Expression of the Anti-apoptotic *bfl-1*/A1 Gene is Regulated by the Epstein Barr Virus Nuclear Antigen 2 (EBNA2)

A dissertation submitted for the degree of Ph.D.

by

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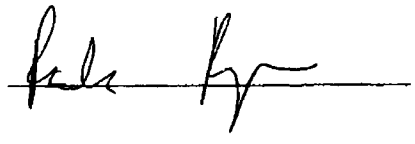
DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph D is entirely my own work 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

Signed 

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Date 16/09/03

This thesis is dedicated to my parents for all the love and encouragement you have always given me.
Thank you.

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ABBREVIATIONS

amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammonium persulphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
β-gal	β-galactosidase
BL	Burkitt's Lymphoma
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
cDNA	complementary DNA
CIP	Calf intestinal phosphatase
CMV	Cytomegalovirus
CTAR	Carboxy terminal activation region
CTP	Cytosine tri-phosphate
DEPC	Diethylpyrocarbonate
dH_20	Distilled water
dNTP	Deoxy nucleoside tri-phosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EA-D	Early antigen-diffuse
EA-R	Early antigen-restricted
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic Mobility Shift Assay
FCS	Fetal calf serum
HA	Haemagglutinin antigen

HEPES	N-[hydroxyethyl]piperazine	N'-[2-ethane sulfonic acid]
Ig	Immunoglobulin	
IL	Interleukin	
IM	Infectious mononucleosis	
LB	Luria-Bertani Broth	
LCL	Lymphoblastoid cell line	
LMP	Latent Membrane Protein	
LP	Leader Protein	
Luc	Luciferase	
mAb	monoclonal antibody	
MAPK	Mitogen Activated Protein Kinase	
MCS	Multiple cloning site	
mRNA	messenger RNA	
NBT	Nitroblue tetrazolium	
NPC	Nasopharyngeal carcinoma	
n s	non-specific	
OA	Okadaic acid	
OD	Optical Density	
OHL	Oral Hairy leukoplakia	
ori	Origin of replication	
p	Plasmid	
PAGE	Polyacrylamide gel electrophoresis	
PBS	Phosphate buffered saline	
PMSF	Phenylmethylsulfonyl fluoride	
pRB	Retinoblastoma protein	
RBP-Jκ/CBF1	Recombination Signal binding protein-Jκ	
RNA	Ribonucleic acid	
RNase	Ribonuclease	
RPA	Ribonuclease Protection assay	
s s	super-shifted complex	
SDS	Sodium dodecyl sulphate	

SSC	Standard sodium citrate
ssDNA	salmon sperm DNA
SV40	Simian Virus 40
T	Thymidine
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBST	TBS + Tween20
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TESS	Transcription factor element search software
TF	Transcription factor
TNF	Tumour necrosis factor
tRNA	Transfer RNA
TRAF	TNF-receptor-associated factors
upH_2O	Ultrapure water
U	Uracil
UV	Ultraviolet
v/v	volume per volume
VA	sodium orthovanadate
w/v	weight per volume

UNITS

bp	Base pairs
Ci	Curies
Kb	Kilobase pairs
KD/KDa	KiloDaltons
µg	microgram
µl	microlitre
°C	Degrees celsius
cm	Centimetres
x g	g force
g	Grams
h	Hours
Kg	Kilograms
L	Litres
M	Molar
mA	Milliamperes
mg	Milligrams
min	Minutes
ml	Millilitres
mM	Millimolar
mol	Moles
ng	Nanograms
nM	Nanomolar
pmole	Picomoles
s	Seconds
U	Enzyme units
V	Volts

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Abstract

The ubiquitous and oncogenic human herpesvirus Epstein-Barr virus (EBV) establishes a latent infection and promotes the long-term survival of the infected host cell by targeting the molecular machinery that controls cell fate decisions (apoptosis, proliferation and differentiation) These host-virus interactions are likely to play a crucial role in the development of EBV-associated malignancies includmg African endemic Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disorders in immunodeficient individuals We have previously shown that (i) EBV-infected Burkitt's lymphoma cell lines exhibit elevated levels of expression of the anti-apoptotic *bfl*-1 gene (also known as *A1*) compared to their uninfected counterparts, (ii) ectopic expression of Bfl-1 can protect a Burkitt's lymphoma (BL) cell line from apoptosis induced by serum deprivation (D'Souza, B , Rowe, M and Walls, D 2000 J Virol , 74, 6652) and (iii) the EBV Latent Membrane Protein 1 (LMP1) stimulates *bfl-1* promoter activity through interactions with components of the cellular Tumour Necrosis Factor Receptor (TNFR/CD40)-signalling pathway by a mechanism which includes an essential role for the transcription factor NFκB (D'Souza et al, submitted) Bfl-1 is an anti-apoptotic protein of the Bcl-2 family, whose preferential expression in hematopoietic and endothelial cells is controlled by inflammatory stimuli This study reports the novel finding that the EBV nuclear antigen 2 (EBNA2), a second major effector of phenotypic change in EBV-infected cells, can independently upregulate *bfl-1* mRNA levels in BL cell lines This thesis presents the novel finding that EBNA2 regulates *bfl-1* promoter activity through interactions with components of the cellular Notch signalling pathway EBNA2-mediated *trans*-activation of *bfl-1* is dependent upon its ability to bind CBF1 (a nuclear component of the Notch signalling pathway) and involves a novel CBF-1-like binding site on the *bfl-1* promoter This EBNA2-mediated effect on *bfl-1* is modulated by other EBV latent proteins that are known to co-operate with EBNA2 (EBNA-LP) or which have been shown to interact with the CBF1-co-repressor complex (EBNA3A, 3B, 3C and RPMS) These findings are relevant to our understanding of EBV persistence, its role in malignant disease, and the B cell developmental process

CHAPTER 1

INTRODUCTION

EPSTEIN BARR VIRUS (EBV)/HUMAN HERPES VIRUS 4 (HHV4)

1.0 CLASSIFICATION.

EBV/HHV4 is a gamma herpesvirus and the only human herpes virus in the gamma herpes virus subfamily The gammaherpesvirus subfamily includes both the lymphocryptovirus and rhadinovirus genera EBV is the prototype lymphocryptovirus Humans are the exclusive natural host for EBV Other lymphocryptoviruses have only been found in old world primates such as chimpanzees The gamma herpes viruses were classified on the basis of their biological properties rather than their genomic organization Thus the gamma herpes virus family consists of viruses, which can establish latent infection in lymphocytes and are associated with cell proliferation Although some members of the beta herpes virus group such as HHV6 and HHV7 are capable of latent infection of lymphocytes they do not corrupt the cell proliferation pathways of the latently infected cells (Kieff 1996)

It is precisely these biological properties, particularly the involvement of the gamma herpes viruses in cell proliferation and thus cancer, which has generated most of the interest and study of this viral subfamily The link between herpes viruses and oncogenic disease however, is restricted to the gamma herpes virus subfamily (Rickinson, 2002) EBV in particular has been associated with several human cancers (See table 1 0 below) and was originally isolated from a human tumor biopsy, Burkitts Lymphoma (BL) (Epstein *et al* , 1964)

1.1 VIRUS STRUCTURE

Similar to other herpes viruses, EBV has a toroid-shaped protein core that is wrapped with double stranded DNA Surrounding this is a nucleocapsid comprised of 62 capsomeres, a protein tegument between the nucleocapsid and the envelope and an outer envelope with external glycoprotein spikes The major EBV capsid proteins are 160,47, and 28kd The most abundant EBV proteins within the envelope and tegument are 350/220 and 152kd respectively EBV also differs from other herpes viruses in the predominance of a single glycoprotein in the outer envelope

FIGURE 1.0. SCHEMATIC REPRESENTATION OF HERPES VIRUS STRUCTURE

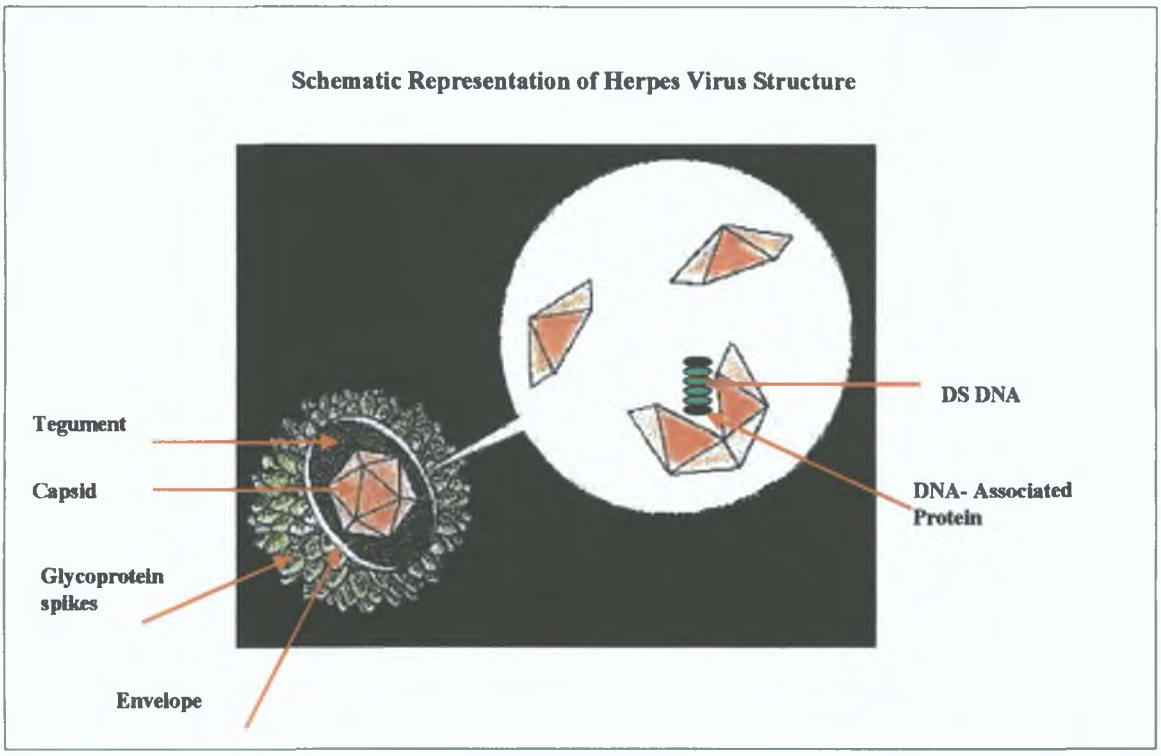


Figure 1.0. Schematic representation of herpes virus structure. All major structural components including the icosotetrahedron shaped capsid, the tegument and the envelope are shown.

1.2 EBV GENOME STRUCTURE

The EBV genome is a linear, double-stranded, 172-kbp DNA with a 60% guanine/cytosine composition (Kieff 1996). The EBV genome includes 85 known genes that occupy most of the viral DNA (Wensing and Farrell 2000). As with many viruses, however, complicated differential splicing of RNA transcripts means the number of proteins transcribed may far exceed the number of genes. The characteristic features of EBV and other lymphocryptovirus genomes include (i) a single overall format and gene arrangement, (ii) reiterated 0.5kbp terminal direct repeats (TR) and, (iii) reiterated 3kbp internal direct repeats that divide the genome into short and long largely unique sequence domains. The major repeat domains serve as landmarks on the EBV genome map however isolates from serial passage may have different numbers of repeat domains thus

repeat domain length is not an invariant feature of EBV DNA, however these differences can be exploited when determining the difference between different isolates or strains In addition since some of these repeat domains may encode particular proteins, differences in protein size on immunoblots can be used to identify particular viral isolates or strains However, each time EBV establishes latent infection in a proliferating cell, the genome persists as an episome that has a number of repeats TR characteristic of that infected cell Thus the number of TRs in latently infected cells are useful in determining whether latently infected cells arose from a common progenitor (Kieff 1996) Figure 1 1 below outlines EBV genome arrangement

FIGURE 1.1 THE EBV GENOME

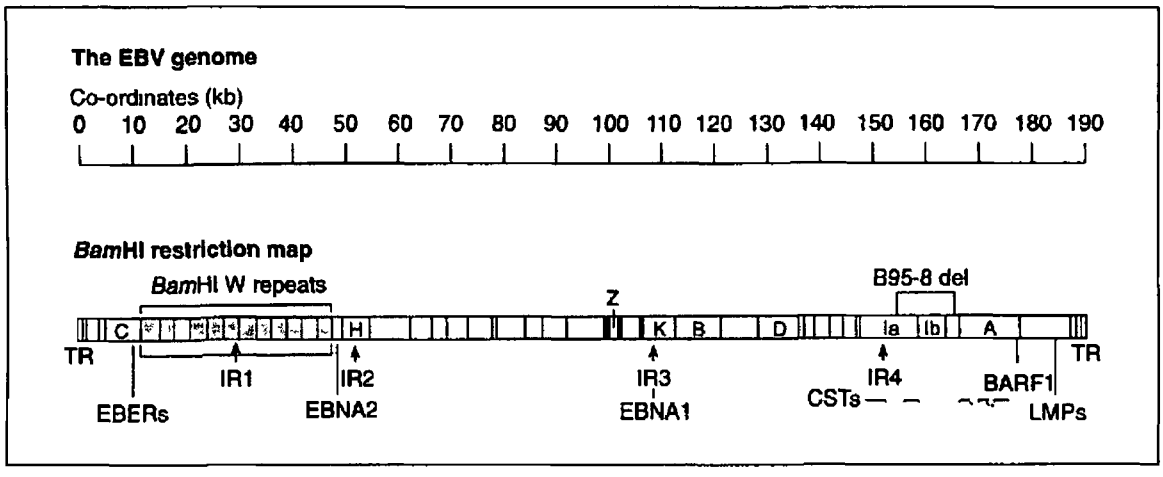


Figure1 1 A schematic representation of the EBV genome. Upper panel coordinates of a linearised version of a composite EBV genome Lower panel in virions EBV exists in a linear form such as shown here, but in infected cells, including tumours, the genome is circularised through terminal repeats, designated TR. Some landmarks on the viral genome include the various families of repetitive elements IR1–IR4 , as well as the terminal repeats TR. The gene locations of EBNA1 and EBNA2, LMP1 and the small RNAs EBERs, as they relate to the *Bam*HI restriction map of the viral genome, are indicated LMP2A and 2B are expressed across the TRs, and thus can only be produced from circularised DNA Also shown are the location of transcription of the BARF1 and the highly spliced RNAs CSTs

EBV was the first herpes virus to be completely sequenced and was sequenced from the EBV strain B95-8 Since this viral strain was initially cloned as a BamH1 fragment library, nomenclature for the open reading frames (ORFs), for transcription or RNA processing are commonly referenced to specific BamH1 fragments (Farrell1992) An example of this is the BARF1 ORF, which is found in the BamH1 A fragment (BA) and is the first ORF (F1) extending in a rightward direction (R) The schematic above shows the location of the various ORFs on the EBV genome (Figure1 1)

