.5) and used in Western blot analysis (Figure 7.4.5).

In the control experiment, EBNA2 was detected 24 hours after induction and remained detectable up to 72 hours over the entire course of the experiment (Figure 7.4.5). However, it can be seen that EBNA2 expression was completely inhibited in the presence of TSA and was not detected at any point over the 72 hours.

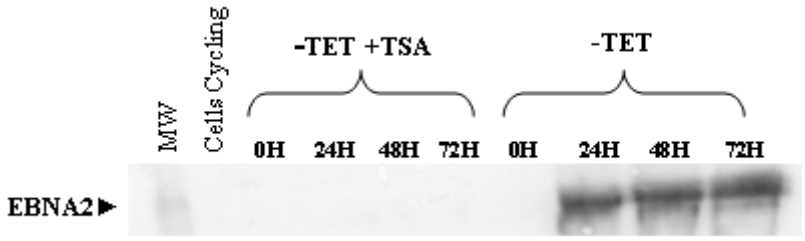


Figure 7.4.5: **Western Blot analysis of EBNA2 protein expression in response to treatment with the HDAC inhibitor TSA in the BL-derived cell line DG75-tTA-EBNA2.**

EBNA2 was induced in DG75-tTA-EBNA2 cells by removal of tetracycline from the cell culture medium. Cells were immdediately divided and treated with TSA or an equal volume of vehicle (ETOH). Total protein was prepared at the indicated time points after induction, and was used in Western blot analysis. Western Blot analysis of EBNA2 protein expression in response to TSA treatment was monitored. Binding of primary antibody was detected using an anti-mouse conjugated alkaline phosphatase secondary antibody.

These results were unexpected and they indicate that expression/activation of exogenous EBNA2 from both ER/EBNA2 expressed in the BJABK3 cell line and wild type EBNA2 expressed in DG75-tTA-EBNA2 was inhibited in the presence of TSA. This experiment revealed a novel affect of TSA, as it appears that EBNA2 expression/activation is potently inhibited by this histone deacetylase inhibitor in these BL-derived cell lines. TSA is known to modulate gene expression, and, since it is a well-established inhibitor of histone deacetylases, a likely mechanism of this effect is the alteration by TSA of chromatin conformation through hyperacetylation of histones. The data presented thus suggests that the inhibition of expression/activation of exogenous EBNA2 by TSA in these engineered cells is linked to its ability to

inhibit HDAC activity thus suggesting a correlation between the acetylation status of histones and EBNA2 expression. Although chromatin reorganisation due to histone hyperacetylation generally correlates positively with gene activation, there are some examples in which histone acetylation has been associated with gene repression (Turner *et al,* 1992; De Rubertis *et al,* 1996; Braunstein *et al,* 1996). There is also an increasing appreciation of the fact that TSA may act through mechanisms other than induction of histone acetylation. Indeed, experimental data has indicated that TSA activates PI3K/AKT signaling (Eickhoff *et al*, 2000). In addition to this, inhibition of protein kinase C abrogates TSA mediated up-regulation of NFκB transcriptional activity and p21 expression that is associated with apoptosis (Maxhimer *et al*, 2005).

Thus, it was not possible to examine the effects of TSA on EBNA2 associated *bik* repression using either of these two different expression systems.

Overall, the results presented here give a preliminary indication that under these conditions, EBV-mediated repression of the *bik* gene occurs by a mechanism that does not involve either of the well known epigenomic mechanisms, DNA methylation or histone acetylation.

7.5 Investigation into a possible role for Proteasomal degradation in the regulation of Bik protein.

Since it has been reported that Bik can be degraded by the proteasome pathway, it was of interest to investigate if the proteasomal degradation of *bik* prevents EBV-infected cells from succumbing to *bik*-dependent apoptosis. Changes in total Bik levels were monitored upon proteasome inhibitor treatment in two EBV positive cell lines. A direct a role for proteasome-mediated protein degradation during EBNA2-associated regulation of *bik* was also investigated.

7.5.1 Effect of proteasome inhibition on Bik expression in EBV-infected cell lines

Accordingly IB4 and AG876 cells were treated with 10μM MG132 or an equal volume of vehicle (DMSO) and protein extracts were prepared at various time points after treatment. The levels of Bik, p53 and β-Actin proteins expressed were analysed by Western blotting (Section 2.2.4.3). Initially, in order to test the effect of proteasome inhibition, the membrane was probed with antibodies against p53, a short-lived protein degraded through the proteasome pathway (Kubbutat *et al*, 1997). Western blotting was also undertaken to analyse Bik protein levels in response to MG132 treatment (Figure 7.5.1) and levels of the endogenous control, β-Actin, were analysed in order to confirm equal quantity loading in each case (Figure 7.5.1A, lower panel). In this experiment, p53 was detected in MG132 and DMSO-treated cells at 0 hours and remained detectable up to 72 hours. An increase in p53 protein expression was observed in both IB4 and AG876 cells and can be seen to coincide with MG132 treatment in a time-dependent manner (Figure 7.5.1) Thus, MG132 treatment led to the accumulation of wild-type p53 in IB4 and AG876 cells in accordance with previously published data (Kubbutat *et al*, 1997). In DMSO-treated cells the levels of p53 expression remained constant over the course of the experiment. As can be seen in Figure 7.5.1A and B, Bik was hardly detectable in cells at 0 hours after treatment and levels remained unchanged after treatment of either IB4 or AG876 cells with MG132. Unlike p53, the failure of Bik to accumulate in the presence of the proteasomal inhibitor argues that the proteasomal degradation of Bik does not play a significant role in maintaining low Bik levels in these EBV-infected B-cell lines.

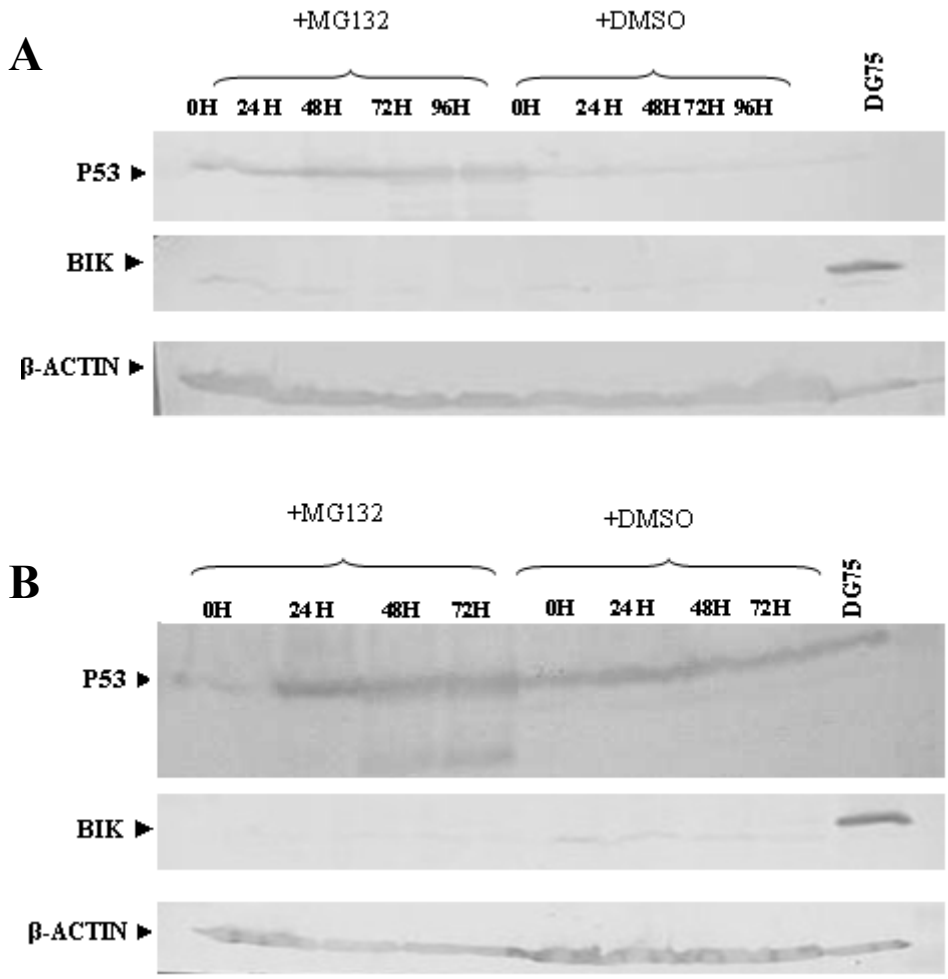


Figure 7.5.1: Western Blot analysis of Bik protein expression in response to treatment with the proteasome inhibitor MG132 in the EBV-infected cell lines IB4 (A) and AG876 (B).

Western blot of DMSO- or MG132-treated IB4 (A) and AG876 (B) cells. Cells were treated with 10μM MG132 or an equal volume of vehicle (DMSO) and total protein was prepared at various time points after treatment and used in Western blot analysis. The level of Bik protein expression was detected using a mouse anti-Bik antibody (BD Biosciences 557040). Bik protein expression in response to MG132 treatment was monitored. Analysis of p53 level is shown in the upper panel. Western blot analysis of Bik protein expression in response to MG132 treatment is illustrated in the middle panel. The lower panel shows the same blot probed for β-Actin. Binding of primary antibodies was detected using an anti-mouse conjugated alkaline phosphatase secondary antibody in each case.

In a second experiment, P493-6 cells that had been previously cycling on the EBV growth programme, were washed four times with PBS and seeded in estrogen-free

medium and subsequently treated with 10μM MG132 or an equal volume of vehicle (DMSO). This was considered time 0. Protein extracts were prepared at various time points following treatment. The levels of EBNA2, Bik and β-Actin protein expressed were monitored by Western blot analysis. Western blotting was used to demonstrate the inactivation of EBNA2 over the course of the experiment and to monitor Bik protein levels in response to EBNA2 repression in the presence of MG132 (Figure 7.5.2). Levels of the endogenous control, β-Actin, were also examined and confirmed equal quantity loading in each case (Figure 7.5.2, lower panel). In this experiment, functional EBNA2 was detected at 0 hours after removal of estrogen from the culture medium. Subsequent to this time point only non-functional EBNA2 was detected in both the MG132 and the DMSO-treated cell pools (Figure 7.5.2). In the DMSO-treated cells, a significant increase in the level of Bik protein can be seen to coincide with the removal of EBNA2 function over the course of the experiment as expected. Bik levels are induced in conjunction with EBNA2 repression by 48 hours, and remain up-regulated at the 72 and 96 hour time points (Figure 7.5.2). MG132 treatment however inhibits the up-regulation of Bik protein in reponse to withdrawal of EBNA2 function. Indeed Bik de-repression is in fact strongly inhibited by 24 hours, and remained suppressed over the 96 hour time course (Figure 7.5.2).

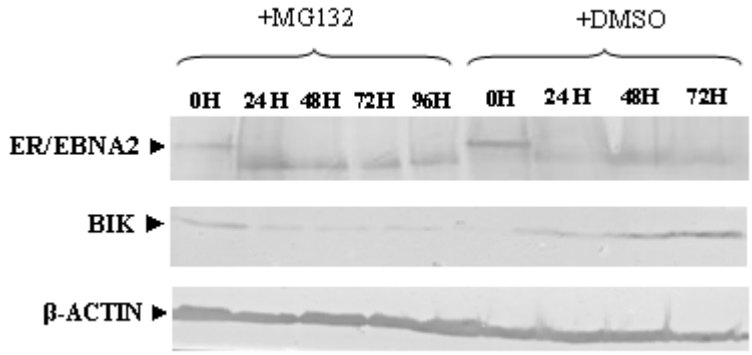


Figure 7.5.2: **Western Blot analysis of Bik protein repression in response to treatment with the proteasome inhibitor MG132 in P493-6.**

ER/EBNA2 function was repressed by removal of estrogen from the growth medium. Cells were treated with 10μM MG132 and total protein was prepared at the indicated time points after repression and used in Western blot analysis. The level of Bik protein expression was detected using a mouse anti-Bik antibody (BD Biosciences 557040). EBNA2-associtaed Bik expression in response to MG132 treatment was monitored by Western Blot analysis. Analysis of ER/EBNA2 levels is indicated in the

upper panel. Western blot analysis of ER/EBNA2-regulated Bik protein repression in response to MG132 treatment is illustrated in the middle panel. The lower panel shows the same blot probed for β-Actin. Binding of primary antibodies was detected using an anti-mouse conjugated alkaline phosphatase secondary antibody in each case.

Thus, decreased proteasomal turnover of Bik is not a significant contributory factor to Bik up-regulation following the loss of EBNA2 activity. Furthermore, inhibition of the proteasome inhibits the relief of repression due to the removal of EBNA2. This interesting and unexpected result could mean that (a) EBV may positively stimulate the turnover of Bik through an intermediary factor, such as c-Myc or IgM, which itself is subject to degradation by the proteasomal pathway or (b) MG132 may inhibit the turnover of a genetic repressor or a protease, thus retarding the re-emergence of Bik following the removal of EBNA2.

7.6 Discussion

Overall, this study suggests that EBNA2 may down-regulate expression of *bik* gene by decreasing transcription from the *bik* promoter and that this down-regulation is independent of the EBNA2-CBF1 interaction. It is not clear as to why co-transfections using EBNA2 and the *bik* promoter-luciferase construct, p-1710/+203-*bik*-luc, failed to elicit a consistently similar EBNA2 response in DG75, although it is clear this effect was not an artefact specific to the assay, since responses from both the *bfl-1* and *981-6* promoters were consistent and readily detectable in the same assay. Nevertheless, in 72% of assays carried out in DG75 cells, EBNA2-mediated down-regulation of the *bik* promoter was evident. While there was the observation that the level of repression varied considerably, EBNA2 expression consistently repressed the *bik* promoter-reporter construct in BL41K3. The *bik*–Luc construct was repressed by EBNA2 in every instance in this context.

The EBNA2 mutant pSG5EBNA2ww323sr which was defective in CBF1-binding was found to reduce *bik* promoter activity to the same extent as wild type (wt) EBNA2 in these experiments. This finding adds additional credence to a role for EBNA2-mediated repression of the *bik* promoter, and also further corroborates results in Chapter 4, which showed transcriptional repression of *bik* in the ΔCBF1 cell line, SM296D3. The lack of CBF1 involvement in the interaction is of significance considering that many of the EBNA2-responsive promoters including those of several EBV latent genes and of the numerous cellular genes have all been shown to contain functional CBF1-binding motifs and to be targets of the EBNA2-CBF1 interaction (discussed in Chapter 1). Elsewhere, EBNA2-associated suppression of transcription of the immunoglobulin μ (IgM) gene has previously been shown to occur via a CBF1-independent mechanism (Jochner *et al*, 1996; Maier *et al*, 2005), and thus is in agreement with the results presented here. To date, this is the only other indication of EBNA2-associated down-regulation of a cellular gene. Interestingly the *c-myc* gene has previously been shown to be only partially CBF1-dependent (Maier *et al*, 2005).

EBNA2 is known to associate with several cellular factors in order to facilitate contact with EBNA2-responsive promoters. Experiments in the laboratory indicate a

potentially important role for Ets family members in the EBNA2-associated activation of the *bfl-1* promoter (Pegman *et al*, 2006). Furthermore, EBNA2 mediated activation of LMP1 is facilitated by several factors including PU.1/Spi-1, a POU domain protein, an ATF/CRE element and the CREB-binding protein, CBP (Laux *et al*, 1994a; Johannsen *et al*, 1995; Sjoblom *et al*, 1995; Sjoblom *et al*, 1998; Cahir-McFarland *et al*, 2000). Furthermore, a CBF2 binding site adjacent to the CBF1 binding site on the Cp promoter contributes to EBNA2 responsiveness in addition to the effect mediated by CBF1 (Ling *et al*, 1993a; Fuentes-Panana *et al*, 2000). Considering this, sequence analysis of the *bik* promoter, identified seven potential CREB binding motifs (including one consensus ATE/CRE motif) along the length of the -1710/+203bp promoter and also two putative Ets binding motifs which were located in close proximity to the consensus CBF1 binding site already identified. The identification of a number of putative Ets binding sites in close proximity to each other and proximal to the CBF1 consensus sequence on the *bik* promoter, is reminiscent of the EBNA2 responsive elements (E2RE) in other EBNA2 responsive promoters including *bfl-1* and *LMP1* (Johannsen *et al*, 1995; Pegman *et al*, 2006). It is possible that EBNA2 mediated repression of the *bik* promoter may require downstream targeting of the putative Ets sites identified on the *bik* promoter.

At least four BH3-only genes are subject to transcriptional control, including *harakiri/hrk/dp5* (Imaizumi *et al*, 1997; Inohara *et al*, 1997), *noxa* (Oda *et al*, 2000), *puma/bbc3* (Han *et al*, 2001; Nakano and Vousden, 2001; Yu *et al*, 2001) and *bim* (Dijkers *et al*, 2000; Harris and Johnson, 2001; Putcha *et al*, 2001; Shinjyo *et al*, 2001; Whitfield *et al*, 2001), and at least five are regulated by different post-translational mechanisms, including *bad* (Zha *et al*, 1996; del Peso *et al*, 1997), *bid* (Zha *et al*, 2000), *bik* (Verma *et al*, 2001), *bim* (Puthalakath *et al*, 1999) and *bmf* (Puthalakath *et al*, 2001).

Analysis of 5' deletions of the *bik* promoter revealed that removal of sequences containing several consensus DNA binding sequences did not affect EBNA2-mediated repression of the *bik* promoter consistently. These results were inconclusive, and did not indicate the significance of any specific region within the *bik* promoter. Hence, with this type of analysis, it was impossible to assign the sequences involved in EBNA2-mediated repression more precisely. When comparing the constructs

themselves, irrespective of EBNA2 expression; it is clear that there is an incremental loss of promoter activity with deletion down to –191bp upstream of the transcription start site, seemingly indicating the loss of elements that are necessary to promote gene transcription in this context.

Although both EBNA2 and c-Myc can independently down-regulate *bik* transcription, and EBNA2 can repress transcription from the *bik* promoter, EBNA2 and c-Myc do not co-operate to down-regulate the transcriptional activity of the *bik* promoter fragment under study nor is there an apparent independent role for c-Myc in this regard. Although it has been reported that the majority of genomic loci occupied by c-Myc in B-cells are located in proximal promoter regions (Zeller *et al*, 2006) regulation of *bik* by c-Myc may be mediated by far upstream or downstream/intronic regulatory elements, or alternatively by changes in RNA stability. It is also conceivable that c-Myc indirectly influences *bik* gene expression. Indeed transcriptional control of *bik* mediated independently of promoter regulation has previously been reported. In a recent study in which *bik* mRNA levels were clearly up-regulated by p53, a direct stimulation of the *bik* promoter could not be detected, even though genomic sequence analysis identified several elements that exhibit homology to potential p53 response elements within -1700bp upstream of the transcriptional start site and within the 13kb intron 1 of the *bik* gene (Mathai *et al*, 2002). In addition, EBNA2-mediated repression of the *bik* promoter is not potentiated by EBNA-LP in DG75.

Limited studies examining the regulation of the *bik* promoter have been published to date. The earliest of these (Verma *et al*, 2000) was the first to identify the 1.9 kb promoter region of the *bik* gene and to establish the minimal *bik* promoter region, localised to a region between −211 to +153bp flanking the transcriptional start site. Since this, it has also been established that DNMT1 knockdown induces the expression of *bik* mRNA, without a change in the DNA methylation state or a change in acetylation status and the effect is thought to be mediated by a Sp1 element on the promoter (Milutinovic *et al*, 2004). In a second report, the chemotherapeutic agent, adriamycin (doxorubicin), provoked an approximately 40-fold induction in *bik* mRNA in SHSY-5Y cancer cells and this effect was the result of transcriptional activation of the *bik* promoter by the E2F proteins; E2F1, E2F2 and E2F3 through a consensus

binding sequence for E2F on the *bik* gene promoter, 104bp upstream from the transcription start site (Real *et al*, 2006). Results presented in this chapter establish that EBNA2 has a role to play in the regulation of transcription from the *bik* promoter.