Two EBV types have been identified in most human populations These two types are generally referred to as EBV type 1 and EBV type 2 (formerly types A and B respectively) The two types differ only by the manner of a few genes however these differences are responsible for consistent type specific genetic and biological activities The genomes for the two strains are identical except for the genes that encode some of the Epstein Barr nuclear antigens (EBNAs) such as EBNA2, EBNA3A,3B 3C and EBNALP, in latently infected cells These differences are reflected in type-specific and type-common EBNA epitopes for antibodies (Young *et al*, 1987) and T-cell recognition (Moss *et al*, 1988) Because type 1 is more common in developed societies, most EBV immune human sera from developed societies react preferentially or exclusively with EBV type 1 EBNA2, 3A, 3B and 3C African sera are more evenly split in their serologic reactivity however the recovery of type-2 virus from blood is unusual (Young *et al*, 1987, Rowe *et al*, 1989)

1.3 Strategy of Infection

Humans provide the natural host for Epstein Barr Virus and it is estimated that over 95% of the world's population carry this herpesvirus (Rowe 2001) EBV target cell tropism is generally accepted to be the B cell Evidence suggesting possible dual-tropism involving epithelial as well as B cells has been debated, though evidence to date suggests EBV mainly infects and replicates in B cells in the healthy host (Faulkner *et al*, 2000, Power and Walls 1993)

Primary infection in vivo occurs at the oropharynx and viral replication at this site amplifies the orally transmitted virus (Rickinson 2002) It is at this site at this stage of primary infection that EBV either gains direct access to B cells by exploiting the structural arrangement of the tonsil epithelium, (which is infiltrated in places by lymphoid cells) or alternatively, accesses B cells at this point by an infection independent mechanism called transcytosis

At a molecular level, EBV B lymphocyte infection *in vitro* involves a number of steps including virus adsorption, viral penetration and uncoating, as well as a number of early intracellular events A number of B cell lines, particularly Burkitts lymphoma (BL) cell lines and lymphoblastoid cell lines (LCLs), are particularly useful for studying EBV infection in vitro

1.4 Early Events in EBV Infection

1.4.1 Viral Adsorption, Penetration and Uncoating

EBV is adsorbed to infected B-lymphocytes by binding to a cell surface protein called CD21 CD21 (also known as CR2) is a member of the immunoglobulin super family and consists of 15 or 16 imperfect repeats of a 60-amino-acid domain, a transmembrane domain and a short carboxy-terminal cytoplasmic domain The EBV binding activity maps to the amino terminal repeat domains The major EBV outer envelope glycoprotein gp350/220 is the CD21 ligand (Kieff 1996) However other modes of entry are also likely to exist since a virus lacking gp350 is still able to infect and immortalize B cells (Janz *et al*, 2000)

Super infection of established BL cell lines is somewhat different in that EBV binding the envelope fuses with the plasma membrane, releasing the nucleocapsid and the tegument into the plasma membrane The differences between the mechanism of infection between primary B-lymphocytes and BL cells are likely to be due to the cytoskeletal abnormalities of the tumor cells (Kieff 1996) Another viral envelope glycoprotein gp85 has been implicated in the fusion of the envelope with the plasma membrane (Miller *et al*, 1988)

At this point the EBV capsid dissolves, the viral genome is transported to the cell nucleus and the viral DNA circularizes The viral DNA circularizes within 16 to 20 hours post infection (Hurley, and Thorley-Lawson 1988) by a process involving fusion of the terminal repeats Details regarding these intracellular post-infection processes are still unclear

1.5 Introduction to the Lytic and Latent Programmes, of EBV Infection.

Two "programmes" of EBV-cellular infection are recognized, these are known as the lytic (or replicative) programme and the latent programme (Thorley-Lawson, and Babcock 1999) The virus lytic programme is the series of events describing the process of active replication and production of infectious progeny EBV virions This cycle involves expression of the "lytic programme genes" in an ordered cascade These genes are involved in viral DNA replication and some encode structural glyco-proteins or proteins that modify the infected cells in order to permit viral envelopment or egress (IARC Monographs 1997) Like several other herpesviruses, EBV also encodes lytic cycle genes that can prevent or delay death of the host cell, which can result from apoptosis triggered by the cell recognizing virus replication as DNA damage (Henderson, *et al*, 1993) Details of the viral lytic genes, and their protein products, involved in the viral lytic programme are given in section 1 9 below The most common outcome of B lymphocyte infection with EBV, however, is a persistent latent infection in memory B cells In fact the virus replicates poorly in recently infected B-lymphocytes (Niedobitek *et al*, 1997), which instead become stably latently infected Latent infection requires expression of a specific set of EBV latent genes encoding nuclear (EBNA/Epstein Barr Nuclear Antigens) and membrane proteins (LMP/Latent Membrane Proteins) and small RNAs (EBERs/Epstein Barr encoded RNAs) Expression of these genes allows viral persistence and ultimately causes continual proliferation of the infected B-lymphocytes (Allday *et al*, 1989, Alfieri *et al*, 1991) a process also known as immortalization Some of these B cells then manage to avoid immunosurveilance and certain death by apoptosis and persist in the host in the memory B cell pool In vivo, only a small number of B cells

enter the memory B cell pool, by a specific selection process, unselected cells die by apoptosis It is thought that EBV can mimic the "selection process" and protect EBV infected cells from apoptosis, while allowing their passage to the memory B cell pool Figure 1 2 below shows the mechanisms whereby EBV can persist in vivo (adapted from Faulkner *et al* , 2000)

FIGURE 1.2. EBV PERSISTENCE IN VIVO

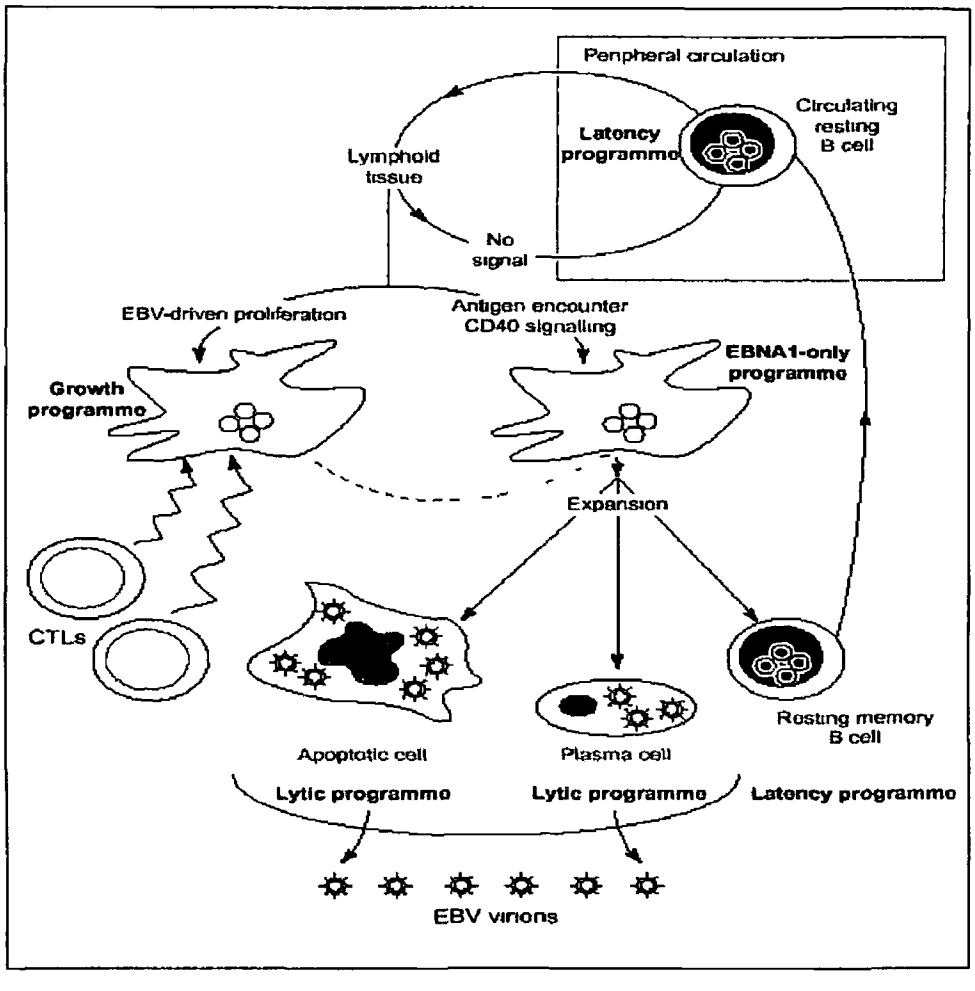


Fig. 1 2 Epstein–Barr virus (EBV) persistence in vivo (reviewed by Faulkner *et al*, 2000) EBV persists in circulating resting B cells, hidden from T-cell immunosurveillance by limited viral gene expression [Epstein–Barr nuclear antigen (EBNA) 1 and latent membrane protein (LMP) 2] and the absence of cellular receptors necessary to elicit a cytotoxic T lymphocyte (CTL) response ('latency programme') Should such a cell enter lymphoid tissue and become activated by encounter with antigen or T cell signaling via CD40, the resulting cell divisions would allow viral genome replication, although still without engendering a CTL response, because viral gene expression is limited to being non-immunogenic ('EBNA1-only programme') The fate of the dividing cells can be as follows to apoptose, but not before the virus has entered the 'lytic programme', releasing infectious virions, to terminally differentiate into a plasma cell with the same effect, or to become a memory B cell, with the virus returning to the latency programme An infected cell can also begin to proliferate under virus-driven mechanisms analogous to B cells infected in vitro These become immortalized owing to the expression of nine viral genes EBNAs 1, 2, 3a, 3b, 3c, LP and LMPs 1, 2a and 2b ('growth programme') Under normal circumstances these cells would be killed by CTLs, but some might enter one of the alternative 'programmes' before this occurs

1.6 EBV LATENT INFECTION

In latent infection, the virus penetrates the cell in the usual manner after binding to CD21 and remains present either as circular episomal DNA or less frequently as linear DNA integrated into the host genomic DNA Episomes present in low copy number in the host cell nucleus are copied by host cell DNA replicating enzyme and are passed to daughter cells by mitosis (Joske and Knecht 1993) The effect of EBV infection on cell growth *in vitro* is immediate and efficient with most cells beginning DNA synthesis 48 to 72 hours post infection At least 11 EBV genes are expressed in latent infection Expression of a subset of 9 of these genes ultimately leads to B cell immortalization These nine genes include six nuclear proteins (EBNA1, 2, 3A, 3B, 3C and EBNA-LP, three membrane proteins (LMP1, LMP2A and LMP2B), and two small non-polyadenylated nuclear RNAs (EBER1 and EBER2) Five of these genes are essential for primary B cell immortalization these are, EBNA1, 2, 3A, 3C, and LMP1 It was originally though that EBNALP was also essential for B cell immortalization however recent studies showed that although it is not essential, it substantially enhances proliferation (Rowe 2001, Izumi 2001) Table 1 0 below, adapted from (Wensing and Farrell 2000, Rowe 2001), summarizes the functions of the EBV latent proteins and their requirement for immortalization The first viral proteins to be expressed in B lymphocytes after EBV infection are EBNA2 and EBNALP, these are transcribed from the Wp promoter in a rightward direction (See Figure 1 3A below), and protein can be detected in the cells within 12-16 hours post infection The Wp promoter is present in multiple copies in the major internal repeat Forty-eight hours after EBV infection, expression of EBNA1, EBNA3s and LMP1 is induced, this is then followed by expression of EBERs and LMP2 such that by 72 hours post infection all 11 latent proteins are at detectable levels Transcription of nuclear proteins is initiated at RNA polymerase II –dependent promoters in the BamH1 C (Cp) and the BamH1 W (Wp) regions in the viral genome Once immortalization has been reached, transcription of the EBNA2 and EBNALP proteins from the strong Wp promoter declines and transcription of the genes switches to the Cp promoter (Woisetschlaeger *et al*, 1990) The switching mechanism is not fully understood at the molecular level However Cp activity is highly dependent on several

cellular transcription factors, including nuclear factor Y (NF-Y) and Sp1 (Borestrom *et al*, 2003) All EBNA mRNAs are derived from the same transcriptional unit by alternative splicing and alternative polyadenylation This can be seen by following the outer long arrowed red line in Figure 1 3A below, where all the EBNA genes are transcribed from either the Cp or Wp promoter Further detail can be seen in figure 1 3 B below The coding exons for most of the EBNAs are towards the 3' end and are preceded by the highly spliced leader exons, which are encoded within the major internal repeat of the genome (Farrell 1995)

FIGURE 1.3A. LOCATION OF EBV GENES ON THE DOUBLE STRANDED VIRAL EPISOME

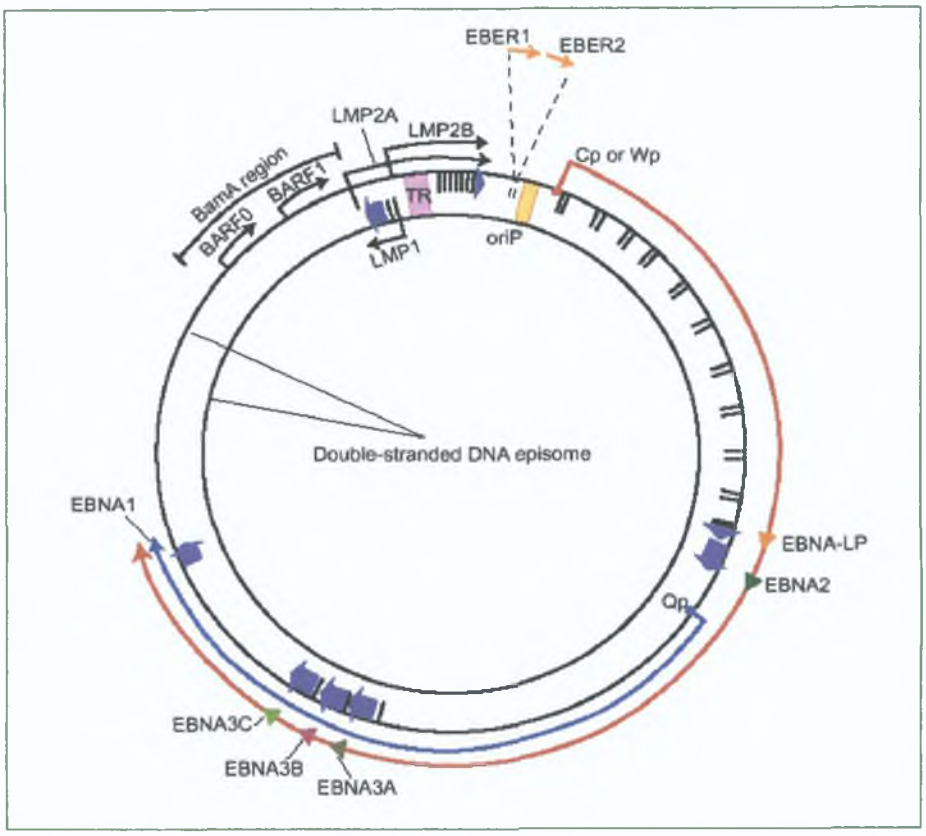


Figure 1.3A The Epstein–Barr virus (EBV) genome. The Diagram below, adapted from (Murray, and Young 2001) shows (a) the location of the EBV latent genes on the double-stranded viral DNA episome. The origin of plasmid replication (oriP) is shown in orange. The large solid blocks (in purple) represent coding exons for each of the latent proteins and the arrows indicate the direction in which they are transcribed. EBNA2 is transcribed from the Wp promoter until cells become immortalized where after transcription is initiated from the Cp promoter. All EBNA mRNAs are transcribed from the same transcriptional unit by alternative spicing and polyadenylation (Details on this are shown in 1.xB below.) EBNA-LP is transcribed from variable numbers of repetitive exons. LMP2A and LMP2B are composed of multiple exons located either side of the terminal repeat (TR) region, which is formed during the circularisation of the linear DNA to produce the viral episome. The orange arrows at the top represent the highly transcribed non-polyadenylated RNAs EBER1 and EBER2. Transcripts from the BamA region can be detected during latent infection, but no protein arising from this region has been definitively identified. Shown here are the locations of the BARF0 and BARF1 coding regions.