Since the observed decrease in the levels of *bik* mRNA upon activation of EBNA2 could also be attributable to the stability of previously transcribed mRNA in the cell, it was therefore of interest to investigate if an altered mRNA stability was involved in the regulation *bik* expression in B-cells in response to EBNA2. The findings presented here indicate that actinomycin D, an inhibitor of DNA-dependent RNA synthesis, substantially increased the level of *bik* mRNA in the presence of EBNA2. Actinomycin D has been shown to increase the level of several mRNAs, a phenomenon termed "superinduction" (Thompson *et al*, 1966; Tomkins *et al*, 1972; Chatterjee *et al*, 1979). Since actinomycin D inhibits mRNA synthesis, it may obstruct the ability of a repressor molecule, (such as c-Myc) to down-regulate the expression of *bik.* Although a role for mRNA stabilisation in the regulation of the *bik* gene cannot be determined from the results presented here, it is clear that the degradation pathway for *bik* mRNA is different in the presence of EBNA2. Little is known about the role of RNA stability in the regulation of the *bik* gene. It has recently been established that the intracellular stability of *bik* mRNA is not affected during its induction by fulvestrant in MCF7 cells, and that fulvestrant induces *bik* mRNA in MCF7 cells primarily by enhancing transcription through *cis*-elements located outside the 2.0kb proximal promoter (Hur *et al*, 2006).

The genomic organisation of *bik* reveals that it has an unusually long 5′ untranslated (UTR) sequence in exon 1 (Verma *et al*, 2000). In this regard, the structure of the *bik* gene resembles that of the anti-apoptotic *bcl-2* proto-oncogene in which the first exon is also untranslated (Seto *et al*, 1988). Interestingly numerous reports have indicated a role for mRNA in *bcl-2* regulation (Bevilacqua *et al*, 2003; Donnini *et al*, 2004; Lee *et al*, 2004; Otake *et al*, 2004; Otake *et al*, 2005). Two separate degradation systems have been revealed to play a key role in the regulation of the half life of mammalian mRNAs; deadenylation decapping-mediated decay (Gao *et al*, 2001; Bail and Kiledjian, 2006); and decay mediated by the ARE motifs (Guhaniyogi and Brewer, 2001; Raijmakers *et al*, 2004). Both mechanisms, require specific motifs for degradation and these are frequently situated in the 3'-UTR and 5'-UTR (Hughes,

2006). Indeed, several oncogenes, tumour suppressors and other genes associated with cell proliferation have been found to express atypically long and complex 5' UTRs that frequently contain regulatory elements (Hughes, 2006). Thus, the presence of unusually long UTR in these *bcl-2* family members may indicate a role in regulation. Of note, it has recently been reported that cytokines direct the regulation of the BH3-only *bim* gene through enhancing the stability of mRNA by the action of the heat-shock cognate protein 70 (Hsc70) (Matsui *et al*, 2007) and furthermore, investigations into the mechanism of up-regulation of *bfl-1* mRNA levels by LMP-1 in our laboratory revealed the importance of a role for both mRNA stabilisation and increased promoter activity (D'Souza *et al*, 2000; D'Souza *et al*, 2004). However in contrast to this, experiments in the laboratory have revealed no significant change in the stability of *bfl-1* mRNA in response to EBNA2 induction (Pegman *et al*, 2006).

Aberrant promoter methylation of tumor suppressor genes is a common feature in human malignancies, and this fact has stimulated the development of DNA methylation inhibitors as potential cancer therapies (Lyko and Brown, 2005). Since sequence analysis indicated the presence of a CpG island around the transcriptional start site of the *bik* promoter, it was thus hypothesised that the loss of *bik* expression in EBV-infected cell lines might be due to methylation. However treatment with 5-azaC failed to lead to increased amounts of Bik protein in either AG876 or IB4 arguing that *bik* is not repressed by a methylation-dependent mechanism in this context.

Histone deacetylase inhibitors, such as trichostatin A (TSA) and sodium butyrate (NaB), increase the level of histone acetylation in many types of cells (Yoshida *et al*, 1990; Yoshida *et al*, 1995; Monneret, 2005). The involvement of histone acetylation in the regulation of *bik* has been implied by the ability of sodium butyrate to induce the *bik* gene when added to the human hepatoma cell line, HuH-6 (Ogawa *et al*, 2004) (reviewed in Chapter 1). In order to test whether the *bik* promoter is actively repressed by a similar mechanism in EBV-infected cell lines, AG876 and IB4 cells were treated with the HDAC inhibitors, trichostatin A and Sodium Butyrate separately. Treatment with either molecule did not affect the steady-state level of Bik, arguing that the acetylation status and chromatin structure of the *bik* gene are unlikely to be dominant regulators of transcription in this cell context.

The effect of TSA on the expression of *bik* during EBNA2 induction was also investigated. Unlike sodium butyrate which elicits a broad range of effects on cells, TSA is a highly specific, reversible inhibitor of histone deacetylase. However, both the activation of ER/EBNA2 and the induction of EBNA2 from a tet-regulated heterologous promoter was inhibited in the presence of TSA and it was therefore not possible to examine the mechanism of EBNA2-mediated *bik* repression using the chosen cell lines.

Treatment of IB4 and AG876 cells with the proteasome inhibitor MG132 did not increase the level of Bik protein. Significantly, however, the up-regulation of *bik* during EBNA2 repression was strongly inhibited by MG132, suggesting an indirect role for the ubiquitin proteasome pathway in the EBV-associated modulation of the *bik* gene. While the possibility of an indirect role for the involvement of the proteasome in the degradation of Bik protein remains, a direct role for the involvement of the proteasome in the degradation of Bik could not be established from this data.

It appears that inhibition of the proteasome inhibits the relief of repression due to the removal of EBNA2. Thus, EBV may positively stimulate the turnover of Bik through an intermediary factor, such as c-Myc or IgM, which itself is subject to degradation by the proteasomal pathway or alternatively MG132 may inhibit the turnover of a genetic repressor or a protease, thus inhibiting the re-emergence of Bik following the removal of EBNA2. This hypothesis is substantiated by the premise that EBNA2-mediated Bik regulation may involve the modulation of IgM and/ or c-Myc as suggested in Chapters 4 and 5, and is further supported by the fact that both IgM and c-Myc are subject to degradation by the ubiquitin-proteasome pathway (Elkabetz *et al*, 2003; Mallette *et al*, 2007).

The targeting of proteins for ubiquitin-dependent degradation is often controlled by phosphorylation or dephosphorylation of the target protein (Musti *et al*, 1997; Weissman, 1997). In this regard, the proteasome pathway has been shown previously to be involved in the degradation of Bcl-2 following its phosphorylation (Breitschopf *et al*, 2000) and indeed, it has also been reported that in various cell types, including BL cell lines, Bim_{EL} degradation is strictly dependent on the phosphorylation of

Bim_{EL} by ERK1/2 (Ley *et al*, 2003; Luciano *et al*, 2003; Mouhamad *et al*, 2004). It is plausible then, that in an EBV infected cell line EBNA2 expression mediates a conformational change in an intermediary factor, possibly by phosphorylation/dephosphorylation, and this facilitates its degradation by the proteasome. Thus, while EBNA2 is being expressed this molecule is constantly degraded. Upon removal of EBNA2 expression, the intermediary molecule maybe phosphorylated/dephosphorylated and thus become adverse to degradation by the proteasome. These observations suggest that the disappearance of Bik protein in Lat III cells may at least in part be an indirect effect of degradation by an MG132-sensitive proteolytic activity in the infected cell.

Taken together, the data presented here indicates that the mechanism by which EBNA2 represses *bik* is complex. The mechanism is primarily transcriptional, with the likelihood that a decrease in promoter activity is important, and that this repressive effect may be cell line-dependent. While, EBV-mediated *bik* repression may occur by a mechanism that does not involve either of the well known epigenomic mechanisms, DNA methylation or histone acetylation, observations suggest that the mechanism by which Bik protein is turned over is different in the presence of EBNA2. It has been previously shown that the infection of BL cells with EBV results in a significant down-regulation in the expression of the BH3-only *bim* gene (Clybouw *et al*, 2005; Leao *et al*, 2007) and that *bim* expression and function are regulated at both transcriptional and post-translational levels in this context (Clybouw *et al*, 2005; Anderton *et al*, 2007). Therefore it seems that tight control of BH3-only gene expression by EBV is necessary and consequently complex. Regulation may occur on many levels, providing multiple means for the virus to keep these killer proteins in check.

CHAPTER 8

General Discussion

Many viruses exploit and manipulate the differentiation state of infected cells, promote or block cell cycling and make use of a variety of mechanisms to evade innate cellular anti-viral responses and encourage cell survival (Hayward, 2004). Since apoptosis is regulated by functional pro-apoptotic and anti-apoptotic proteins, it is not surprising that EBV controls the cellular response to apoptosis by regulating both pro- and anti-apoptotic genes and their products. EBV immortalises B-cells in culture to generate continually proliferating B lymphoblastoid cell lines. EBNA2, EBNA3A and EBNA3C are essential for this process, as is LMP1 (Kieff, 1996). LMP1 is the viral protein predominantly responsible for the promotion of cell survival through activation of NF-κB, Bcl-2 family members, A20 and Akt activity (Hayward, 2004). EBNA2, in addition clearly stimulates B-cell survival and growth proliferation in the setting of EBV infection (reviewed in Hayward, 2004). This viral transcription factor acts as a pleiotropic activator of viral and cellular genes involved in growth control and plays a decisive role in the induction and maintenance of proliferation of the infected cell. EBNA2 is the master transcription factor that regulates the expression of EBV genes during the growth programme. It also regulates the expression of genes encoding cell surface and soluble proteins that modulate the cell's interaction with the environment, like the receptors CD21, CD23, and CCR7 and the cytokines TNF-α, LT-α and IL-18 (Cordier *et al*, 1990; Burgstahler *et al*, 1995; Spender *et al*, 2001; Pages *et al*, 2005) in addition to cellular genes that are implicated in intrinsic growth and survival regulation, like *cyclinD2*, *c-myc, bfl-1* and *c-fgr* (Knutson, 1990; Sinclair *et al*, 1994; Kaiser *et al*, 1999; Pegman *et al*, 2006). Furthermore, in a study using Sindbis virus infection to induce Nur77 synthesis and apoptosis, it was demonstrated that EBNA2 could bind to Nur77 and protect against Nur77-induced cell death (Lee *et al*, 2002).

The ability of EBV latent proteins to manipulate cell survival by directly regulating members of the Bcl-2 family is well established. As discussed in Chapter 1, EBV LMP1 is known to induce the expression of several anti-apoptotic Bcl-2 family members such as Bcl-2, Bfl-1, Mcl-1 and recently has been shown to down-regulate the expression of pro-apoptotic Bax and Bim (Laherty *et al*, 1992; Fries *et al*, 1996; Wang *et al*, 1996; D'Souza *et al*, 2000; Clybouw *et al*, 2005; Grimm *et al*, 2005; Pegman *et al*, 2006; Anderton *et al*, 2007). EBNA2 in particular has previously been implicated in the modulation of the anti-apoptotic *bcl-2* (Finke *et al*, 1992).

Significantly the over-expression of Bcl-2 in group I BL cell lines resulted in diminished apoptosis in response to a number of stimuli as compared with group III BL cell lines, and cell death inhibition was directly correlated to the amount of Bcl-2 expressed (Henderson *et al*, 1991; Milner *et al*, 1992). It has also been shown in the laboratory that EBNA2 up-regulates the anti-apoptotic *bfl-1* gene. EBNA2 *trans*-activation of *bfl-1* requires CBF1, a nuclear component of the Notch signaling pathway, and *bfl-1* expression is induced and maintained at high levels by the EBV growth programme in an LCL (Pegman *et al*, 2006).

In our laboratory, preliminary RNA multiriboprobe nuclease assays had indicated a possible correlation between the EBV growth programme and the down-regulation of the pro-apoptotic *bik* gene in BL-derived cell lines and LCLs. Since Bik has been implicated in several cancers and given that repression of *bik* or inactivation of its product is thought to play a role in tumour development (Reviewed in Chapter 1), these observations prompted an investigation to determine the role and mechanism of *bik* down-regulation in this context.

Here, evidence is presented in Chapter 3 that loss of Bik protein appears to be a common feature of LCLs and BL type IIIs expressing the full compliment of EBV latent genes. The fact that both native EBNA2 (expressed in the Dg75-tTA-EBNA2 cell line) and active chimeric ER/EBNA2 (in the BL41K3 and BJABK3 cell lines) down-regulated *bik* in the absence of other EBV latent proteins provides compelling evidence that *bik* can be regulated by EBNA2 alone (Chapter 4). Further evidence of this is seen in EBV-negative BL cells superinfected with the EBNA2-defective P3HR1 virus (BL41P3HR1) and the EBNA2-deficient EBV-positive DAUDI cell line, which both show elevated Bik expression (Chapter 3). Repression of *bik* by EBNA2 demonstrates for the first time that EBNA2 targets the transcriptional regulation of a pro-apoptotic protein and may provide an explanation for the observation that BL type I cells in culture tend to have higher levels of *bik* expression than their type III counterparts. Overall it appears that repression of *bik* at the level of transcription, with an associated loss of Bik protein appears to be a feature of LCLs relative to BL-derived cell lines. However, transcriptional regulation of *bik* by EBV may vary between cell lines since it is less apparent in BL type III cells.

The detection of Bik in BL type I cells was not consistent with a pro-apoptotic role in this cell context. However, no mutations leading to loss-of-function were identified nor was a lack of Bax expression implicated in the apparent survival of BL cell lines to this pro-apoptotic gene. Interestingly, whereas the Bik protein expressed in BL Type I cells in the absence of EBNA2 expression always appeared as 27/26kDa double bands during Western blotting, the low level of Bik detected in LCLs and BL Type III cells always appeared as a faint 26kDa single band. Evidence indicates that Bik is phosphorylated, and as such may become active (Verma *et al*, 2001). Thus, it appears that there is a loss of total Bik expression upon induction of EBNA2, and the low level of Bik that remains is not phosphorylatyed. Bik phosphorylation has previously been detected in SW480 cells (human, colon, adenocarcinoma) (Verma *et al*, 2001). In addition, the PI3K pathway is required for both sIgM-mediated sustained Bik protein expression and apoptosis in B104 cells (Jiang and Clark, 2001). Conceivably then, phosphorylation of Bik may confer conformational changes that expose the BH3 domain rendering the protein active in BL Type I cells and the low levels of unphosphorylated Bik which is found in LCLs is inactive. While this model is consistent with the role for EBV in protecting infected cells from apoptosis, it does not explain the apparent resistance of BL Type I cell lines to Bik expression. As was reviewed in Chapter 1, several lines of evidence suggest that the balance between pro-apoptotic Bik and the anti-apoptotic Bcl-$_{XL}$ protein may play a key role in determining B-cell fate (Saltzman *et al*, 1998; Jiang and Clark, 2001). Thus, the relative proportion of Bik not associated with Bcl-$_{XL}$ may play a crucial factor in the ability of these cells to tolerate the expression of this pro-apoptotic molecule. This hypothesis is consistent with the stable transfection experiments described in Chapter 6, where neither DG75 nor BJAB were able to sustain ectopic Bik expression. Bik may also be counteracted by a so far undefined inhibitor in type I cells (EBV latent programme) as compared with type III cells and LCLs (EBV growth programme).

The evidence presented in Chapter 6 indicates that ectopic expression of Bik causes IB4 cells to die by apoptosis and a clear dose dependent increase in apoptotic activity occurred in response to addition of increasing quantities of the Bik expression plasmid pcDNA3-HA-*bik*. The toxic effect of Bik relative to the BH3-deleted Bik indicates that the death-promoting activity of Bik is dependent on a functional BH3 domain. In addition, induction of cell death by Bik was strongly inhibited by the pan-caspase

inhibitor zVAD-fmk, indicating that Bik induces apoptosis by a pathway that is upstream of the caspases. Together, these results indicate for the first time that down-regulation of the pro-apoptotic *bik* gene promotes B-cell survival during the EBV growth programme. Since it is clear that EBNA2 plays a key role in repressing *bik* it is now apparent that this host-virus interaction contributes to the survival of EBV-infected B-cells that are proliferating on the viral growth programme, thus implicating a contributory role for Bik in setting the elevated threshold of resistance of these cells during EBV infection.

As discussed in Chapter 6 down-regulation of pro-apoptotic Bcl-2 family members has been shown previously to protect malignant cells from apoptosis. Loss of Bax increases tumourigenicity and reduces apoptosis (McCurrach *et al*, 1997; Yin *et al*, 1997; Ionov *et al*, 2000) and the absence of both Bax and Bak can enhance transformation, to a greater extent than the loss of either protein alone (Zong *et al*, 2001). Preliminary data also indicates that the absence of *bim* accelerates *Myc*-induced lymphomagenesis (Cory and Adams, 2002) and there is also evidence that the p53 targets, Noxa and Puma, might mediate p53 apoptotic function and that Bmf might inhibit metastasis (Puthalakath *et al*, 2001).

In the EREB 2-5 conditional LCL, withdrawal of estrogen results in cessation of cell growth and a significant proportion of the cells die by apoptosis (Kempkes *et al*, 1995a). Loss of expression of EBNA2-regulated genes such as cellular *c-myc*, *cyclinD2* and *cdk4* (Kempkes *et al*, 1995a; Kaiser *et al*, 1999) and EBV *LMP1* and *LMP2* (*TP*) (Kempkes *et al*, 1995b) contribute substantially to this outcome. Experiments presented in Chapter 5 demonstrate a role for EBNA2 in regulation of the *bik* gene in this context. Conceivably, since EBNA2 modulates both IgM, and c-Myc expression (Jochner *et al*, 1996; Kaiser *et al*, 1999) and since both of these genes can affect *bik* expression (Jiang and Clark, 2001 and Chapter 5) it is possible that EBNA2 mediates the regulation of *bik* through both IgM-dependent and c-Myc-dependent pathways. The *c-myc* gene is transcriptionally activated by EBNA2 in a CBF1-independent manner (Maier *et al*, 2005) and is known to share similar functions with EBNA2 as a transcriptional regulator and hence, was a good candidate to mediate Bik suppression by EBNA2. c-Myc over-expression in P493-6 cells did down-regulate *bik* in the absence of EBNA2 (Chapter 5), but the steady state level of

bik mRNA/Bik protein was considerably lower when cells were cycling due to EBV Lat III. Thus, since EBNA2 contributes not only to the activation of c-Myc-mediated *bik* repression (as would be the case in c-Myc-only-driven *bik* repression) but also to the activation of the IgM-associated pathway, and since EBNA2-mediated regulation of both of these genes is only partially CBF1 dependent (Maier *et al*, 2005) the hypothesis that EBNA2 mediates *bik* repression through both *c-myc* and *IgM* may explain the considerable differences observed in the levels of *bik* expression when cells are cycling due to EBNA2 in contrast to cells proliferating due to c-Myc expression. The data presented here demonstrates for the first time that the *bik* gene is repressed through a mechanism driven by EBNA2, and that c-Myc can also drive *bik* repression independently of functional EBNA2 expression during the EBV growth programme.

In BL type I cells, however, c-Myc is overexpressed due to chromosomal translocation (Brady *et al*, 2007), and yet these cells express detectable Bik. Introduction of EBNA2 into these cells leads to repression of both c-Myc and Bik, arguing against a role for c-Myc as a negative regulator of Bik. Furthermore, in BJABK3, a non BL cell line derived from a lymphoblastoid B-cell line that does not carry the t(8;14) translocation, c-Myc expression is transiently up-regulated in response to EBNA2 (Maier *et al*, 2005) and yet Bik is still down-regulated. Thus, it appears that EBNA2 mediated repression of Bik is not dependent on c-Myc in this context.

Genomic deletion of CBF1 in DG75 cells did not abrogate the ability of EBNA2 to down-regulate *bik* in these cells (Chapter 4). In contrast, it has previously been shown in the laboratory that EBNA2-mediated *trans*-activation of the *bfl-1* gene is abolished by the inhibition of EBNA2/CBF1 signaling (Pegman *et al*, 2006). This is interesting as CBF1 is the major rate-limiting factor for EBNA2 activation of cellular target genes (Maier *et al*, 2006). This finding is further supported by observations made using the non-CBF1-binding EBNA2www323sr (promoter regulation studies in Chapter 7).