FIGURE 1.3 B. DETAILS OF EBNA MRNA TRANSCRIPTION.

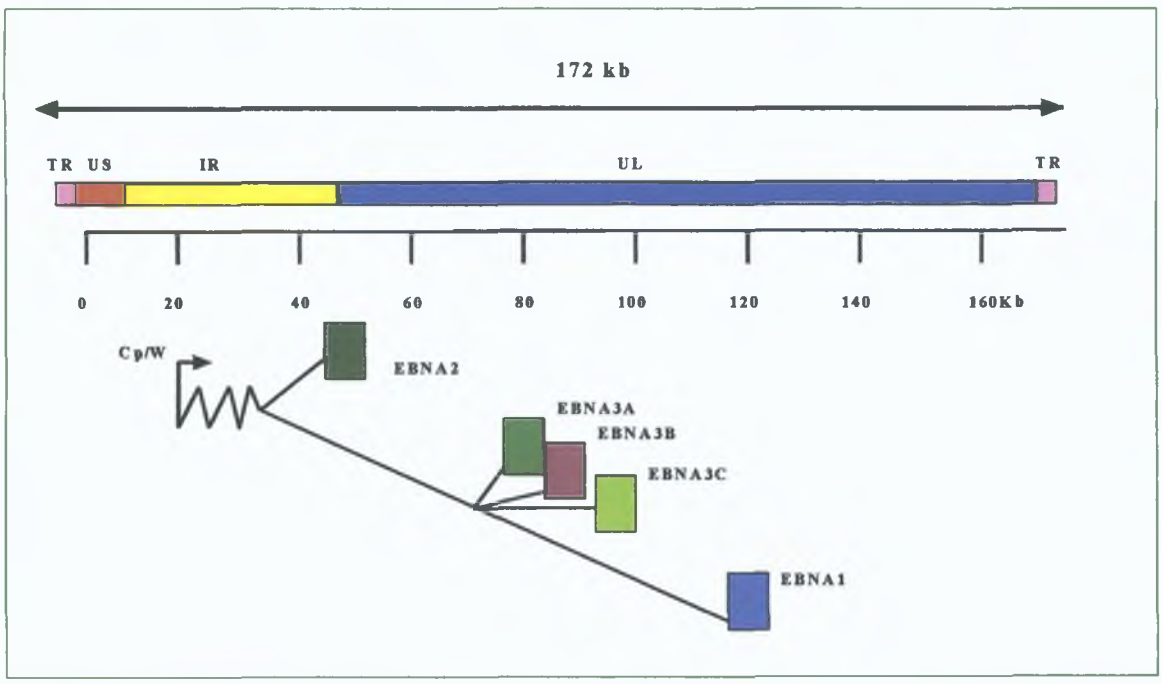


Figure 1.3B. A simplified outline of the splicing of the EBV nuclear antigen coding mRNAs. All the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcript.

1.7 THREE FORMS OF LATENCY, LATENCY I, II AND III

Three forms of latent infection have been characterized in EBV carrying B cell lines and EBV tumor carrying biopsies. These are frequently described as latency I, II and III (Kerr *et al.*, 1992). These different forms of viral latency were suggested on the basis of differential expression of viral latent genes and promoter usage in EBV associated tumors and immortalized B cell lines. The locations of the latency genes in the viral genome and their patterns of expression in the different latency states are shown in Figure 1.3A above (Murray and Young 2001). The outer long arrowed red line represents EBV transcription during a form of latency known as latency III (Lat III) where all the EBNA mRNAs are transcribed from either the Wp or Cp promoter. The inner shorter arrowed blue line represents the EBNA1 transcript originating from the Qp promoter during Lat I and Lat II.

In latency I defined by BL, only the EBV EBNA1 antigen is expressed as well as high copy numbers of EBER1 and 2 Also known as the EBNA1 only programme, this describes the form of latency in B cells driven to proliferate in response to physiological signals All the EBV latent proteins elicit a strong immune response except EBNA1 thus the EBNA1 only programme may explain the ability of the virus to persist undetected by the immune system of infected individuals In latency II as defined by Nasopharyngeal carcinoma (NPC) and Hodgkins Lymphoma (HD) both LMP1 and LMP2 are expressed in addition to EBNA1 and the EBER RNAs Latency III, also known as the growth phase is designed to drive the limited proliferation of B cells prior to differentiation and the establishment of persistent infection in memory B cells Latency III is the best characterized latency programme and is found in LCLs, immunoblastic B cell lymphomas of the immunosuppressed and some B cell blasts in patients with infectious mononucleosis All the known latency genes are expressed Other less well-characterized patterns of EBV gene expression have been identified in other 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 more recent studies indicate that breast and hepatic cancers are not EBV associated diseases Reviewed by (Herrmann and Niedobitek 2003) EBV latent gene expression and the form of latency identified in a number of EBV-associated diseases are detailed in table 1 0 below

TABLE 1.0

Type of Latency	Gene Product	Examples	Reference
I	EBERs, EBNA1	Burkitts Lymphoma, Gastric Carcinoma	(Rowe *et al.*, 1987) (Imai *et al.*, 1994)
II	EBERs, EBNA1,LMP1,2A, 2B, BARF0	Hodgkins Disease, Nasopharyngeal Carcinoma	(Deacon *et al.*, 1993) (Hitt *et al.*, 1989) (Brooks *et al.*, 1992)
III	All EBV Latent Genes	PTLD*, IM*	(Young *et al.*, 1989) (Tierney *et al.*, 1994)
Other	EBNA1, 2, LMP1	T cell Lymphomas	(Harabuchi *et al.*, 1990)
Other	EBERs, EBNA1,2	Smooth Muscle tumors	(Lee *et al.*, 1995)
Other	EBNA1, 2, LP, LMP1	OHL*	(Webster-Cyriaque *et al.*, 2000)

*PTLD- Post Transplant Lymphoproliferative Disorder, *IM- Infectious Mononucleosis, *OHL- Oral Hairy Leukoplakia.

1.8 EBV LATENT GENES

1.8.1 EBNA1

EBV Nuclear Anitgen 1 (EBNA1) is a multifunctional 73kDa phosphoprotein of 641 amino acid residues. The protein consists of a short amino terminal region, a, 20-40kDa glycine-alanine repetitive sequence flanked by arginine rich regions and a highly charged acidic carboxy terminal sequence (Hennessy and Kieff 1983). The EBNA1 protein plays a number of important roles during latent infection of human host cells during EBV infection. Firstly, EBNA1 activates replication of the episomal viral genome once every cellular S phase (Adams 1987; Yates and Guan 1991). Secondly, EBNA1 governs segregation of the viral episomes during cell division so that the EBV genome is stably maintained. Thirdly, EBNA1 trans-activates transcription of other EBV latent gene products. All three of these functions require a direct interaction with oriP, the origin of viral replication (See Figure 1.1 for position on the EBV genome). EBNA1 binds as a dimer to a specific sequence within the oriP, TGGATAGCATATGCTATCCA. OriP is comprised of two non-contiguous functional elements, a dyad symmetry element (DS) and a family of repeats (FR). The DS contains four DNA binding sites and is the likely

site for initiation of DNA synthesis The FR contains 20 EBNA1 binding sites and when bound by EBNA1 plays an important role in activating DNA replication from the DS, activating expression of other EBV latent genes and governing stable segregation of the EBV genome (Chittenden *et al* 1989, Sugden and Warren 1989, Rawlins *et al* 1985, Wysokenski and Yates 1989) The diagram below (Figure 1 4) Adapted from (Avolio-Hunter and Frappier 1998) shows the various functional domains of the EBNA1 protein

FIGURE 1.4. EBNA1 FUNCTIONAL DOMAINS

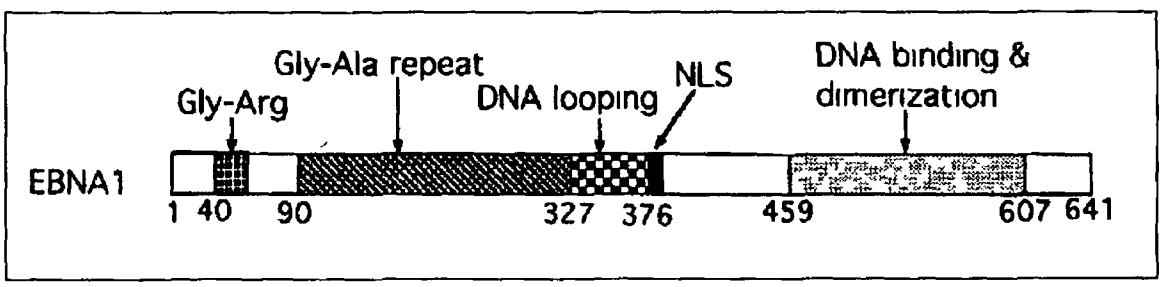


Figure1 4 Functional domains of EBV nuclear antigen1 (EBNA1). The gly-Ala box is a repetitive region composed entirely of glycine and alanine, it varies in length between viral strains Adapted from Avolio-Hunter and Frappier 1998

OriP was originally interpreted as being the replication origin of EBV but it is now thought that in the whole, EBV plasmid replication may initiate at other locations in the plasmid (Little and Schildkraut 1995) The main role of EBNA1/oriP may be to ensure stability of the replicated DNA and segregation into the daughter cells at division (Mackey and Sugden 1999) EBNA1 binding to oriP also activates a transcription enhancer activity in oriP that can activate expression of linked viral genes EBNA1 also activates expression of the lymphoid recombinase genes (RAGs), through an as yet unidentified mechanism (Srinivas and Sixbey 1995) Activation of the RAGs could promote chromosomal rearrangements and translocations and possibly also facilitate viral integration Expression of EBNA1 in EBV negative cell lines has no obvious effect on cellular growth characteristics However the expression of EBNA1 in the B cells of transgenic mice has been shown to be associated with the development of lymphocytic

lymphoma and leukaemia suggesting that EBNA1 predisposes the mouse lymphocytes to oncogenic change (Wilson and Levine 1992)

EBNA-1 can also act as a strong RNA binding protein and can bind RNA in vitro through arginine/glycine containing (so-called 'RGG') motifs These motifs have been found in an increasing number of proteins that interact with RNA The EBV antigen contains three potential 'RGG' motifs located around an internal glycine/alanine-rich repetitive sequence in the protein (Avolio-Hunter and Frappier 1998)

1.8 2 EBNA2

1.8.2.1 Identification and Characterization of EBNA2.

In vitro infection of B cells with EBV leads to the immortalization of these cells and the establishment of permanently growing lymphoblastoid cell lines (LCLs) Thirty-four years ago, a mutant form of EBV, the so-called P3HR1 virus was isolated, which had lost the ability to transform B cells in vitro (Hinuma *et al*, 1967, Miller *et al*, 1974) In this transformation-incompetent strain a deletion had been mapped which spans the open reading frame (ORF) of EBNA2 and also parts of EBNALP (Bornkamm *et al*, 1982, Dambaugh *et al*, 1984, Hennessy and Kieff 1985, Dillner *et al*, 1986) The immortalizing capacity of the virus was restored when the DNA fragments spanning the EBNA2 ORF were reintroduced (Hammerschmidt and Sugden 1989) EBNA2 has found to be essential not just for initiation of B cell immortalization but also for maintaining the transformation process (Kempkes *et al*, 1995a)

1.8.2.2 Structure of EBNA2

The EBNA2 gene encodes an 83kDa protein that localizes in large nuclear granules and is associated with nucleoplasmic chromatin and nuclear matrix fractions (Petti *et al* 1990) EBNA2 differs extensively between type1 and type 2 EBV isolates (Aitken *et al*, 1994) and is responsible for the biological difference that enables the type1 strains to transform B lymphocytes more efficiently (Rickinson *et al* 1987) The EBNA2 proteins identified in type1 and 2 EBV called EBNA2A and 2B respectively only share about 50%

sequence homology (Adldmger *et al*, 1985) Characteristic structures of the EBNA2 protein are (i) a negatively charged region at the amino-terminus, thought to play a role in homodimerization (Zimber-Strobl and Strobl 2001) (ii) a polyproline region consisting of 10-40 consecutive prolines depending on the virus strain, (iii) a diversity region in the middle of the protein where the homology between EBNA2A and 2B is very low, (iv) a domain responsible for the interaction with the DNA binding protein RBP-jk/CBF1, (v) an arginine-glycine rich stretch of around 18aa, (vi) a negatively charged region, which harbors a trans-activation domain, and (vii) a nuclear localization signal at the carboxyterminus (Zimber-Strobl and Strobl 2001)

FIGURE 1.5. STRUCTURE OF THE EBNA2 PROTEIN

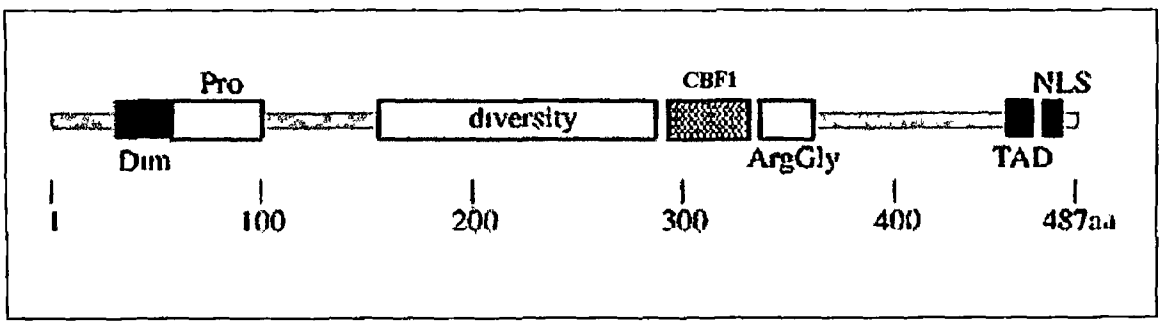


Figure 1 5 Structure of the EBNA2 protein of the B95 8 virus strain Characteristic parts of the EBNA2 protein are a negatively charged region at the amino-terminus, which likely plays a role in homodimerization (Dim), a polyproline stretch (Pro), a diversity region, where the homology between EBNA2A and EBNA2B is very low, a domain responsible for the interaction with CBF1/RBP-j (CBF1), an arginine–glycine rich stretch (ArgGly), a trans-activation domain (TAD) and a nuclear localization signal (NLS) at the carboxyterminus