EBV down-regulates the expression of two further pro-apoptotic members of the Bcl-2 family; *bax* and *bim* (discussed in Chapter 1) (Clybouw *et al*, 2005; Grimm *et al*, 2005; Anderton *et al*, 2007). Aberrations in the expression of several pro-apoptotic

proteins would increase the variety of apoptotic stimuli against which the host cell can protect itself, since the range of apoptotic stimuli to which the individual pro-apoptotic proteins can respond is not always absolute; different proteins are known to respond to different types of stimulus. For example, Bid-deficient mice appear normal, but their hepatocytes (but not their lymphocytes) are resistant to anti-Fas antibody-induced killing while in contrast, Bim is required for haematopoietic cell homeostasis and as a barrier against autoimmune disease (reviewed in Bouillet and Strasser, 2002). In addition, the fact that EBV mediates an elevated level of several anti-apoptotic proteins including Bcl-2, Bfl-1 and Mcl-1 (Henderson *et al*, 1991; Wang *et al*, 1996; Pegman *et al*, 2006) provides compelling evidence the regulation of both pro- and anti-apoptotic genes is central to the overall strategy that EBV employs to control the cellular response to apoptosis in infected cells.

The cellular proteins Notch and c-Myc and the EBV LMP1 are known to have overlapping functions with EBNA2, particularly as transcriptional regulators. LMP1 can partially substitute for EBNA2 in mediating cell survival (Zimber-Strobl *et al*, 1996) and in the up-regulation of the surface markers CD21, CD23, intercellular adhesion molecule 1 (ICAM-1), and lymphocyte function antigen-1 (LFA-1) (Peng and Lundgren, 1992; Peng and Lundgren, 1993). It is presented in Chapter 4 that LMP1 does not influence the expression of *bik* in DG75 cells. In the presence of the EBNA2-deleted P3HR1 genome, Notch can partly, and c-Myc fully replace EBNA2 in the maintenance of B-cell proliferation (Pajic *et al*, 2000; Hofelmayr *et al*, 2001). While findings in Chapters 4 and 7 established that the EBNA2-CBF1 interaction was not required for EBNA2-mediated *bik* repression it should be noted that EBNA2 and Notch have overlapping functions beyond their capacity to interact with CBF1. Significantly, as discussed in Chapter 5, it has also been shown in the laboratory that high levels of Notch 1IC can substitute for EBNA2 to repress *bik,* although not to the same extent. Thus, EBNA2 may execute notch-like functions to facilitate the regulation of pro-apoptotic *bik* and additional common interaction partners of both Notch and EBNA2, such as SKIP, Nur77, PU.1, Spi-1 or Spi-B could potentially contribute to this function (Laux *et al*, 1994a; Johannsen *et al*, 1995; Zhou *et al*, 2000a; Zhou *et al*, 2000b; Lee *et al*, 2002). Interestingly Myc is also a direct transcriptional target of Notch1 participating as an indispensable downstream effector

in Notch 1IC-induced tumourigenic action (Efstratiadis *et al*, 2007) a further indication that EBNA2 is mimicking the Notch cellular pathway.

The down-regulation of *bik* by EBNA2 may be a key event in the rescue of EBV-infected B-cells from apoptosis and an important route by which EBV gains access to the memory B-cell pool and contributes to oncogenesis. In Chapter 6 it was shown that the reintroduction of Bik causes IB4 cells to die by apoptosis. The anti-apoptotic function of EBNA2 may complement the LMP1-induced survival signals mediated through NF-κB and provide initial protection prior to LMP1 expression during primary EBV infection of B-cells (as suggested in Lee *et al*, 2002). While most EBV-associated tumours do not express EBNA2, this viral protein is expressed in EBV-associated tumours arising in immuno-compromised patients such as PTLD and in primary central nervous system lymphomas in AIDS patients (Cen *et al*, 1993; Auperin *et al*, 1994; Delecluse *et al*, 1995) and its anti-apoptotic activity may be a factor in the development of these malignancies. Thus, dual proliferative and anti-apoptotic roles for EBNA2 may emulate the activities attributed to cellular Notch (Miele and Osborne, 1999; Shelly *et al*, 1999). Indeed, EBNA2 mimicry of Notch IC has previously been extended beyond the recognised shared mechanism of regulation through CBF1 (Zimber-Strobl and Strobl, 2001). In this regard EBNA2 has been shown to inhibit Nur77-mediated apoptosis demonstrating a mutual mechanism for anti-apoptotic activity (Lee *et al*, 2002).

Taken together the data presented in Chapter 7 indicates that the mechanism by which EBNA2 represses *bik* is complex. The findings demonstrate that EBNA2 may down-regulate expression of *bik* by decreasing transcription from the *bik* promoter and that this down-regulation involves a mechanism that is independent of CBF1 and is not potentiated by EBNA-LP. EBNA2 and c-Myc do not co-operate in the down-regulation of the *bik* promoter fragment nor is there an apparent independent role for c-Myc although these conclusions are limited to the 1.9kb promoter fragment used. An abundance of genes have been shown to interact with Myc in this context; in fact one group recently identified 668 direct Myc-regulated gene targets, including 48 transcription factors in the P493-6 cell line, indicating that Myc is a central transcriptional hub in growth and proliferation control (Zeller *et al*, 2006). Analysis in

chapter 7 indicated that there were 14 putative Myc-related binding sites spread throughout the -1710/+203 region of the *bik* promoter. However, recent studies have suggested that binding sites for transcription factors are not necessarily near the transcriptional start sites of genes or in CpG islands. In this regard, it has been reported that only 18% of Myc-binding sites are within 1kb of a 5'-exon and only 24% are within 1kb of a CpG island (Cawley *et al*, 2004). Indeed a recent genomics study found that approximately half of all Myc-bound sites were intergenic (>10 kb away from transcriptional start sites) (Bieda *et al*, 2006). In another recent study c-Myc binding sites on DNA from the P493-6 cell line were analysed, and showed that c-Myc may regulate up to 10-15% of all genes in this B-cell context, and could occupy approximately 4,296 genomic loci. The majority of these (63%) are located within a 10kb range around known gene regions with a strong binding preference toward 5' proximal promoter regions (Zeller *et al*, 2006). Significantly only 36% of Myc binding loci are within 5kb of a CpG island in this context also. Thus, *bik* regulation may be effected through a consensus *c-myc* binding element located either far upstream (or downstream) of the -1710/+203 region of the promoter. A global binding model for Myc has recently been suggested (Bieda *et al*, 2006). In this model Myc binding is widespread encompassing possibly thousands of sites per chromosome and including both genic and intergenic sites. This global binding regulates general chromatin structure and in that way directs cell biology through influencing functions such as cell cycling and fate that depend on overall chromatin structure (Bieda *et al*, 2006).

Interestingly in support of the hypothesis presented in Chapter 4, findings in Chapter 7 indicate that EBV may stimulate the turnover of Bik through either the modulation of an intermediary factor such as c-Myc or IgM or through the inhibition of the turnover of a genetic repressor or a protease. In the presence of ER/EBNA2, the level of *bik* mRNA is affected by actinomycin D, to a greater extent than in the absence of EBNA2. Following ER/EBNA2 activation there is a substantial accumulation in *bik* mRNA levels. Since actinomycin D inhibits mRNA synthesis, an accumulation in mRNA levels after treatment with this drug indicates an apparent contradiction, the resolution of which may offer important evidence concerning the mechanism of gene regulation in question (Chatterjee *et al*, 1979). The steady-state level of mRNA is determined by the dynamic interaction between its rate of synthesis and its rate of

degradation. In the presence of transcriptional inhibitors, the rate of mRNA synthesis is diminished and a lower steady-state level of the mRNA would normally result. It is assumed that quantitating the decreasing amounts of mRNA as a function of time in the presence of transcriptional inhibitors would be a measure of the degradation rate. However, this assumption is based on the conditions that (1) mRNA synthesis is successfully blocked by the inhibitor and (2) the inhibitor does not interfere (directly or indirectly) with the degradation mechanism of the mRNA in question. Indeed actinomycin D has previously been shown to lead to an accumulation in the levels of several mRNAs including alpha 2u and P-Glycoprotein mRNAs, a phenomenon termed "superinduction" (Chatterjee *et al*, 1979; Lee and Ling, 2003). When superinduction is observed in the presence of transcriptional inhibitors, one or both of these criteria presumably do not hold. It has been proposed that the superinduction in the presence of transcriptional inhibitors could be due to the inhibition of expression of an intermediary factor involved in mRNA regulation. It is clear from the result presented in Chapter 7, that the degradation pathway for *bik* mRNA is different in the presence of EBNA2. On the basis of these observations, treatment of BL41K3 cells (in which ER/EBNA2 is activated), with actinomycin D may result in the loss of an intermediary factor. This intermediary factor(s) may be preferentially eliminated by actinomycin D due to a short half life, thus leading to an accumulation of the *bik* mRNA. Significantly, this is supported by findings in Chapter 5, which indicate that the up-regulation of *bik* during EBNA2 repression was significantly inhibited by MG132, suggesting that EBNA2-mediated repression of the *bik* gene requires an intermediary factor and is further substantiated by the premise that EBNA2-mediated Bik regulation may involve the modulation of IgM and/ or c-Myc (Chapter 4). This result is complex to interrupt, yet significantly, *gapdh* mRNA levels appear stable and were similar in estrogen-treated and estrogen-untreated samples. Overall, it is clear from the result presented in Chapter 7, that the degradation pathway for *bik* mRNA is different in the presence of EBNA2.

A protein similar to Bik has also been discovered in rodents (Hegde *et al*, 1998) this gene, designated *bik*-like killer (*blk),* is considered the murine ortholog of human *bik* on the basis of their location in syntenic regions, gene organisation, and nucleic acid as well as amino acid sequence homology (Coultas *et al*, 2004). However, B-cells from *blk* $^{-/-}$ knockout mice developed and reproduced normally (Coultas *et al*, 2004)

and deletion of this gene was shown to have a little effect on the sensitivity of murine cells to apoptotic stimuli (Coultas *et al*, 2004) including p53 over-expression (Mathai *et al*, 2005). Remarkably, therefore, murine *blk* and human *bik* respond differently to stress stimuli (Coultas *et al*, 2004; Mathai *et al*, 2005). This apparent distinction between the function of Bik and its murine ortholog, Blk may be explained by the substantial differences in structure and expression from its human counterpart. In this regard, the human and mouse *bik* orthologs are only 43% identical, despite having very similar gene structures (Hegde *et al*, 1998) and furthermore unlike Bik, *Blk* is largely restricted to hematopoietic and endothelial cells (Coultas *et al*, 2004).

Viral infection represents a cellular stress that induces host cell pro-apoptotic responses. To overcome this barrier to productive infection, viral polypeptides modulate a variety of host cell pathways. The general strategy of some viruses to down-regulate pro-apoptotic genes is well established, and in this regard the human cytomegalovirus encodes "viral mitochondria-localised inhibitor of apoptosis" (vMIA), which functions as a potent cell death suppressor by binding to and inhibiting pro-apoptotic Bcl-2 family members Bax and Bak (Arnoult *et al*, 2004). The inactivation of the pro-apoptotic tumour suppressor p53 by the papillomavirus E6 oncogene is an important mechanism by which E6 promotes cell growth (Li *et al*, 2005). In addition, adenovirus L4-100K assembly protein is a granzyme B substrate that potently inhibits granzyme B-mediated cell death (Andrade *et al*, 2001).

The inhibition of pro-apoptotic pathways is a fundamental step in tumour development and may be responsible for therapy resistance. Apoptosis-based strategies are important tools for the development of effective anticancer strategies. Such strategies may seek to correct specific genetic defects as in the case of mutations, or substitute for deregulated tumour suppressor genes (Whartenby *et al*, 1995). Indeed studies have shown that over-expression of several pro-apoptotic genes, including Bax, Bcl-$_{XS}$, apoptin, and TRAIL, can display an anti-tumour effect (Ealovega *et al*, 1996; Ashkenazi *et al*, 1999; Walczak *et al*, 1999; Huh *et al*, 2001; Li *et al*, 2001; Backendorf *et al*, 2008). Conceivably the apoptosis deficiency of EBV-associated malignancies could be overcome by targeted enforced expression of pro-apoptotic Bcl-2 proteins such as Bik or Bim or targeted knockdown of anti-apoptotic proteins such as Bfl-1. Potentially, high expression of Bik alone might kill cells, and

lower expression levels of anti-apototic proteins could increase cellular sensitivity to radiation or chemotherapy. In view of the fact that expression of Bik served to induce apoptosis in an EBV-infected cell line, this avenue may be of considerable interest for the future treatment of malignancies in which *bik* is repressed, possibly in tandem with low-dose chemotherapy. The anti-tumour activity of Bik in melanoma has previously been demonstrated *in vivo* in a human xenotransplant nude mouse model. Induction of Bik resulted in significantly reduced tumour size and delayed tumour growth. Comparable results were also reported for xenotransplanted colon and prostate carcinoma, for which tumour formation could be prevented by intratumoral injection of a Bik-expressing Adenovirus (Tong *et al*, 2001).

Bik has also been found to sensitise tumour cells to apoptosis mediated by several chemotherapeutic agents including etoposide, epirubicin, paclitaxel, pamidronate and doxorubicin (Daniel *et al*, 1999; Panaretakis *et al*, 2002; Oppermann *et al*, 2005) and also to CD95/Fas-triggered apoptosis (Daniel *et al*, 1999; Oppermann *et al*, 2005). Additionally undecylprodigiosin (UP), a bacterial bioactive metabolite with anticancer properties, enhanced the levels of Bik protein in several human breast cancer cell lines (Ho *et al*, 2007) as did treatment with neurotoxins Maneb and MPP^+ in a human neuroblastoma cell line (Fei and Ethell, 2008). *Bik* is also induced in estrogen-dependent MCF7 breast cancer cells in response to inhibition of estrogen signaling (Hur *et al*, 2004) and has been identified as one of a set of genes classified as 'molecular markers' for thermoradiosensitivity in cervical cancer (Harima *et al*, 2004). Furthermore, delivery of a liposome and *bik* gene complex dramatically inhibited tumour growth and prolonged survival in a mouse orthotopic breast cancer model (Zou *et al*, 2002). One recent study showed that *bik* can function as a mediator of apoptosis triggered by viral infection-induced shutoff of protein synthesis (Shimazu *et al*, 2007).

Indeed, several molecular therapeutic approaches against cancer are targeted to overcome drug resistance, and are based on the combination with chemotherapeutic drugs. Since most chemotherapeutic agents require the activation of caspases to induce apoptosis, chemoresistance may be caused by a defect in apoptosis-inducing pathways. In this context, it has been reported that ectopic Bik expression can

overcome drug resistance in breast cancer and in lymphoma and melanoma cells (Daniel *et al*, 1999; Radetzki *et al*, 2002; Oppermann *et al*, 2005). Thus, triggering Bik in EBV-associated malignancies may support the pro-apoptotic activities exerted by the immune system or those resulting from chemotherapies. In this regard, Bik can be transcriptionally induced by BCR-triggering in B104 cells (Jiang and Clark, 2001) and an increase in *bik* mRNA was also observed in TGF-β-mediated apoptosis of the Ramos B-cell line (Saltzman *et al*, 1998). Interestingly, certain chemotherapeutic agents have been shown to induce Bik expression, which in turn contributes to the apoptotic response of cancer cells to chemotherapy including adriamycin, antiestrogen fulvestrant, doxorubicin and γ-radiation (Thiantanawat *et al*, 2003; Mathai *et al*, 2005; Real *et al*, 2006).

Undoubtedly, the efficacy of *bik* would depend largely on its targeting to the infected cell in question, a practice which requires a delivery system that can both withstand intravenous conditions and can also transport the therapeutic gene to the target cell. Ad-mediated gene transfer is a clinically relevant cancer treatment and has been shown to induce apoptosis of tumour cells while sparing the 'normal' adjacent cells (Liu *et al*, 1995). Indeed intratumoural injection of adenoviral wt *bik* led to tumour regression in one study (Tong *et al*, 2001). Nonviral gene delivery by liposomes is another promising strategy since the liposome vehicle has very low immunogenicity and toxicity. In one study, a modified cationic liposome formulation (SN) containing a surface-protection polymer to stabilize the liposome-DNA particles for intravenous injection was employed and SN-*bik* was seen to successfully inhibit tumour growth in nude mice (Zou *et al*, 2002). Delivery of genes that encode apoptosis-related proteins could potentially have many advantageous effects on malignancy. Firstly not only would tumour cell proliferation be correctly balanced by an increase in cell death, but the barriers to the immune response would collapse, tumour invasiveness would be hindered, and sensitivity cytotoxicity would be restored (as reviewed in Gomez-Navarro *et al*, 2000). Importantly, the need that tumour cells have for mechanisms that allow them to avoid apoptosis is universal, shared by all tumour localisations in the body. This evasion represents a common ground that can be targeted in the vast environment of tumour heterogeneity. With the increasing identification oncogenes or tumour suppressor genes involved in the apoptotic pathway, gene therapy has emerged as a promising strategy in the attempted modulation of apoptosis. (As reviewed in (Gomez-Navarro *et al*, 2000).

Recently interest has been growing in the potential of anticancer drugs that, like the pro-apoptotic BH3 domain, bind one or more Bcl-2 homologs and trigger apoptosis. It is thought that such 'BH3 mimetics' may prove to be more effective than anti-tumour agents acting further upstream. Furthermore, BH3 selectivity holds the ability of specifically targeting the Bcl-2 homolog(s) required to maintain a particular tumour type, potentially avoiding injury of the healthy cell population. Indeed the ability of a Bid BH3 peptide to activate Bax *in vitro* has previously been shown and may mean that Bax (or Bak) could also be targeted. Moreover, a Bik BH3 peptide can effectively compete with natural myristoylated (myr) Bid protein for binding Bcl-2, thus abrogating the anti-apoptotic activity of Bcl-2 and enabling myrBid induced cytochrome *c* release (Letai *et al*, 2002). BH3 mimetics may also prove to be important adjuvants in conventional therapy, sensitising cells to diverse chemotherapy agents, and potentially facilitating lower doses of a conventional agent or more durable responses (reviewed in Adams and Cory, 2007).

In this study it has been established that the EBNA2 mediated suppression of *bik* has a potential role to play in the regulation of B-cell apoptosis by EBV. It is possible that a combinatorial strategy will be key for the treatment of lymphomas, and by understanding the interactions of viral and cellular signaling pathways involved in tumourigenicity, the most effective strategy may be developed. A combinatorial approach involving the enhanced expression of a pro-apoptotic molecule such as *bik*, (either through direct activation for example via BCR-triggering or direct delivery of a BH3 mimetic), in tandem with the targeted down-regulation of expression of anti-apoptotic genes involved in the pathway such as *bfl-1* (by means of a functional blockade or inhibition of expression) may be most effective in restoring the apoptotic machinery in the cell and sensitising EBV-associated malignancies to chemo- or radiotherapies. In addition, the identification of viral proteins that provide survival signals to naturally infected tumour cells provides us with an opportunity to link pathway deregulation with potential therapeutic strategies.

In summary, this thesis presents the novel finding that EBV down-regulates the cellular pro-apoptotic *bik* gene. Significantly, restoration of Bik expression in IB4 cells induces a pathway upstream of the caspases stimulating the induction of apoptosis that is dependent on a functional BH3 domain. The EBV latent transcription

factor EBNA2 plays a key role, and possibly c-Myc and this down-regulation is independent of the cellular transcription factor CBF1. This is the first indication of the down-regulation of expression of a pro-apoptotic gene by EBNA2 and to date EBNA2 is known to transcriptionally repress the activity of only one other cellular gene, the immunoglobulin heavy chain locus (IgM). A more complete understanding of the role and regulation of the *bik* gene in BL-derived cells has benefits for the biology of EBV and will undoubtedly further the understanding of malignant disease. Collectively, these findings suggest that functional deactivation of *bik* expression in EBV-infected cells and modulation of its expression by EBNA2 may be a contributory factor in the development of EBV-associated B-lymphomas.