Sequence comparison between EBNA2A, EBNA2B and the homologous protein from the closely related baboon Herpes virus Papio (HPV), revealed a series of nine conserved regions (CR) within EBNA2 (Ling *et al*, 1993b) Subsequent analyses identified regions within EBNA2, which were essential for its immortalization and transcriptional activities Regions essential for the transforming capacity of EBNA2 have been mapped by extensive mutational analysis (Cohen *et al*, 1991, Yalamanchili *et al*, 1994, Harada *et al*, 1998) At the amino-terminus, the region between 94-110, encompassing a

polyproline region (PPR) was found to be essential for B cell transformation. However, the role of this highly unusual PPR motif for immortalization remains enigmatic as recent studies (Gordadze *et al*, 2002) suggest that the EBNA2 Polyproline Region is dispensable for EBV-mediated immortalization maintenance, postulating that the loss of the polyprolme region increases LMP transcription which can be cytostatic for B cells when over expressed thereby explaining previous studies which showed loss of immortalization function of a PPR negative EBNA2 Two other regions absolutely necessary for B cell transformation were identified (i) between aa 280-337 in which CR5 and CR6 are located and (ii) in the region between amino acids 425-462 (See Figures 1 5 above and 1 7 below) Subsequent analyses revealed that the region between 425 and 462 contains the EBNA2 acidic trans-activation domain (TAD) (Cohen *et al*, 1991, Cohen, *et al*, 1991) and that the sequence between 280-337 contains sequences which allow EBNA2 bind to CBF1 and SKIP, members of a repression complex which when bound by EBNA2 free up EBNA2 responsive promoters to trans-activation by the EBNA2 TAD (Ling *et al*, 1993a) Also present in this region is part of the arginine glycine region EBNA2 binds to the survival motor neuron protein (SMN) via the methylated arginine-glycine region and this may be a critical step in B cell transformation (Barth *et al*, 2003)

1.8 2.3 FUNCTION OF EBNA2

EBNA2 is essential for the immortalization of primary B-lymphocytes and along with EBNALP is the first protein expressed during this process EBNA2 is also essential for the maintenance of the transformed state Using an LCL conditional for functional EBNA2 expression in the presence of estrogen, it was shown that cells deprived of functional EBNA2 entered a quiescent non-proliferative state or die by apoptosis (Kempkes *et al*, 1995a)

The other vital function of EBNA2 is as a specific trans-activator of latent viral genes, and a number of cellular genes EBNA2 activates the transcription of all other viral proteins expressed in LCLs by trans-activating (i) the BamHI-C promoter Cp (Woisetschlaeger *et al*, 1990, Sung *et al*, 1991), from which transcription of all EBNA genes is controlled (See Figure 1 3 above), and (ii) the promoters of the latent membrane

proteins LMP1 and LMP2 (Abbot *et al*, 1990, Fahraeus *et al*, 1990, Wang *et al*, 1990, Zimber-Strobl *et al*, 1991, Laux *et al*, 1994) EBNA2 also activates the transcription of cellular genes including CD21, the B lymphocyte differentiation marker (Cordier *et al*, 1990), CD23, the B cell activation marker (Wang *et al*, 1987, Wang *et al*, 1990, Wang, *et al*, 1991), the oncogene, c-fgr (Knutson, J C 1990, Patel *et al*, 1990), BATF which induces expression of a B cell specific transcription factor of the same name (Johansen *et al*, 2003) as well as the chemokine receptor BLR2/EBII (Burgstahler *et al*, 1995] EBNA2 also acts as a transcription repressor and represses transcription of the immunoglobulin heavy chain locus (Jochner *et al*, 1996) resulting in the down-regulation of the proto-oncogene c-myc (Kaiser *et al*, 1999)

EBNA2 has also been shown to play an important role in the maintenance of proliferation of LCLs and this effect is mediated by its ability to regulate the expression of the cell cycle related proteins, cyclin D2, and cdk4 (which drive cell cycle progression from G0 to G1) (Kempkes *et al*, 1995c, Zimber-Strobl *et al*, 1996)

1.8.2.4 EBNA2, CBF1 AND THE CBF1 REPRESSION COMPLEX

EBNA2 does not bind to DNA directly but is tethered to responsive promoters by a ubiquitously expressed cellular DNA binding protein CBF1 (also called RBP-jk, KBF2, RBP-j, or CSL) (Henkel *et al*, 1994, Grossman *et* al, 1994, Waltzer *et al*, 1994, Zimber-Strobl *et al*, 1994) The cognate DNA sequence element to which CBF1 binds is 5'-CGTGGGAA-3' and this sequence was first described in the EBNA2 responsive element (ERE) of the LMP2A promoter (Zimber-Strobl *et al*, 1993) Binding sites for CBF1 have subsequently been identified in other known EREs of promoters activated by EBNA2 including the Cp, LMP1 and CD23 promoters (Ling *et al*, 1993, Laux *et al*, 1994, Ling *et al*, 1994)

CBF1 was initially cloned by T Honjos group (Hamaguchi *et al*, 1989, Matsunami, *et al*, 1989) CBF1 is a transcriptional repressor and repression is effected in part by tethering a histone deacetylase (HDAC) co repressor complex to the promoter (Henkel *et al*, 1994, Hsieh and Hayward 1995, Waltzer *et al*, 1995, Kao *et al*, 1998) The co repressor proteins SKIP, Sin3A, SMRT, CIR, SAP30, HDAC1 and HDAC2 have been

shown to be components of this complex (Zhou *et al.*, 2000a; 2000b; Hsieh *et al.*, 1999; Kao *et al.*, 1998). Repression occurs as a result of histone deacetylation, which leads to chromatin remodeling and a loss of transcription factor access to the nucleosome-associated promoter sequences. It has also been demonstrated that CBF1 binds to elements of the basal transcription machinery, disturbing the TFIIA-TFIID interaction, which is essential to transcription initiation (Olave *et al.*, 1998).

EBNA2 trans-activates promoters by binding to the repression domain of CBF1 to relieve repression and bringing a transcriptional activation domain to the promoter (Hsieh and Hayward 1995). Shown in Figure 1.6 below is a model for EBNA2 activation of CBF1-repressed promoters. The various members of the CBF1 repression complex are also shown.

FIGURE 1.6. MODEL FOR EBNA2 ACTIVATION OF CBF1 REPRESSED PROMOTERS

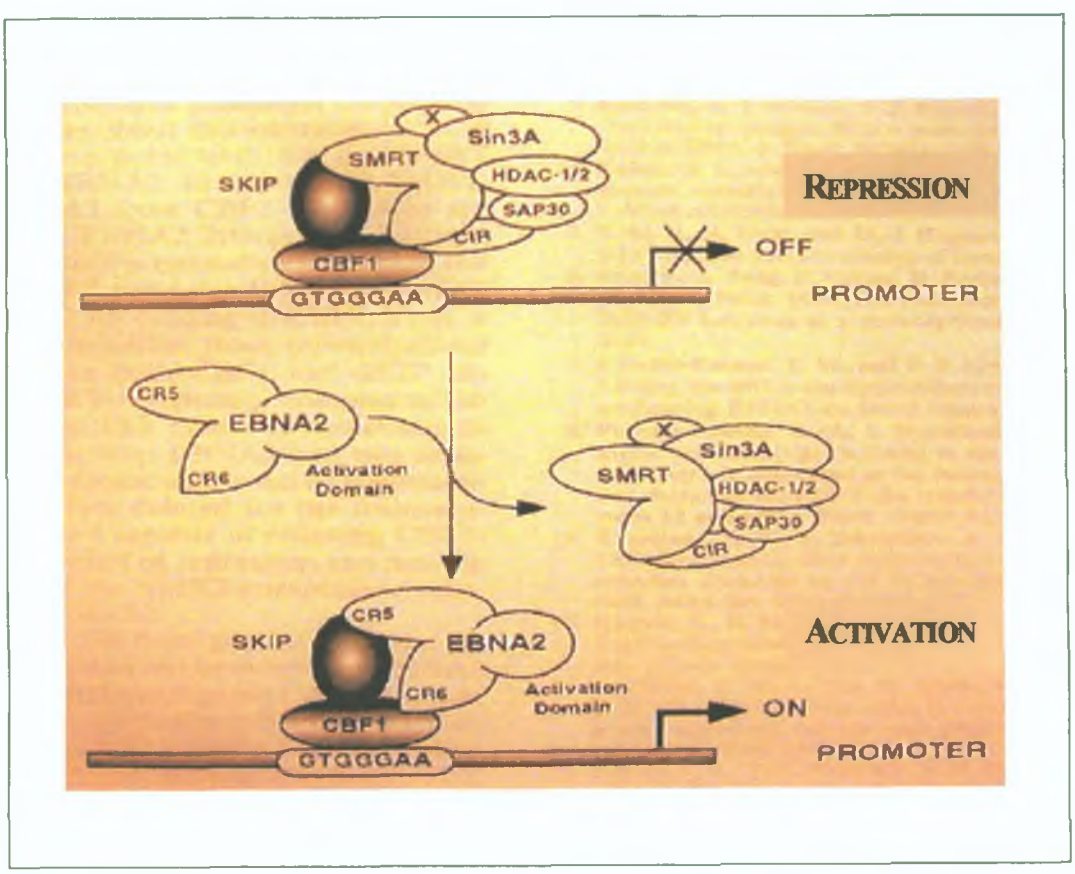


Figure 1 6 Model for EBNA2 activation of CBF1-repressed promoters CBF1 binds to the DNA sequence GTGGGAA in responsive promoters SKIP is bound to CBF1 SMRT contacts both SKIP and CBF1 SMRT is a component of a co repressor complex that includes Sin3A, SAP30, CIR, HDAC1, and HDAC2 and potentially other Sin3-associated proteins (indicated by x) This complex mediates promoter repression through chromatin remodelling EBNA2 competes with SMRT for contacts on both SKIP and CBF1 Displacement of the SMRT-co repressor complex relieves repression, and introduction of the EBNA2 activation domain induces transcriptional activation. EBNA2 mutants in CR5 and CR6 lose the ability to interact with SKIP and CBF1, respectively Loss of either interaction impairs the ability of EBNA2 to activate CBF1-repressed promoters

1.8 2.5 Co-Activating Proteins Interact with EBNA2.

The acidic domain of EBNA2 interacts with factors of the basal transcription machinery including TFIIH, TATA, box binding protein (TBP), associated binding factor TAF40, TFIIB, and the co-activator p100 that interacts with TFIIE (Tong *et al*, 1995a 1995b 1995c) The trans-activation domain also interacts with the histone acetyltransferases (HATs) p300/CBP and PCAF (Wang *et al*, 2000) and with the viral protein EBNALP that acts as a co-activator of EBNA2 (Nitsche *et al*, 1997, Harada *et al*, 1997) Apart from the activation domain, EBNA2 interacts with the chromatin remodeling complex hSW1-SNF, which converts the chromatin structure to facilitate transcription (Wu *et al*, 1996, 2000) Another protein DP103, the dead box protein, which is complexed to the survival neuron protein SMN, also binds to EBNA2 and these can cooperatively trans-activate the LMP1 promoter (Voss *et al*, 2001)

The portions of EBNA2 which are important for facilitating contact between EBNA2 and the CBF1 repression complex have been identified in the CR5 and CR6 region (See Figure 1 7 below) between amino acids 280-337 Interactions with both CBF1 and SKIP have been shown to be required for EBNA2-mediated trans-activation for CBF1 repressed promoters (Zhou *et al*, 2000a) CR6 in the EBNA2 protein proved to be the CBF1 targeting domain Mutation of two tryptophan residues in this region abolished CBF1 interaction (Ling *et al*, 1995, Ling *et al*, 1993) This mutation, (creating a mutant EBNA2 named WW323SR) also abolished the activity of EBNA2 to activate reporters carrying CBF1 binding sites (Hsieh and Hayward 1995, Ling *et al*, 1993) and when

transferred into the open reading frame for the EBV genome resulted in a non-immortalizing strain of the virus (Yalamanchili *et al.*, 1994). Mutation of the CR5 region resulted in an EBNA2 that retained CBF1 interaction but had a diminished ability to activate a Cp promoter driven reporter (Ling *et al.*, 1995). In the context of the EBV genome, deletion of the CR5 region results in a non-immortalizing EBV mutant (Harada *et al.*, 1998). The deletion in CR5 prevented the binding of SKIP, a member of the CBF1 repression complex.

FIGURE 1.7. SCHEMATIC REPRESENTATION OF EBNA2 AND THE POSITIONS AT WHICH IT BINDS TO CBF1 AND SKIP

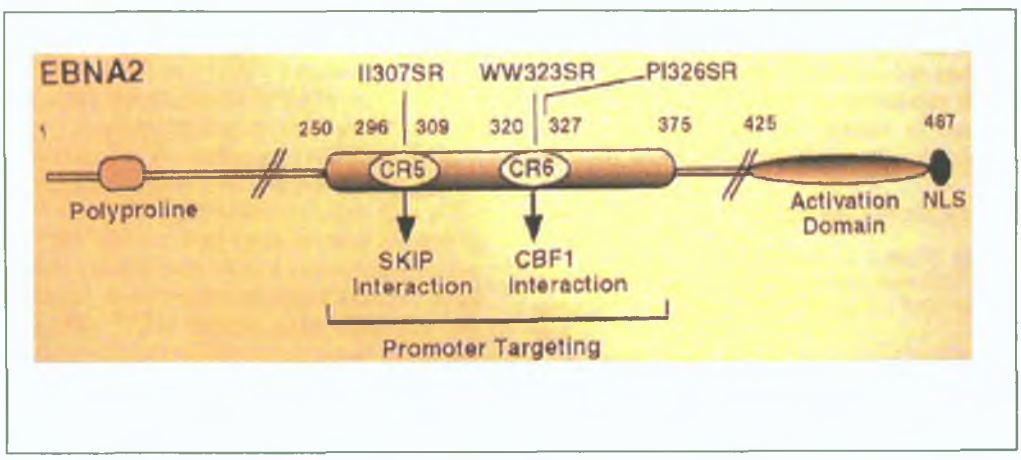


Figure 1.7 Schematic representation of EBNA2 illustrating the relative locations of characterized functional domains and of the positioning of the WW323SR mutation used in this study. The amino acid numbers are indicated. CR5, CR6 and a nuclear localization signal (NLS) are also indicated.

The behavior of the CR5 and CR6 EBNA2 mutants suggests that contacts on both CBF1 and SKIP are required for EBNA2 mediated trans-activation for CBF1 repressed promoters (Zhou *et al.*, 2000a). Figure 1.7 above shows a schematic illustration of EBNA2 and its functional domains as well as the location of the various mutations which identified the essential domains for relieving CBF1 mediated promoter repression. In the studies described in this thesis, the mutant WW323SR EBNA2 was employed as a tool for determining the region of EBNA2 required to trans-activate the promoter of a cellular apoptosis related gene *bfl-1*.

1 8.2.6 EBNA2 Responsive Promoter Elements

EBNA2 regulates expression of the viral Cp latency promoter that drives expression of the EBNA family of nuclear proteins in type III latency and also contributes to the positive regulation of the LMP2A, LMP2B and LMP1 promoters (Jin and Speck 1992, Sung *et al*, 1991, Tsang *et al*, 1991, Zimber-Strobl *et al*, 1993) As well as the cellular CD23, CD21 and cyclin D2 promoters The EBNA2 responsive elements for the Cp, LMP1, LMP2 CD21 and CD23 promoters have all been characterized (Woisetschlaeger *et al*, 1990, Sung *et al*, 1991, Abbot *et al*, 1990, Fahraeus *et al*, 1990, Wang *et al*, 1990b) as orientation independent sequences, which contain at least one CBF1 binding site (Zimber-Strobl and Strobl 2001) Although the EBNA2-CBF1 interaction is important for regulation of these target genes, optimal activation by EBNA2 also requires cooperation with other transcription factors (Meitinger *et al*, 1994)

In the LMP1 promoter, multiple transcription factor binding sites have been identified One transcription factor, PU 1/Spi-B, which also interacts directly with EBNA2, has been found to be essential in mediating EBNA2 activation of LMP1 (Johannsen *et al*, 1995, Laux *et al* 1994) PU 1, a DNA binding protein which is a member of the ets oncogene family, recognizes a purine rich sequence 5'-GAGGAA-3' (known as a PU Box) The PU 1 protein was shown to be a tissue specific transcriptional activator that is expressed in macrophages and B cells (Klemsz *et al*, 1990) PU 1 protein complexes are required for Normal B Cell Receptor–Mediated Signal Transduction (Garrett-Sinha *et al*, 1999) and regulation of its expression is essential for mature B cell survival (Hu *et al*, 2001) It has also been shown that a POU domain protein is involved in the EBNA2 mediated trans-activation of LMP1 (Sjoblom *et al*, 1995)

In the Cp promoter, the EBNA2 responsive element also contains a CBF2 binding site adjacent to the CBF1 binding site and this also contributes to EBNA2 responsiveness (Fuentes-Panana and Ling 1998) The cellular proteins that comprise CBF2, include the hnRNP protein AUF1 (Fuentes-Panana *et al*, 2000) In the LMP2A promoter, beside

CBF1, two other as yet unidentified proteins bind to the EBNA2 responsive element to contribute to EBNA2 responsiveness (Hofelmayr *et al*, 1999)

Although the regulation of many of the EBNA2 target genes such as those listed above, and also including CD23, interleukin 1 and beta-interferon have been shown to be mediated by CBF1 (Kanda *et al*, 1999, Krauer *et al*, 1998, Ling *et al*, 1994, Wang *et al*, 1991), there are many other responsive genes such as the cyclin D2 which lack CBF1 binding sites in their promoter regions (Spender *et al*, 2001) These genes may be activated either directly or indirectly as part of a downstream response cascade (Kaiser *et al*, 1999)

1 8.2.7 Viral and Cellular Proteins Counterregulate Trans-activation Through CBF1.

EBNA2 mediated trans-activation through CBF1 can be disrupted by a number of viral and cellular genes The viral EBNA3A, and 3C proteins repress EBNA2 mediated trans-activation by bindmg directly and exclusively to CBF1 thereby preventing CBF1 binding to DNA (Le Roux *et al*, 1994, Johannsen *et al*, 1996, Robertson *et al*, 1995, Waltzer *et al*, 1996, Zhao *et al*, 1996) Furthermore EBNA3C can form complexes with a member of the CBF1 repression complex, namely HDAC1 HDAC1 and CBF1/RBP-Jkappa bind to the same or adjacent regions of EBNA3C (Radkov *et al*, 1999) (See Figure 1 9) One of the Epstein-Barr Virus *Bam*HI-A Rightward Transcripts- (BARTs) contains an open reading frame designated RPMS1 This encodes a nuclear protein RPMS (Smith *et al*, 2000) RPMS interacts with CBF1 and the CBF1-associated co repressor CIR to stabilize the CBF1 co repressor complex, thereby negatively regulating the trans-activational effect of EBNA2 (Zhang *et al*, 2001) (See Figure 1 14 adapted from Zhang *et al*, 2001) A cellular LIM protein KyoT2 has also been identified which dislocates CBF1 from DNA by competing with EBNA2 for binding to CBF1 (Taniguchi *et al*, 1998)

1.8.2.8 CBF1 LINKS EBNA2 AND THE CELLULAR NOTCH PATHWAY.

CBF1 is a ubiquitously expressed cellular DNA binding protein, which is well conserved throughout evolution In Drosophila this protein is known as Suppressor of Hairless Su (H) Notch receptors are a family of cell surface receptor proteins which are activated after ligand binding, by a series of cleavage events which lead to translocation of the intracellular domain of the receptor (N^{IC}) to target cell nuclei (Schroeter *et al*, 1998) After translocation to the nucleus, N^{IC} activates genes by binding to CBF1 (Struhl and Adachi 1998, Lecourtois and Schweisguth 1995) Thus CBF1 is a downstream target of the Notch signaling pathway Since both EBNA2 and Notch associate 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)

Studies have shown that the Notch signaling pathway operates to regulate cell differentiation in a variety of vertebrate developmental and cell maturation processes including, muscle cell differentiation, pancreatic cell differentiation, granulocyte differentiation, early embryogenesis, neural development and lineage commitment of lymphocytes (Swiatek *et al*, 1994, Hamada *et al*, 1999, Nye *et al*, 1994, Kopan *et al*, 1994, Apelqvist *et al*, 1999, Milner *et al*, 1996, Schroeder and Just 2000, Pui *et al*, 1999, Radtke *et al*, 1999) Recent studies indicate that in addition to cell differentiation, Notch signaling has direct effects on proliferation and programmed cell death (Artavanis-Tsakonas *et al*, 1999), reviewed by (Miele and Osbourne 1999)

1.8.2 9 STRUCTURE OF NOTCH RECEPTORS

In mammals four Notch genes have been identified (Notch1-4) these all encode receptors consisting of a single trans-membrane domain (TM) The amino-terminus contains a signal peptide followed by multiple epidermal growth factor-like repeats (EGF), which functions as a ligand binding domain, and a cysteine-rich Lin12/Notch repeats (L/N) These repeats are highly conserved in mammalian Notch receptors, particularly Notch-1

and -2, and represent the putative main ligand-binding site The intracellular part contains the RAM domain a high-affinity-binding site for transcription factors of the Suppressor of Hairless/CBF-1 group (see Figure 1 8 below, Tamura *et al*, 1995, Miele and Osbourne 1999) and ankyrin repeats which are also important for CBF1 interaction Also in the intracellular region is located the NLS a nuclear localization domain, and a C terminal PEST region which regulates protein stability A possible trans-activation domain has also been identified at the carboxy-terminus (Kurooka *et al*, 1998, Dumont *et al*, 2000) (Notch structure is reviewed by Weinmaster 1997 and Miele and Osbourne 1999)

FIGURE 1.8 STRUCTURE OF THE NOTCH RECEPTOR

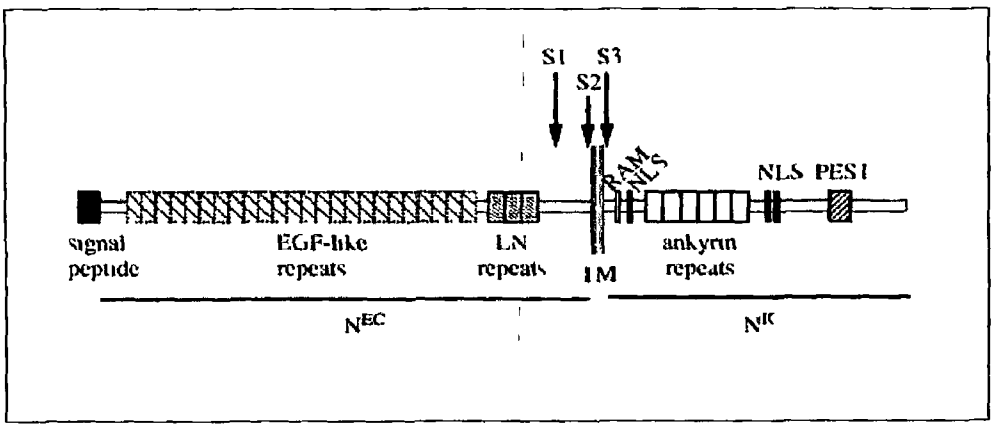


Figure 1 8 Structure of the Notch proteins The amino-terminal part contains a signal peptide followed by multiple epidermal growth factor (EGF)-like repeats, that function as ligand-binding domain, and cysteine rich Lin12/Notch repeats (LNR) The intracellular part (N IC) harbors the RAM domain and a series of ankyrin repeats, which both interact with RBP-J, 3 nuclear localization signals (NLS), and a C-terminal PEST region The arrows (S1 to S3) indicate the sites of proteolytic cleavage during protein maturation and after ligand binding

1.82.10 ACTIVATION OF NOTCH RECEPTORS

Notch receptors are activated by binding membrane-bound ligands Numerous Notch ligands have been identified in mammals and these are homologous to the Drosophila Su(H) ligands Delta and Serrate and the *C Elegans* ligand Lag-1, ollectively referred to as DSL from the initials of Delta, Serrate, and Lag-1 (Weinmaster, 1997) Mammalian Delta homologues are denominated "Delta-like" and mammalian Serrate homologues are

denominated "Jagged" (Miele and Osbourne 1999) Notch proteins are produced as single 300kDa polypeptide precursors that are processed to a mature heterodimeric form via a series of post ligand binding proteolytic cleavage events

Notch receptors have three sites termed S1 to S3 at which cleavage occurs A furin-like convertase (Logeat *et al*, 1998) catalyzes the cleavage of the Notch pre-protein at S1 generating an ammo terminal and a carboxy terminal fragment The amino terminal subunit contains the extracellular element (N^{EC}), which consists of multiple epidermal growth factor (EGF)-like repeats The carboxy terminal subunit contains a short extracellular region, the trans-membrane domain (TM) and the intracellular region (N^{IC}) (Blaumueller *et al*,1997)

Two further ligand-binding induced cleavages occur at S2 and S3 TACE, tumour necrosis factor α-converting enzyme catalyses the cleavage at S2 which removes most of the extracellular part of the carboxyterminal subunit (Mumm *et al*, 2000, Brou *et al*, 2000) Removal of this region then induces cleavage at S3 which releases the activated Notch receptor N^{IC} This final cleavage at S3 is catalyzed by a γ-secretase-like protease whose activity requires presenilin (De Strooper *et al*, 1999, Wolfe *et al*, 1999) The intracellular domain is then translocated to the nucleus where it binds to CBF1 and regulates target gene expression (Struhl and Adachi 1998, Jarriault *et al*, 1995)

1.8.2.11 Notch1-IC Also Modulates Gene Expression by Association with the CBF1 Co-repressor Complex.

Like EBNA2, Notch-IC binds to the repression domain of CBF1 , disturbing it from its position on DNA as a repression complex As described above, one particular region containing sequential tryptophan residues in CR6 is required to mediate EBNA2 interaction with CBF1 (Yalamanchili *et al*, 1994) Two domains within Notch-IC mediate its interaction with CBF1 these are the RAM domain and the ankyrin repeats (Tamura *et al*, 1995, Aster *et al*, 1997) EBNA2 binds to SKIP as well as CBF1 in order to relieve repression and bring its activation domain into contact with target promoters, Notch-IC also interacts with SKIP but via its ankyrin repeats (Zhou *et al*, 2000b)

Different amino acids within the CBF1 protein may be critical for the binding of either EBNA2 or Notch-IC (Fuchs *et al*, 2001)

1.8.2.12 NOTCH-IC CO-ACTIVATING PROTEINS

As with EBNA2, Notch-IC mediated transactivation via CBF1 is also enhanced by a number of co-activating proteins The Notch-IC transactivation domain and ankyrin repeats also interact with two co-activating histone acetyltransferases (HATs), PCAF and GCN5 (Kurooka and Honjo 2000) Another protein MAML1, which interacts with Notch-IC and CBF1 to enhance the Notch-IC transactivational effect has also been described (Wu, L *et al*, 2000)

1.8.2.13 EBNA2 AND NOTCH-IC OVERLAP IN THEIR FUNCTIONS AND IN THEIR TARGET GENES.

Since both EBNA2 and Notch-IC proteins share a common mechanism for regulating target gene expression it is then unsurprising that they also overlap in the range of functional activities and in the genes that they regulate

EBNA2 is essential for the immortalization of EBV infected B cells, as already outlined above (section 1 8 2 1) Notch-IC, also has the ability to immortalize cells Constitutive activation of any of the four Notch receptors can lead to neoplastic transformation in a number of different cell types Expression of the human homologue of Notch1, TAN1 can be oncogenic for T cells (Ellisen *et al*, 1991) Constitutive activation of the Notch2 gene is implicated in thymic lymphomas in cats (Rohn *et al*, 1996) Similarly transgenic mice expressing constitutively activated Notch3 developed T cell leukemias (Bellavia *et al*, 2000) while expression of high levels of Notch 4 are associated with mammary adenocarcinomas in mice (Jhappan *et al*, 1992) The exact mechanisms by which the different activated Notch receptors exert their transforming function is not fully understood however, two oncogenes c-myc and E1A have been shown to cooperate with activated notch receptors in neoplastic transformation (Girard *et al*, 1996, Capobianco *et*

al, 1997) The ankyrin repeats have been identified as essential in conferring the transforming capacity to Notch in E1A immortalized kidney cells while the other CBF1 interaction domain RAM, as well as the Notch trans-activation domain were not required (Jeffries *et al*, 2000) In T cell leukaemiogenesis, however, the ankyrin repeat and trans-activation domains were essential, while the PEST and RAM domains were not crucial (Aster *et al*, 2000)

Both EBNA2 and Notch also act as trans-activators of viral and cellular genes Transient transfection assays showed Notch1-IC could also regulate EBNA2 target genes in B cells Transient transfection assays involving promoter reporter constructs for various viral promoters, showed that Notch1-IC trans-activated the Cp, LMP1 and LMP2A promoters albeit to a lesser extent than EBNA2 Transfections involving stably expressed Notch1-IC resulted in the up-regulation of LMP2A but not LMP1 Studies by the same group also showed that Notch1-IC can induce CD23 but not CD21 expression (Hofelmayr *et al*, 1999)

Another EBNA2 target gene, IgH, the only gene known so far to be down-regulated by EBNA2, has also been identified as a gene which is down-regulated by Notch1-IC in human and chicken B cells (Strobl *et al*, 2000, Morimura *et al*, 2000) Although Notch1-IC can regulate certain EBNA2 responsive genes it cannot replace EBNA2 in maintaining B cell immortalization as unlike EBNA2, it cannot up-regulate LMP1 expression However in an LCL where LMP1 was expressed in an EBNA2-independent manner, Notch1-IC along with LMP1 was able to maintain the B cells in an immortalized state, thus Notch1-IC can transiently substitute for EBNA2 in the maintenance of proliferation in LMP1 expressing immortalized B cells (Hofelmayr *et al*, 2001) Very recent studies have also showed that both EBNA2 and NotchIC activate a cellular gene named *BATF*, which induces expression of a B cell specific transcription factor of the same name [Johansen, 2003] BATF is a member of the AP-1/basic leucine zipper family of transcription factors, which form heterodimers with the Jun proteins to bind preferentially to AP-1 consensus sites BATF may have a role in negatively regulating AP-1 activity since the BATF-Jun heterodimers display a reduced activity relative to Fos-Jun heterodimers and thus inhibit the expression of AP-1 target genes and retard AP-1

mediated growth Also both EBNA2 and Notch-IC have been shown to protect cells by binding to the cellular Nur77 (TR3/NGF1B), transcription factor that is a member of the nuclear hormone receptor super family (Jehn *et al*, 1999, Lee *et al*, 2002)