CHAPTER 9

Summary and Concluding Remarks

Bik is expressed in BL-derived cell lines

- Loss of Bik protein appears to be a common feature of LCLs and BL type IIIs expressing the full compliment of EBV latent genes.
- Loss-of-function mutations in the *bik* gene are unlikely to be a feature of BL cells since no mutations leading to changes in the primary amino acid sequence were found.
- Bax was expressed in a range of cell lines including BL Type I (except DG75), BL type III and LCLs.

Expression of exogenous *bik* in EBV infected cell lines

- Ectopic Bik triggers apoptosis in the EBV-infected cell line, IB4, by a mechanism which dependent on the BH3 domain.
- Bik-induced cell death was inhibited by the pan-caspase inhibitor zVAD-fmk.
- Expression of exogenous Bik but not BH3-domain deleted Bik led to reduced cell viability in a range of EBV infected cell lines using Luciferase and GFP expression assays.
- A dose dependent apoptotic response to increasing quantities of HA-Bik expression was observed.

Regulation of *bik* by EBNA2 in BL-derived cell lines

- The EBV latent protein EBNA2 can transcriptionally repress *bik* expression in EBV-negative BL-derived cell lines DG75-tTA-EBNA2, BL41K3 and BJABK3 and in the EBV positive cell line BL419A.
- Neither the Notch pathway associated protein CBF1/RBPJκ nor the EBNA2-CBF1 interaction are necessary for EBNA2-associated repression of *bik.*
- EBNA2 mediated repression of the *bik* gene is not dependent on *c-myc* expression in BL.
- The induction of LMP1 did not influence the expression of the *bik* gene in response to tetracycline-regulated induction of this protein, indicating no role for LMP1 in its regulation.

Regulation of *bik* by EBNA2 in LCLs.

- Inactivation of the EBV growth programme in the conditional Lymphoblastoid cell line EREB2-5 leads to the de-repression of *bik* mRNA and protein.
- c-Myc can trigger *bik* repression in P493-6 cells in the absence of functional EBNA2.
- The steady state level of *bik* was considerably lower when cells were cycling due to the EBV growth programme when compared to cells proliferating on the c-Myc programme, indicating a possible co-operative role for both EBNA2 and its cellular target gene *c-myc* in *bik* repression in this context.

Regulation of the *bik* promoter in BL-derived cell lines

- Transient transfection assays using *bik* promoter reporter constructs indicate repression of the *bik* promoter by EBNA2.
- EBNA2-mediated repression of the *bik* promoter in DG75 cells does not require RBP-Jκ/CBF1 and is not potentiated by EBNA-LP.
- EBNA2 and c-Myc do not co-operate in the down-regulation of the *bik* promoter nor is there an apparent independent role for c-Myc in the regulation of the 1.9kb *bik* promoter fragment.

Other Mechanisms

- Actinomycin D substantially increased the concentration of *bik* mRNA in the presence of EBNA2.
- Treatment of EBV infected cell lines with 5-azaC failed to lead to increased amounts of Bik protein, arguing that Bik is not repressed by a methylation-dependent mechanism in this context.
- Treatment of EBV infected cell lines with the HDAC inhibitors, TSA and Sodium Butyrate did not affect the steady state level of Bik, suggesting that the acetylation status and chromatin structure of the *bik* gene are unlikely to be dominant regulators of transcription in this cell context.

- Trichostain A, a HDAC inhibitor potently inhibited the activation of ER/EBNA2 and the induction of EBNA2 from a tet-regulated heterologous promoter.
- EBV may indirectly stimulate the turnover of Bik through a) the modulation of an intermediary factor such as c-Myc or IgM or b) through the inhibition of the turnover of a genetic repressor or a protease. No direct role for the involvement of the proteasome in the degradation of Bik was established.

Concluding Remarks

In view of the fact that expression of Bik served to induce apoptosis in an EBV-infected cell line this avenue may be of considerable interest for the future treatment of EBV-associated malignancies possibly in tandem with low-dose chemotherapy. The antitumor activity of Bik in melanoma has previously been demonstrated *in vivo* in a human xenotransplant nude mouse model. Induction of Bik resulted in significantly reduced tumour size and delayed tumour growth. Comparable results were also reported for xenotransplanted colon and prostate carcinoma, for which tumour formation could be prevented by intratumoral injection of Bik adenovirus (Tong *et al*, 2001).

It is now clear that EBNA2 mediated suppression of *bik* has a potential role to play in the regulation of B-cell apoptosis. Importantly, EBNA2-mediated down-regulation of *bik* may contribute to the survival of EBV-infected B-cells *in vivo*, and the effect on *bik* may contribute to the development of EBV-associated B-cell malignancies in which EBNA2 is expressed.

Of interest, the EBV anti-apoptotic vBcl-2 homologues, BHRF1 and BALF1, are transiently expressed immediately following EBV infection, and are essential for B-cell immortalisation (Altmann and Hammerschmidt, 2005). BHRF1 has been shown to interact with and inhibit Bik (Boyd et al, 1995; Elangovan and Chinnadurai, 1997), and hence negative transcriptional modulation of *bik* by the EBV growth programme may supersede these early events to extend this survival advantage, thus favouring immortalisation and potentially lymphomagenesis.

CHAPTER 10

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APPENDIX I

Solutions for DNA Manipulation

Storage of DNA

0.5 M EDTA (pH 8.0)

186.1g	EDTA
800ml	dH_2O

The pH was adjusted to 8.0 by addition of NaOH pellets and the volume adjusted to 1L with dH_2O. The solution was sterilised by autoclaving and stored at room temperature.

TE buffer (pH 8.0)

10mM	Tris-Cl (pH 8.0)
1mM	EDTA (pH 8.0)

Solution for Bacterial Growth Media

Ampicillin stock solution

A stock solution of ampicillin was made up to a concentration of 100mg/ml in dH_2O. The stock solution was filter sterilised and stored at -20˚C.

LB broth

5g	Tryptone
2.5g	Yeast extract
5g	NaCl

The volume was adjusted to 500ml, followed by autoclaving for 20 minutes at 15lb/sq. and storage at 4˚C.

LB broth with ampicllin

Ampicillin was added to autoclaved LB broth to a final concentration of 100µg/ml and stored at 4˚C.

LB agar

5g	Tryptone
2.5g	Yeast extract
5g	NaCl
7.5g	Agar

The volume was adjusted to 500ml with dH_20, followed by autoclaving and agar plates were stored at 4˚C.

LB agar with antibiotics

Antibiotics were added to autoclaved LB broth to a final concentration of 100µg/ml (Ampicillin), after cooling the LB agar to ~50˚C. Plates were stored at 4˚C.

SOB medium

10g	Tryptone
2.5g	Yeast extract
0.025g	NaCl
5ml	KCl (250 mM)

The pH was adjusted to 7.0 with 5M NaOH.
The volume was adjusted to 500ml with dH_20 and the medium autoclaved.
2.5ml of 2M $MgCl_2$ was added after cooling the broth to 5˚C and the medium was stored at 4˚C.

SOC medium

98ml	SOB medium
2ml	1M glucose (filter sterilised)

Stored at 4˚C.

Solutions for Preparation of Competent Cells

1M $MgSO_4$

24.65g	MgSO4.7H20
100ml	dH20

Sterilised by filtering and stored at room temperature.

TFB1

30mM	potassium acetate
10mM	$CaCl_2$
50mM	$MnCl_2$
100mM	RbCl
15%	glycerol

The pH was adjusted to 5.8 with 1M acetic acid, and the solution filter sterilised and stored at room temperature.

TFB2

100mM	MOPS (pH 6.5)
75mM	$CaCl_2$
10mM	RbCl
15%	Glycerol

The pH was adjusted to 6.5 with 1M KOH, and the solution filter sterilised and stored at room temperature.

Solutions for DNA preparations

Buffer P1 (Re-suspension buffer)

50mM	Tris-Cl (pH 8.0)
10mM	EDTA (pH8.0)
100µg/ml	RNase A

Stored at 4˚C following addition of RNase A.

DNase-free RNase

RNase A (1mg/ml) in upH_2O.
Heated to 100˚C for 30 minutes.
Cooled slowly and stored at -20˚C.

Buffer P2 (Lysis buffer)

200mM	NaOH
1% (w/v)	SDS

Stored at room temperature.

Buffer P3 (Neutralisation buffer; (3M potassium acetate)

29.6g	potassium acetate
50ml	dH_2O
11.5ml	glacial acetic acid

Adjust volume to 100ml with dH_2O.
The resulting solution is 3 M with respect to potassium and 5M with respect to acetate.

Stored at room temperature.

Buffer QBT (Equilibration buffer)

750mM	NaCl
50mM	MOPS (pH 7.0)
15% (v/v)	Isopropanol
0.15% (v/v)	Triton-X 100

Stored at room temperature.

Buffer QC (Wash buffer)

1M	NaCl
50mM	MOPS (pH 7.0)
15% (v/v)	Isopropanol

Stored at room temperature.

Buffer QF (Elution buffer)

1.25M	NaCl
50mM	Tris-Cl (pH 8.5)
15%	Isopropanol

Stored at room temperature.

50% (v/v) Glycerol

25ml	glycerol
25ml	dH_2O

The solution was autoclaved and stored at room temperature.

Annealing buffer

100mM	Potassium acetate
30mM	Hepes-KOH, pH 7.4
2mM	Magnesium acetate

Stored at 4 – 8 ˚C.

Solutions for Agarose Gel Electrophoresis

50X TAE (Tris-acetate/EDTA electrophoresis buffer)

242g	Tris base
57.1ml	Glacial acetic acid
100ml	0.5M EDTA (pH 8.0)

The volume was adjusted to 1L with dH_2O and the buffer was stored at room temperature.

1X TAE (Working solution)

20ml	50X TAE
980ml	dH_2O

Stored at room temperature.

Agarose gel loading dye

40% (w/v)	Sucrose
0.25% (w/v)	Bromophenol blue

Stored at room temperature.

Ethidium bromide

10mg	Ethidium bromide
1ml	dH_2O

The solution was stored in the dark at 4 ºC. 100µl of stock solution was added to 1L of dH_2O for TAE gel staining.