1.8.3 EBNA3A, 3B AND 3C.

The EBNA3 family consists of 3 nuclear proteins, EBNA-3A, -3B and -3C encoded by tandemly arranged genes located in the middle of the linear viral genome They are encoded by alternatively spliced transcripts initiated at the Cp (See Figure1 3B) and are each composed of a small 5'-and a large 3'-exon (Ricksten *et al*, 1988a, Bornkamm and Hammerschmidt 2001) The proteins themselves are composed of 944, 937 and 992 amino-acid residues, respectively and are located in large nuclear clumps in the nuclear matrix, chromatin and nucleoplasmid fractions (Petti *et al*, 1990) The mRNAs encoding these proteins are the least abundant EBNA mRNAs occurring in latently infected cells (Kallin *et al*, 1986)

Although the EBNA-3A, -3B and -3C proteins differ in their amino acid sequences in type 1 and type 2 strains of EBV (Sample *et al*, 1990b) Studies have shown both EBNA-3A and -3C are required for immortalization whereas EBNA-3B is not (Tomkinson *et al*, 1993) EBNA3A is essential for the initiation but not for the maintenance of B cell transformation (Kempkes *et al*, 1995c) Since EBNA2 is the transcription factor that activates the expression of EBNA3A, it could well be that EBNA3A induced by EBNA2 is the key protein for the initiation of immortalisation of B cells (Manet *et al*, 1998)

EBNA-3B is one of the dominant primary targets for recognition of immortalized cells by cytotoxic T cells (Rickinson and Moss 1997) and as such represents a potential target for CTL treatment of EBV associated malignancies Evidence for this is available from studies in patients with post-transplant lymphoproliferative disease (PTLD) treated with EBV-specific T cells Treatment with EBV-specific T cells failed to arrest proliferation,

as the CTLs could not recognize an EBNA3B deleted virus strain (Gottschalk *et al*, 2001)

EBNA3B has also been shown to modulate the expression of the cellular CD40, CD77 and vimentin genes (Silins and Sculley 1994)

Similarly to EBNA2, EBNA3C may be regarded as a trans-activator of viral and cellular genes, transfection assays demonstrate that expression of EBNA3C up-regulates CD21 in an EBV-negative BL cell line while expression of EBNA3C in Raji cells (where the EBN3C ORF is deleted) up-regulates the levels of LMP1, CD21 and vimentin proteins (Wang *et al*, 1990a, Allday *et al*, 1993, Ring 1994) EBNA3C can also trans-activate genes via a CBF1-independent mechanism, studies by (Zhao *et al*, 2000) showed that EBNA3C could cooperate with EBNA2 to trans-activate the LMP1 promoter through an Spi-1/(Pu 1)/SpiB binding site in the promoter Binding of the two viral proteins together forms a stable complex, which allows trans-activation of LMP1
EBNA-3C has furthermore been described to counter the action of cyclin-dependent kinase inhibitor p16/INK4A, to functionally inactivate Rb in a similar way to the viral proteins human papillomavirus E7 and adenovirus E1A, and to override a number of cell-cycle checkpoints (Parker *et al*, 1996, Parker *et al*, 2000, Wade and Allday 2000)

The most well documented function of the EBNA-3A, -3B and -3C proteins is that they inhibit transcriptional activation of EBNA-2-responsive promoters including the LMP2A, LMP1 and Cp promoters EBNA3A, 3B and 3C bind to CBF1, (Le Roux *et al*, 1994, Robertson *et al*, 1995, Waltzer *et al*, 1995) thereby preventing CBF1 and CBF1-EBNA-2 complexes from binding to their cognate CBF1-binding sites (Bain *et al*, 1996, Johannsen *et al*, 1996, Radkov *et al*, 1997, Radkov *et al*, 1999), Waltzer *et al*, 1995, Zhao *et al*, 1996) Binding of EBNA3A to CBF1 results in down-regulation of c-myc and EBV transformed lymphoblast growth (Cooper *et al*, 2003) A model showing EBNA3A/3B and 3C repression of EBNA2-CBF1 mediated trans-activation is shown below (adapted from Zhao *et al*, 2000)

Figure 1.9. Model for EBNA3A/3B/3C Repression of EBNA2-CBF1 Mediated Trans-activation.

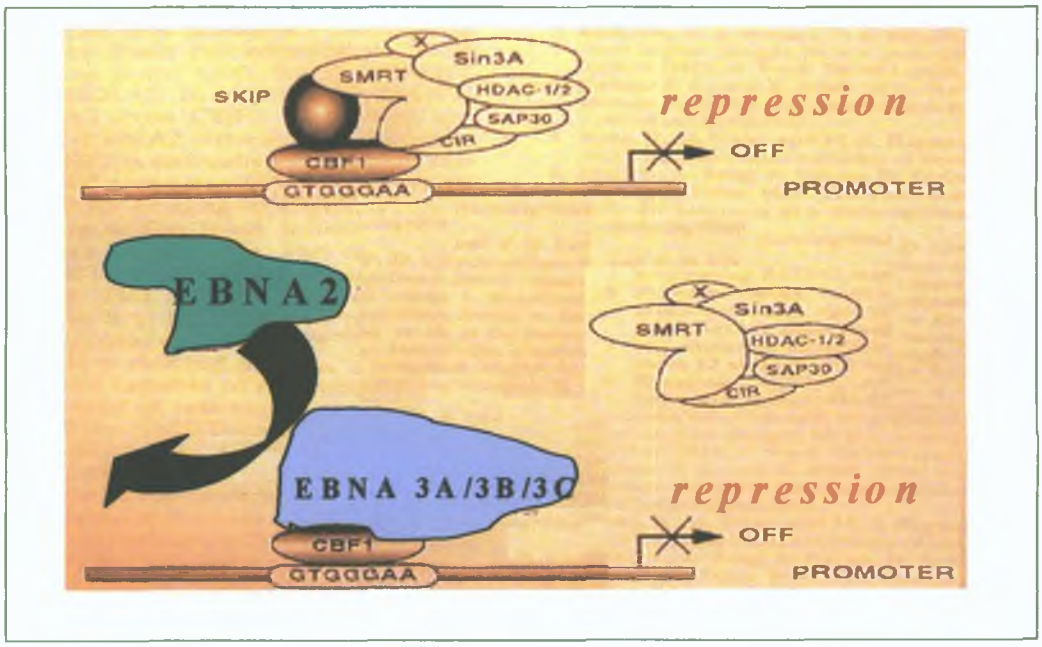


Figure 1.10. A model for EBNA3 mediated repression of EBNA2-CBF1 associated trans-activation. All three EBNA3s bind directly to CBF1 thereby preventing EBNA2 associating with CBF1 and the other members of the repression complex. Thus preventing EBNA2 mediated trans-activation of target promoters

The EBNA3 proteins are thus understood to counterbalance and fine-tune the action of EBNA2. By inhibiting CBF1-EBNA2 mediated trans-activation of the Cp promoter, the EBNA3 proteins also inhibit their own synthesis thus setting up an auto regulatory loop controlling their own expression (Waltzer et *al.*, 1995; Robertson *et al.*, 1995).

1.8.4 EBNA-LP

EBNA-LP (also referred to as EBNA5) is encoded by the 5' leader sequence of bicistronic mRNAs, which encode the other EBNAs (See Figure 1.3B). The ATG translation initiation codon for LP is created by a splicing event that occurs near the 5' end of the message (Wang *et al.*, 1987). EBNA-LP consists of a multirepeat domain (W1W2) and a unique carboxyl-terminal domain (Y1Y2) (Fig.1.10) and is therefore

detected as a protein ladder in immunoblot analyses, possibly as the result of heterologous polypeptides with different numbers of W1/W2 repeats

Figure 1.10. EBNA2 and EBNA-LP are Generated by Alternate Splicing

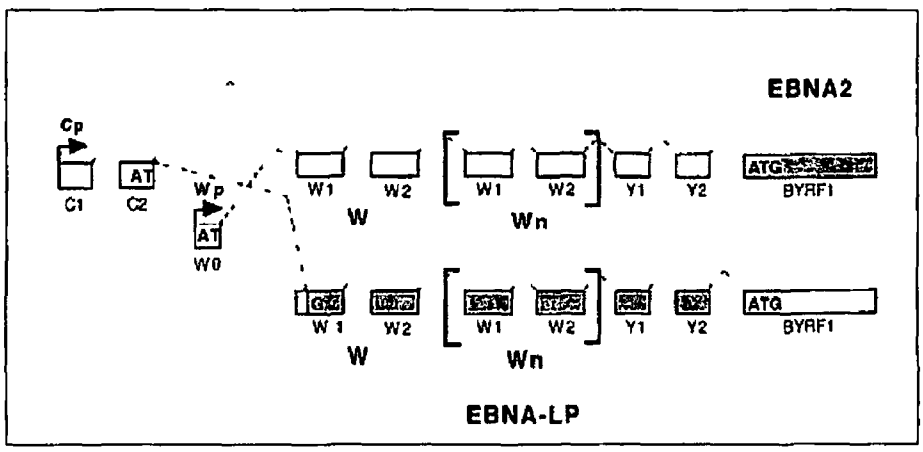


Figure 1.10 Detailed representation of how EBNA2 and EBNA-LP mRNAs are generated from a unique RNA precursor by facultative splicing. Boxes represent exon sequences and broken lines represent intron sequences The exons coding for EBNA2 or EBNALP respectively, are indicated by the grey boxes The EBNA-LP 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 (adapted from Manet *et al*, 1998)

In freshly infected cells, EBNA-LP and EBNA-2 appear as the first detectable virus-encoded proteins (Alfieri *et al*, 1991) EBNA-LP localizes to the nucleus in distinct foci now recognized as nuclear domain 10 (ND10) bodies or promyelocytic leukemia-associated protein (PML) oncogenic domains (PODs) (Jiang *et al*, 1991, Petti *et al*, 1990) Genetic studies, using recombinant EBNA-LP mutants lacking the Y1 and Y2 exons, or mutants which had a stop codon inserted after the first amino acid in Y1, showed reduced, but still detectable, transforming ability. Thus EBNA2 is not essential to but greatly enhances the process of transformation (Hammerschmidt and Sugden1989, Mannick *et al*, 1991) Although the actual role of EBNA-LP in EBV-induced B-cell immortalization remains unclear, other studies suggest two major biological functions of EBNA-LP Firstly as a co-activator of EBNA2 and secondly as a protein which associates with several cellular proteins and structures

EBNA-LP is primarily known as a co activator of EBNA-2 It has been reported that EBNA-LP and EBNA-2 cooperatively stimulate the gene expression of cellular and viral proteins such as cyclin D2 (Sinclair *et al*, 1994) LMP-1 and LMP2B (Hammerschmidt and Sugden 1989, Nitsche *et al*, 1997, Harada *et al*, 1997) The mechanism by which EBNA-LP functions as a co-activator to EBNA2 is unknown, however, conserved regions within EBNA-LP required for co-operation with EBNA2 are also important for nuclear localization and/or nuclear matrix local-ization (Peng *et al*, 2000b, Yokoyama *et al*, 2001)

Apart from enhancing EBNA2 trans-activational effects, EBNA-LP also interacts with various cellular proteins and structures EBNA-LP was shown to bind to p53 and pRb in vitro binding assays (Szekely *et al*, 1993) and to be co-localized with an antigenically distinct form of pRb in a nuclear domain named ND10 (Jiang *et al*, 1991, Szekely *et al*, 1996) Although EBNA-LP binds to p53, results from transient transfections demonstrated that neither p53, pRb nor E2F transcription was up-regulated thus the in vivo relevance of EBNA-LP-p53/pRB interaction is unknown (Inman and Farrell 1995) EBNA-LP also associates with the 70-kDa family of heat shock proteins (hsp70s) in vivo, which co localize in ND10, and trans-located to the nucleolus under conditions of cellular stress (Kitay *et al*, 1996b, Mannick *et al*, 1995 and Szekely *et al*, 1995)
EBNA-LP is also localized in the cytoplasm as well as the nucleus of EBV-infected cells and interacts with a cellular cytoplasmic protein, HAX-1 (Kawaguchi *et al*, 2000) which is involved in B-cell signal transduction and apoptosis Although the biological significance of the interaction between EBNA-LP and the cellular proteins is unclear at present, these results suggest that EBNA-LP is not only a co-activator of EBNA-2 but also a multifunctional protein that modulates various components of cellular machinery and that the functions of EBNA-LP in EBV-induced B-cell immortalisation result from the sum of these interactions with viral and cellular proteins

Like the other EBV regulatory proteins, EBNA-LP is phosphorylated in EBV-infected cells (Petti *et al*, 1990, Sauter *et al*, 1988) The pattern of EBNA-LP phosphorylation is dependent on the cell cycle stage in that EBNA-LP is hyperphosphorylated in G2 /M

phase and hypophosphorylated in G1 /S phase (Kitay *et al*, 1996a) Studies by the same group revealed that EBNA-LP is phosphorylated at multiple sites, and p34 *cdc2* and casein kinase II mediates the phosphorylation of EBNA-LP in vitro

A serine residue at position 35 in the W2 repeat has been identified as the major phosphorylation site for EBNALP in vivo, and replacement of this residue with alanine abolishes EBNA-LP co-operation with EBNA2 in the induction of LMP1 Further, replacement of the serine residue with glutamic acid (which mimics constitutive phosphorylation), restored the EBNA2 cooperative function (Yokoyama *et al*, 2001) Taken together these results support the hypothesis that the phosphorylation of EBNA-LP is crucial to at least its EBNA2 cooperative function in infected cells

1.8.5 LMP1

Among the proteins involved in B-cell immortalization, LMP1 is the only latent protein that exhibits oncogenic properties The expression of LMP1 leads to the transformation of rodent fibroblast cell lines and renders them tumorigenic in nude mice (Wang *et al*, 1985) The expression of LMP1 in Burkitt's lymphoma cell lines induces many of the phenotypic changes observed in EBV infection including the up-regulation of B cell activation markers, cell adhesion molecules and an increased resistance to stimuli that induce apoptosis (Rowe *et al*, 1994) In epithelial cells, LMP1 expression blocks the normal process of differentiation, reminiscent of the undifferentiated phenotype frequently observed in NPC (Dawson *et al*, 1990) In keeping with these *in vitro* findings, targeted expression of LMP1 in the skin or B cell compartment of transgenic mice leads to the induction of epithelial hyperproliferation and lymphomagenesis respectively (Kulwichit *et al*, 1998) The detection of LMP1 expression in many EBV-associated malignancies such as Hodgkin's disease, immunoblastic lymphomas and nasopharyngeal carcinomas suggests that this protein contributes to EBV-associated tumorigenesis

1.8.5.1 STRUCTURE AND FUNCTIONS OF LMP1

LMP1 is an integral membrane protein of the Tumour Necrosis Factor Receptor (TNFR)/CD40 super family and is composed of a short cytoplasmic N-terminus of 24 amino acids, six trans-membrane domains that total 186 amino acids and a cytoplasmic C-terminus of, 200 amino acids (Baker and Reddy 1996, Clausse *et al*, 1997) LMP1 functions as a constitutively active receptor and signals principally from intracellular compartments (Gires *et al*, 1999, Lam and Sugden 2003b) Both oligomerisation and localization within glycosphingolipid-rich membrane rafts are essential for the initiation of signaling (Higuchi *et al*, 2001, Ehopoulos and Young 2001) (See Figure 1 11 below)