Solutions for Cell Culture

Media and Supplements

Supplemented RPMI 1640 (200 ml)

176ml	RPMI 1640
20ml	Foetal bovine serum (decomplemented at 50˚C for 30 minutes)
2ml	200mM L-glutamine
2ml	penicillin/streptomycin (10mg/ml)

Estrogen (β-Estradiol)

A 20mM solution was prepared in 100% ethanol and stored at -20˚C.

Tetracycline

A 5mg/ml stock was prepared in 100% ethanol and stored at -20˚C.

Geneticin (G418)

A 50mg/ml stock was prepared in cell culture medium and stored at -20˚C

MG132

A 5mM stock was prepared in in DMSO and stored at -20˚C.

zVAD-fmk

A 20mM solution was prepared in DMSO and stored at -20˚C.

Phosphate buffered saline (PBS)

5 tablets were dissolved in 500ml dH_2O to give a 1X working concentration of PBS, and the solution was sterilised by autoclaving.

Thiol supplements

The following were added to 200 ml of supplemented media:

200µl	α-Thiolglycerol
2ml	Sodium pyruvate
2ml	HEPES

Bathocuproine disulfonic acid (BCS) 10mM stock solution

36.4mg	BCS
10ml	1X PBS

Aliquoted and stored at -20^0C.

α-Thiolglycerol

A stock solution of 50mM in PBS containing 20μM BCS was prepared.

20μl	10mM BCS
10ml	1 X PBS
43.3 μl	100% α-thiolglycerol

Aliquoted and stored at -20^0C.

Solutions for DEAE-Dextran Tranfections Protocol

Tris buffered saline (TBS)

25mM	Tris-Cl (pH 7.4)
137mM	NaCl
5mM	KCL
0.7mM	$CaCl_2$
0.5mM	$MgCl_2$
0.6mM	Na_2HPO_4

To make 200ml TBS:

5ml	1M Tris-Cl (pH 7.4)
5.48ml	5M NaCl
0.5ml	2M KCl
1.4ml	100mM $CaCl_2$
0.1ml	1M $MgCl_2$
0.2ml	0.6M Na_2HPO_4
187.32ml	upH_2O

TBS was prepared from autoclaved stocks, aliquoted and filter sterilised before use.

DEAE dextran

DEAE dextran was prepared at a concentration of 1mg/ml in TBS on the day of use and filter sterilised.

β-Galactosidase Assay

100X Mg solution

0.1M	$MgCl_2$
4.5M	2-mercaptoethanol

Stored at -20˚C.

0.1M sodium phosphate buffer

41ml	0.2M $Na_2HPO_4.2H_2O$
9ml	0,2M $NaH_2PO_4.2H_2O$
50ml	dH_2O

1X ONPG substrate

ONPG was prepared at a concentration of 4mg/ml in 0.1M sodium phosphate buffer (pH 7.5) and stored at -20˚C.

1M sodium carbonate

10.59g	Sodium Carbonate
100ml	dH_2O

Stored at room temperature.

Propidium Iodide Solution

69μM	Propidium Iodide
38MM	NACITRATE (PH 7.4)

Solutions for RNA Analysis

DEPC-treated H_2O

1ml	DEPC
1000ml	dH_2O

The mixture was left in a fume cupboard overnight, followed by autoclaving.

RNA loading buffer

50%	Glycerol
1mM	EDTA (pH 8.0)
0.25%	Bromophenol blue
0.25%	Xylene cyanol FF
1µg/ul	Ethidium bromide

Solutions for Protein Analysis

Solutions for Protein Isolation

Suspension buffer

0.1M	NaCl
0.01M	Tris-Cl (pH 7.6)
0.001M	EDTA (pH 8.0)
1µg/ml	leupeptin
1µg/ml	Aprotinin
100µg/ml	PMSF

Stored at 4˚C

Leupeptin

A stock solution of leupeptin was made to a concentration 2mg/ml in dH_2O and stored at –20˚C.

Aprotinin

0.1M stock solution of aprotinin was made up in dH_2O and stored at -20˚C.

PMSF
A stock solution of PMSF was made up in isopropanol and stored at -20˚C in the dark.

2X SDS loading buffer

100mM	Tris-Cl (pH 7.6)
4% (w/v)	SDS
20% (w/v)	Glycerol
10% (v/v)	2-mercaptoethanol
0.2%	Bromophenol blue

Stored at room temperature.

Solutions for SDS-PAGE/ Western Blotting

Table A: 10% (v/v) resolving gels and 5% (v/v) stacking polyacrlyamide gels

Component	10% Resolving Gel (ml)	5% Stacking Gel (ml)
Acrylagel	3.33	0.42
Bis-Acrylagel	1.35	0.168
1.5M Tris (pH 8.8)	2.5	0
1M Tris (pH 6.8)	0	0.312
dH_2O	2.61	1.5475
10% (v/v) SDS	0.10	0.025
10% (v/v) APS	0.10	0.025
TEMED	0.01	0.0025
Total	**10 ml**	**2.5 ml**

5X Tris-glycine running buffer

15.1g	Tris base
95.4g	glycine (pH 8.3)
50ml	10% (w/v) SDS

Made up to 1L with dH_2O and stored at room temperature.

1X Tris-glycine running buffer

200ml	5X Tris-glycine running buffer
800ml	dH_2O

Transfer buffer

750ml	dH_2O
2.9g	Glycine
5.8g	Tris base
3.7ml	10% (w/v) SDS
200ml	Methanol

Adjusted volume to 1L with dH_2O and stored at 4˚C.

1X Tris buffered saline (TBS)

6.1g	Tris base
8.8g	NaCl
800ml	dH_2O

The pH was adjusted to 7.5 with HCl and the volume adjusted to 1L. Stored at room temperature.

TBS-T

1L	1X TBS
1ml	Tween 20

Stored at room temperature.

Blocking Buffer

5g	non fat dry milk powder
100ml	TBS-T

Stored at 4˚C.

Appendix II

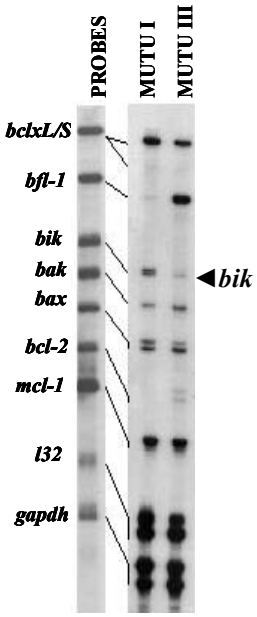

Figure A: EBV-associated modulation of the steady-state levels of mRNAs from *bcl-2* related genes in the MUTU I and MUTU III cell lines.

mRNA levels from the apoptosis related *genes* $bcl_{XL/S}$, *bfl-1, bik, bak, bax, bcl-2* and *mcl-1* were analysed in a range of BL cell lines and LCLs by RPA (18 hour exposure). Unprotected [32-P]-labeled antisense riboprobes (5000cpm, per lane) were loaded alongside RPA-processed samples and are shown linked to their smaller RNase-protected fragments which correspond to steady state levels of mRNA in each sample. (Adapted from D'Souza *et al*, 2000).

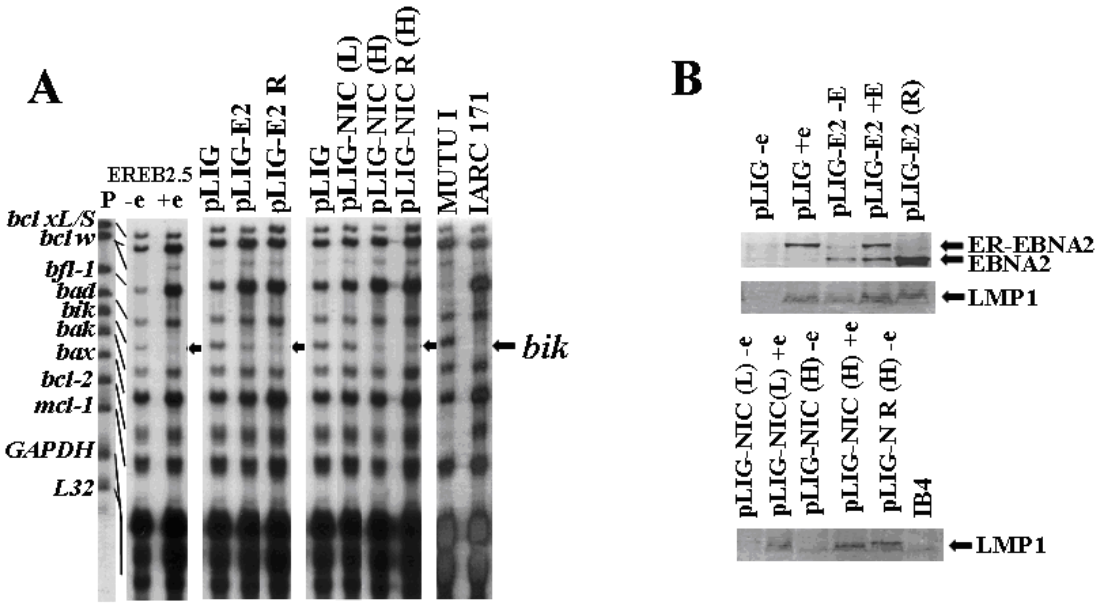

Figure B: High Levels of Notch 1IC can substitute for EBNA2 to repress *bik*

(A) Transduction of estrogen-deprived EREB 2-5 with HIV-based defective lentiviruses expressing either EBNA2 (pLIG-E2) or high levels of human Notch 1IC (pLIG-NIC(H)) (Gordadze *et al*, 2001). mRNA levels from the apoptosis related *genes bcl-$_{XL/S}$, bfl-1, bik, bak, bax, bcl-2* and *mcl-1* were analysed by RPA. Unprotected [32-P]-labeled antisense riboprobes (5000cpm, per lane) were loaded alongside RPA-processed samples and are shown linked to their smaller RNase-protected fragments which correspond to steady state levels of mRNA in each sample. (B) Levels of EBNA2, ER/EBNA2 and LMP1 in the experiment shown in (A)+/- e (plus/minus estrogen) (courtesy of Dr. Brendan D'Souza).