FIGURE 1.11. SCHEMATIC DIAGRAM OF THE STRUCTURE OF LMP1

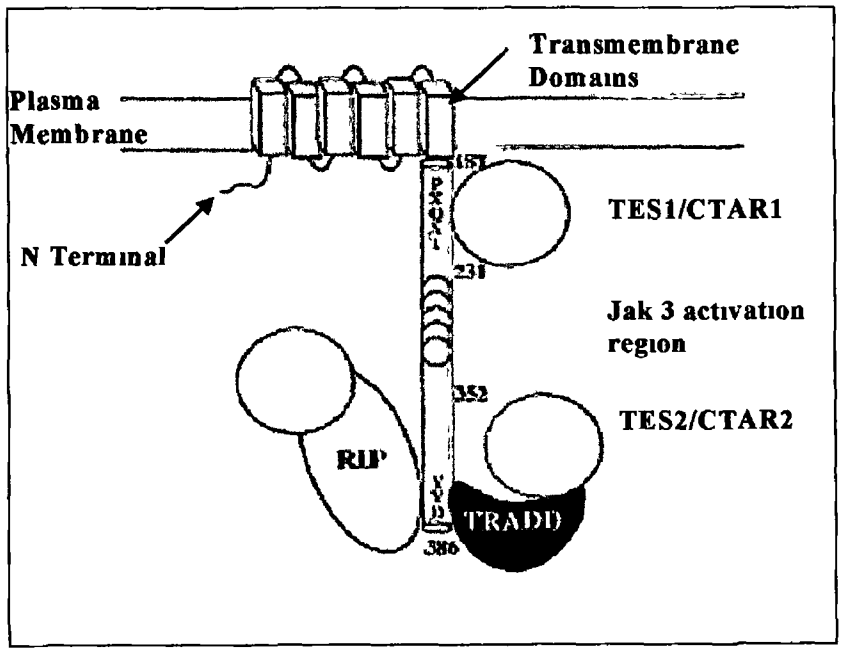


Figure 1 11 Schematic Diagram of LMP1 LMP1 consists of a 24 amino acid N terminal, cytoplasmic domain, six transmembrane domains and a, 200 amino acid C terminal cytoplasmic tail LMP1 aggregates to lipid rafts in the plasma membrane and associates with TRAFs via a PxQxT motif in Transformation Effector site 1(TES1) or Carboxyterminal activation region1 (CTAR1), (residues 187 to 231) and with TRADD and RIP via a YYD motif in Tes2/CTAR2 (residues 352-386) The region between amino acids 275 and 307of LMP1 is responsible for activation of Jak3 (Adapted from Cahir-McFarland, 1999)

The cytoplasmic carboxyl-terminal tail of LMP1 contains two major effector domains, C-terminal activating region 1 (CTAR1), also known as Transformation Effector Site 1 (TES1), and CTAR/TES2 (Figure 1 12) CTAR1 is located proximal to the membrane, binds the Tumor Necrosis Factor (TNF) receptor-associated factors (TRAFs) (Devergne *et al*, 1996, Huen *et al*, 1995) and is essential for EBV-mediated B-cell immortalization (Kaye *et al*, 1999, Kaye *et al*, 1995, Izumi *et al*, 1997) CTAR2/TES2, which is located near the C terminus, supports the long-term growth of immortalized B cells (Izumi and Kieff 1997) and recruits the TNF receptor-associated death domain protein (TRADD) and receptor-interacting protein (RIP) (Ehopoulos *et al*, 1999, Huen *et al*, 1995, Izumi *et al*, 1999) (Figure 1 11) Consequently, LMP1 triggers several signaling pathways that lead to the activation of several transcription factors, including NF-κB, STATs, AP-1, and ATF2 (Brennan 2001, Richardson *et al*, 2003, Kieser *et al*, 1997, Eliopoulos *et al*, 1999a, Eliopoulis *et al*, 1999b, Ehopoulos and Young 2001, Gires *et al*, 1999, Huen *et al*, 1995) NF-κB plays a key role in most LMP1-stimulated gene expression (Devergne *et al*, 1998, He *et al*, 2000, Mehl *et al*, 2001, Pai *et al*, 2002, Zhang *et al*, 2001) (Figure 1 12)

FIGURE 1.12 SCHEMATIC REPRESENTATION OF THE MOLECULAR INTERACTIONS AND SIGNALLING PATHWAYS ENGAGED BY LMP1

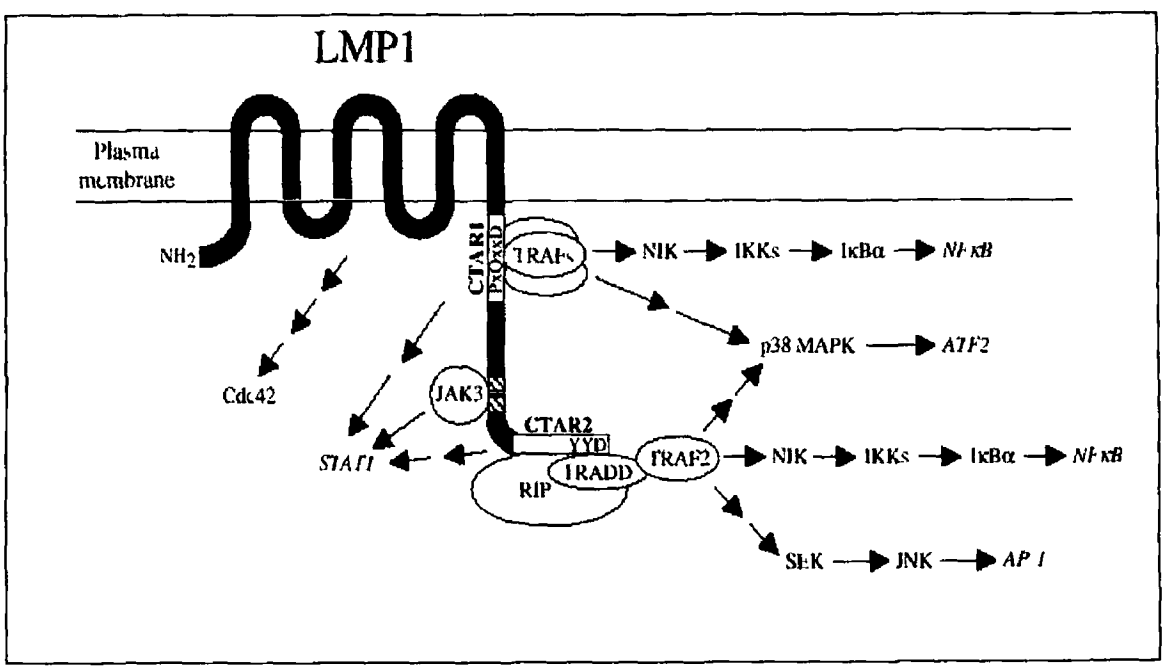


Figure 1 12 Schematic representation of the molecular interactions and signaling pathways engaged by LMP1. LMP1 has a short cytoplasmic N-terminus, six trans-membrane segments which confer aggregation and oligomerization at the plasma membrane and a long cytoplasmic C-tail containing two effector domains, CTAR1/TES1 (aa, 194–232) and CTAR2/TES2 (aa 351–386) CTAR1, which is essential for EBV-mediated B cell immortalization, binds TRAF1, TRAF2, TRAF3 and TRAF5 through a P204 xQ206 xxD209 motif and activates the NF-κB and p38 signaling pathways CTAR2 supports the long-term growth of immortalized B cells and recruits TRADD through a YYD386 motif to activate downstream signals, such as NF-κB, JNK and p38 Both LMP1 C-terminal domains also mediate the activation of the JAK/STAT pathway, although an intermediate region enclosing two copies of a proline-rich PxxPxP sequence has been shown to bind JAK3 and induce STAT binding activity independently of CTAR1/CTAR2 The trans-membrane domains of LMP1 are responsible for the activation of the small GTPase Cdc42 leading to cytoskeletal changes (adapted from Brennan, 2001)

LMP1 mimics a constitutively active CD40 receptor in many respects and both molecules can activate overlapping signaling pathways (Gires *et al*, 1999, Baker and Reddy 1996) CD40 also recruits TRAFs and can activate the NF-κB, JNK and p38MAPK pathways (Kehry 1996, Aicher *et al*, 1999, Hatzivassiliou *et al*, 1998) Signaling pathways initiated from LMP1 and CD40 regulate cell fate decisions including proliferation, differentiation and apoptosis and involve the modulation of similar sets of genes including those encoding B-cell surface markers (CD54, CD23, LFA-1), cytokines (IL-6) and anti-apoptotic proteins (A20, Bcl-x) (Eliopoulos *et al*, 1997, Kilger *et al*, 1998, Van Kooten and Banchereau 1997, Zimber-Strobl *et al*, 1996, Kehry 1996) Despite many similarities shared between CD40 and LMP1, they also differ substantively (reviewed by Lam and Sugden 2003) In this regard, it has been demonstrated that although LMP1 and CD40 can independently bind several of the same TRAF molecules (namely TRAF2, 3 and 5), TRAF6 binds to CD40 but not to LMP1 and TRAF1 and TRADD have been reported to bind to LMP1 but not to bind CD40 directly (Busch and Bishop1999)

The suppression of apoptotic death is a function of LMP1 that contributes to its oncogenicity One well-documented mechanism by which LMP1 can protect against apoptosis is by up regulating the expression of several anti-apoptotic proteins including Bcl-2, A20 and Mcl-1 (Milner *et al*, 1992, Henderson *et al*, 1991, Fries *et al*, 1996,

Wang *et al*, 1996, Laherty *et al*, 1992) thus raising the apoptotic threshold of the infected cell and also providing protection against a range of apoptosis-inducing stimuli We have previously shown that elevated mRNA levels from an additional *bcl-2* family member, *bfl-1/a1*, are a feature of EBV-infected B lymphocytes exhibiting type 3 latency, and that the expression of LMP1 in an EBV-negative BL cell line coincided with a dramatic increase in *bfl-1/a* mRNA levels (D'Souza *et al*, 2000) In that study, Bfl-1 protected against serum-depletion-mduced apoptosis when expressed in the same cell context Bfl-1 is an anti-apoptotic protein whose preferential expression in hematopoietic and endothelial cells is controlled by inflammatory stimuli such as TNF and interleukin-1 (Choi *et al*, 1995, Karsan *et al*, 1996)

1.8.6 LMP2A/LMP2B

The latent membrane protein 2 (LMP2) gene is expressed in latently infected B cells and encodes two messages of 2 0 and 1 7 kb in length, which are produced by alternative promoter usage (Laux *et al*, 1988, Sample *et al*, 1989) The longer message encodes LMP2A, while the shorter message is initiated from a promoter, approximately 5 KB downstream, that is bi-directional and responsible for LMP1 expression in the leftward direction (Laux *et al*, 1988) and LMP2B expression in the rightward direction Both LMP2A and 2B are integral membrane proteins but the difference between the two isoforms is that LMP2A has an N- terminal 119 aa cytoplasmic signaling domain that is not present in LMP2B The main function of LMP2A and 2B is that they are capable of preventing viral lytic cycle activation (Wensing and Farrell 2000) LMP2A, co-localizes in patches in B cell membranes with LMP1 (Longnecker and Kieff 1990)

In B-lymphocytes antigen cross-linking leads to the activation of the B cell receptor (BCR) (De-Franco 1997) A particular domain of the BCR, the immunoglobulin α/β immunoreceptor tyrosine-based activation motif (ITAM) is phosphorylated and it and sequences adjacent to it act as binding sites for Syk kinase and members of the Src family of tyrosine kinases including Lyn (De-Franco 1997) The kinases sequestered by the BCR are then activated and stimulate a cascade of phosphorylations and kinase activity which

eventually results in B cell differentiation and proliferation (De-Franco, 1997) Interest in LMP2A began with the discovery that BCR mediated signal transduction is abolished by LMP2A expression (Miller *et al*, 1993) Mutational analyses of the LMP2A protein explains this finding A functional ITAM region has been identified in the LMP2A N terminal around tyrosine residues 74 and 85 The ITAM domain in LMP2A has been identified as crucial for association with the Lyn, Fyn, Syk and Csk kinases as well as for inhibition of BCR signals (Burkhardt *et al*, 1992, Fruehlmg *et al*, 1997, Fruehling *et al*, 1998, Longnecker 2000, Longnecker, *et al*, 1991, Scholle *et al*, 1999 Recent Studies also show that LMP2A associates with lipid rafts (Dykstra *et al*, 2001, Higuchi *et al*, 2001) thus LMP2A blocks BCR signaling by interfering with raft association of the BCR (Dykstra *et al*, 2001) One of the repercussions of BCR activation is the induction of the EBV lytic cycle (Miller *et al*, 1995, Kieff *et al*, 2001) This results in the expression of EBV genes, many of which are highly immunogenic Expression of these genes targets them for destruction by the immune system, and thus by inhibiting BCR activation, LMP2A may have an important function in maintaining EBV latency and helping EBV to avoid detection by the immune system (Kieff *et al*, 2001) Although LMP2A expression is not essential to EBVs immortalizing properties, LMP2A expression seems to mediate signals involved in cellular proliferation and survival in mouse transgenic models (Caldwell *et al*, 1998, Longnecker 2000) Regions within the C terminal of LMP2A are implicated in the molecular association of LMP2A molecules and the association of LMP2A molecules and other molecules carrying the LMP2A C-terminal domain (Matskova *et al*, 2001) Studies by the same group also identified cysteine rich repeats which are targets of palmitoylation, though LMP2A localization to the lipid rafts in the cell membrane is not dependent on palmitoylation (Matskova *et al*, 2001) Because of the number of motifs identified in LMP2A, a putative role for LMP2A in signal transduction has been proposed particularly in light of the ability of LMP2A to enhance LMP1 mediated activation of the AP-1/JNK and NF-κB pathways (Dawson *et al*, 2001, Kieser *et al*, 1997, Murono *et al*, 2000) Recent studies found that LMP2A regulates c-Jun protein through extracellular signal-regulated kinase (ERK) (Chen *et al*, 2002)

Most of the studies to date focus on the LMP2A product, and as such there has been no comprehensive phenotypic analysis of the LMP2B isoform This is partly because of the hydrophobic character of LMP2 proteins Recent studies (Lynch *et al*, 2002) revealed that LMP2B co-localizes with LMP2A in perinuclear regions in transiently transfected cells Although mRNAs for both proteins are expressed in immortalized B cells, genetic analyses of the LMP2 gene have shown that neither product is required for immortalization of B cells by the virus (Longnecker *et al*, 1992, Longnecker et *al*, 1993, Kim and Yates 1993) Later studies with LMP2A-knockout viruses revealed no discernable defect in their ability to generate immortalized cell lines from primary B cells (Konishi *et al*, 2001) Virus mutants that disrupt LMP2A but leave LMP2B intact and, presumably, expressed, indicate that LMP2B possesses no independent role in producing the B cell receptor signaling blockade phenotype of the LMP2 gene While the significance of LMP2B and its role in pathogenesis remains unclear, homology studies comparing the LMP2 gene of EBV with that of rhesus and baboon lymphocryptoviruses have revealed that the ability to make the LMP2B transcript is well conserved (Rivailler et *al*, 1999) This implies that there is an as yet unrecognized role for LMP2B in the EBV life cycle

1.8.7 EBERs

EBV encodes two small non-polyadenylated RNAs termed EBERs EBER1 and EBER2 are the most abundant viral transcripts in latently EBV infected cells and are encoded by the right hand 1000 base pairs of the EcoR1 J fragment of the EBV genome (Lerner *et al*, 1981, Rymo 1979) EBER1 is 167 nucleotides long while EBER 2 is 172 nucleotides long (Rosa *et al*, 1981) The EBER genes are separated by 161 base pairs and are transcribed from left to right on the EBV map by RNA polymerase III Although the primary sequence homology is only about 54%, a high degree of homology is observed in their extensively base paired secondary structures which consist of a series of short stem loop structures As yet no definitive role in EBV infection has been found for the EBERs, but the conservation of the EBER primary sequences within the EBV strains suggests some essential function for these RNAs (Arrand *et al*, 1989)

Following primary infection of B-lymphocytes with EBV, EBNA2 and EBNALP expression is detectable after 6 hours, after this point, the LMPs and EBERs are expressed However, infection with the P3HR1 strain in which the EBNA2 gene is deleted, results in cells expressing only EBNALP and trace amounts of EBER in primary B lymphocytes, meanwhile, infection with the same virus in EBV genome negative BL cell lines leads to the expression of EBNA1, EBNALP, EBNA3 and the EBERs (Rooney *et al*, 1989) Thus EBER expression may be dependent on the host cell, possibly due to the requirement for particular cell cycle products or the state of B cell differentiation (Takada and Nanbo 2001) Although debate surrounds EBER expression in permissively infected cells, it is generally accepted that EBERs are consistently expressed in latently infected cells, and as such, EBER1 detection through in situ hybridization studies has been used as a marker for EBV infection in tissue samples (Chang *et al*, 1992) EBERs are localized in both the cytoplasm and nuclei of interphase cells and exist in ribonucleoprotein (RNP) complexes that can be precipitated by antibodies to a systemic lupus erythematosis protein, La (Chang *et al*, 1992, Howe and Steitz 1986, Schwemmle *et al*, 1992, Lerner *et al*, 1981)

Another highly abundant ribosomal protein EAP/L22 has been identified in the La containing RNP complexes (Toczyski *et al*, 1991, Toczyski *et al*, 1994) L22 binds to the stem loop structure of EBER1 and the putative function of this association is to sequester the cellular L22 molecules (Takada and Nanbo 2001) The L22 gene is translocated in certain leukemias and as such, L22 levels may influence cell transformation, (Nucifora *et al*, 1993) the association of the EBERs with a cell immortalization protein (L22) may indicate a possible role in not only EBV driven immortalization but also in oncogenesis The EBER genes have also been implicated as having an oncogenic role in the Akata cell line in that transfection of the EBERs into EBV negative clones restored the capacity for growth in soft agar, tumourgenicity in SCID mice, resistance to apoptosis inducers and up-regulated expression of *bcl-2* that was originally a feature of EBV infected Akata cell clones but was lost in the negative clones (Komano *et al*, 1999) The role of EBERs in oncogenesis is also supported by findings, which showed the EBERs induce human IL-10 expression, which acts as an autocrine growth factor in BL cells (Kitagawa *et al*, 2000)

1.8.8 CSTs/BARTs

In latently EBV infected cell lines and tumors, expression of a family of alternatively spliced polyadenylated transcripts that arise from the BamH1 region of the EBV genome are consistently observed (Chen *et al*, 1992, Gilligan *et al*, 1990, Gilligan *et al*, 1991, Karran *et al*, 1992) These so-called BamH1A rightward transcripts (BARTS) or complimentary strand transcripts (CSTs) are transcribed in a rightward direction antisense to a group of lytic genes also present in the BamH1-A region The antisense nature of the transcripts relative to the lytic cycle genes suggested a role in regulating the lytic cycle gene expression (Karran *et al*, 1992) Apart from this function however, the discovery of a polyadenylation site at 160,964 on the EBV genome implies that these transcripts may encode functional proteins A number of potential protein products have been identified although protein expression from this family of transcripts is still unclear Analysis of the transcripts has identified putative open reading frames (ORFs) and the best studied of these include BARF0, RKBARF0, A73, and RPMS1 all of which are initiated at an ATG site (Chen *et al*, 1992, Fries *et al*, 1997, Gilligan *et al*, 1991, Karran *et al*, 1992, Sadler and Raab-Traub 1995, Smith *et al*, 2000, Smith *et al*, 1993)

FIGURE 1.13. RELATIVE POSITIONS OF THE RPMS1 AND BARF0 ORFS IN THE EBV BARTS.

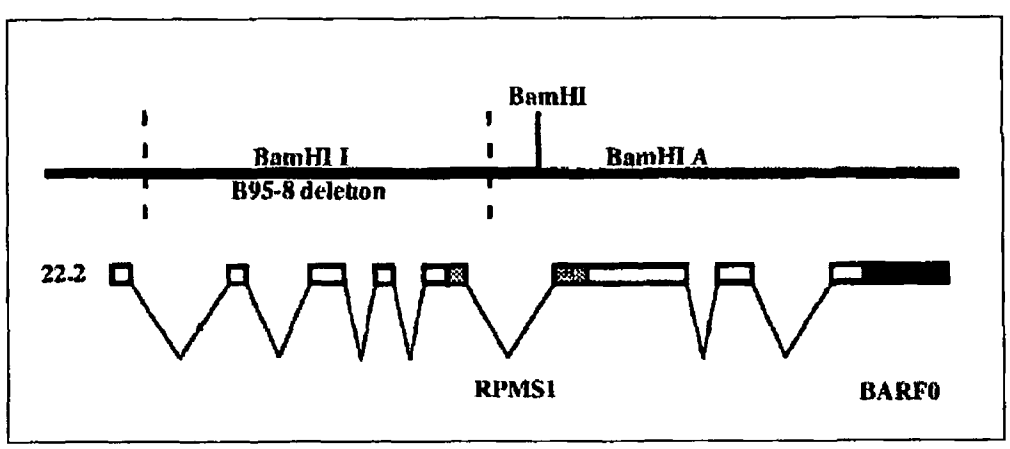


FIG. 1 13 Relative positions of the RPMS1 and BARF0 ORFs in the EBV BARTs are shown These are transcribed in the rightward direction and their relative positions in the BamH1A region of the EBV genome are detailed (adapted from Zhang *et al*, 2001)

1.8.8.1 BARF0 PROTEINS AND A73

The A73 spliced CST was one of the first identified, and RPA analyses suggest that this spliced product is one of the more abundant of the CST proteins expressed (Chen *et al*, 1992, Smith *et al*, 2000) As yet no full-length cDNA clone encoding the A73 protein has been identified The A73 product has been shown to be a cytoplasmic protein which in yeast two hybrid assays interacts with RACK1 (Smith *et al*, 2000), a cellular protein involved in regulating signaling from protein kinase C and Src tyrosine kinases (Smith 2001) Two potential BARf0 encoded proteins have been described in detail, BARF0 a 174 amino acid protein (Koslowski *et al*, 1999), and RK-BARF0, a splice variant of a possible 279 amino acids A complete RKBARf0 clone has not yet been isolated and it has been discovered that the RK-BARF0 cDNA is a chimera of two overlapping cDNAs (Sadler and Raab-Traub1995)

1.8.8.2 RPMS1

The RPMS1 ORF was originally identified from a composite of overlapping cDNA clones (Hitt *et al*, 1989, Smith *et al*, 1993) but the isolation of cDNAs representing complete CSTs facilitated identification of RPMS1 as the most 5' ORF in one of these full length cDNA species (Smith *et al*, 2000) Expression of the RMPS1 protein has only been detected in cell lines transfected with vectors expressing the RPMS1 ORF, and in these transfected cells, the protein is localized to the nucleus (Chen *et al*, 1999, Smith *et al*, 2000) The RPMS1 protein was shown to have a low level of homology to the EBNA2 protein (Smith *et al*, 1993), including the WWP motif required for EBNA2 binding to CBF1 (Ling *et al*, 1995)

The RPMS1-CBF1 interaction was shown to interfere with EBNA2 and Notch mediated trans-activation of promoters containing CBF1 binding sites and hence repressed transcription from these reporter constructs (Smith *et al*, 2000) This effect has been further investigated and it has been shown that RPMS1 also binds Sin3a and CIR, both members of the CBF1 repression complex (Figure 1 14) One possible implication of this is that RPMS1 may function to down-regulate transcription of the other latent proteins

from the Cp promoter in an attempt to prevent recognition of viral antigens by the immune system, thereby assisting EBV persistence (Smith 2001).

FIGURE 1.14. MODEL FOR RPMS MEDIATED REPRESSION OF EBNA2-CBF1 ASSOCIATED TRANS-ACTIVATION OF TARGET GENES

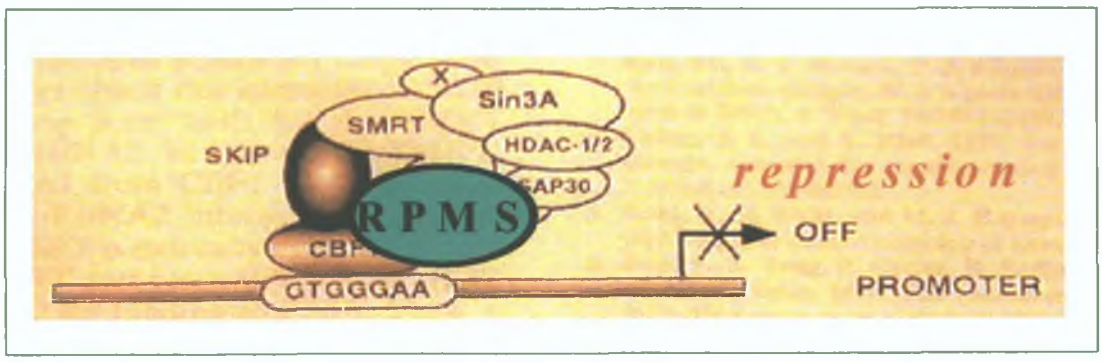


Figure 1.14. A model for RPMS mediated repression of EBNA2-CBF1 associated trans-activation of target genes. RPMS binds to CBF1 and CIR to stabilize the co repression complex thereby preventing EBNA2 mediated trans-activation of target genes.

1.9 GENES OF THE VIRAL LYTIC CYCLE.

The EBV viral lytic cycle is the replicative stage of the virus life cycle and is essential for the production of infectious pathogens, which can be transmitted by virus shedding in the saliva from, infected to EBV naïve individuals. Only a small number of latently infected lymphocytes spontaneously enter the replicative cycle, and in these cells the viral DNA is amplified several hundred fold by a lytic origin of replication ori Lyt (Hammerschmidt and Sugden 1988). In vitro, lytic infection is induced mainly by the addition of phorbol esters, these chemicals drive the infected cells into the lytic cycle and this effect is probably mediated by protein kinase C activation of Jun-fos interactions with AP-1 upstream of the immediate early viral genes (Farrell *et al.*, 1983; Farrell *et al.*, 1989; Laux *et al.*, 1988). Lytic replication has also been induced in the Akata cell line (which carries an LMP2A deleted virus), by cross linking of surface immunoglobulins, studies revealed that greater than 50% of the cells entered the lytic cycle under these conditions (Takada 1984; Takada and Ono 1989). Another approach used to induce lytic replication

of EBV involves super infection of Raji cells with the defective EBV from the P3HR1 cell line (Mueller-Lantzch *et al*, 1980) Raji is an EBV positive BL cell line, which has an unusually high EBV episome copy number The Raji cell line is defective for viral replication and late gene expression and infection is thus tightly latent (Polack *et al*, 1984) EBV virions from P3HR1 contain rearranged DNA molecules in which the intermediate early trans-activators of the lytic cycle are expressed after super infection (Cho *et al*, 1984, Miller *et al*, 1984) Thus super infection of the Raji cell line with the P3HR1 EBV induces lytic replication in the Raji cell line Studies using these cell lines permitted the identification of the EBV replicative proteins and their classification into early antigens (EA), membrane antigens (MA), and viral capsid antigens (VCA) The early antigens have been further subdivided into diffuse early antigens (EA-D) and restricted early antigens (EA-R), on the basis of their sensitivity to methanol fixation (Henle *et al*, 1971)

Following induction of the lytic cycle, cells that have become permissive to viral replication, undergo cytopathic changes characteristic of herpesviruses, including migration 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 (IARC Monographs 1997) Lytic cycle gene expression follows a temporal and sequential order (Farrell 1992, Takada and Ono 1989) and some viral genes are expressed independently of new protein synthesis, early after induction, such genes are classified immediate early genes, the early lytic viral genes are expressed after the immediate early genes and their expression is not affected by inhibition of viral DNA synthesis (Kieff 1996)

FIGURE 1.15 A SCHEMATIC REPRESENTATION OF EARLY AND LATE EBV GENE EXPRESSION

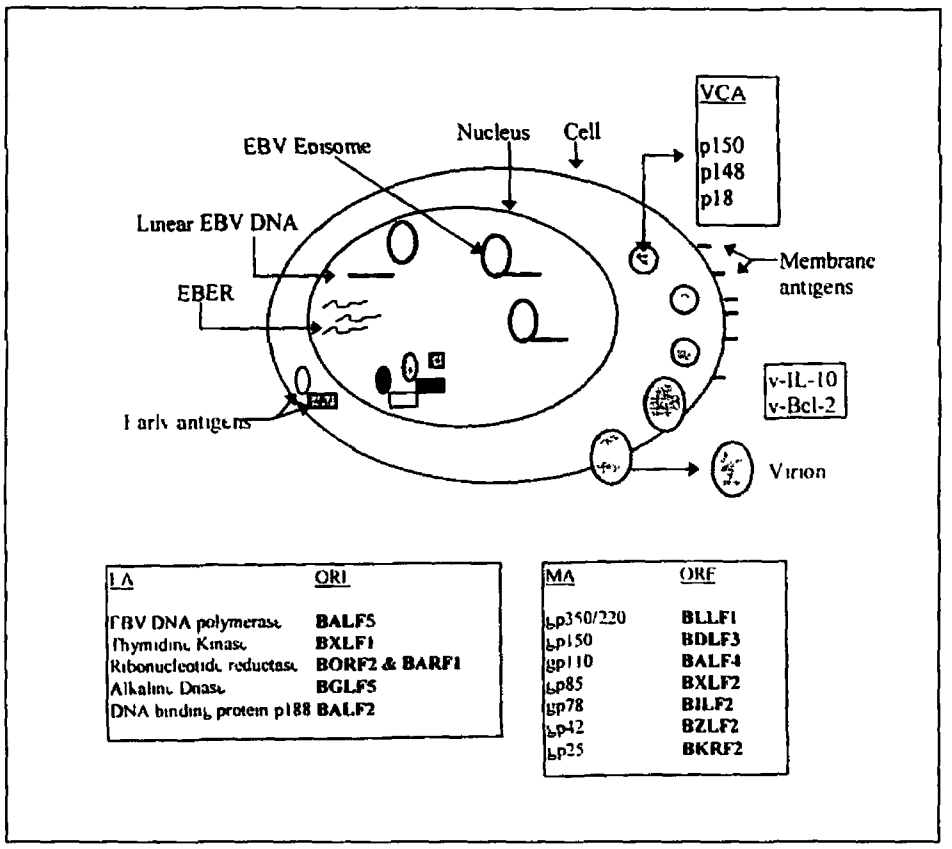


Figure 1.15 A schematic representation of early and late EBV gene expression The VCA, the MA and the EA are illustrated and their open reading frames are written in bold

1.9.1 IMMEDIATE EARLY GENES

After induction of the lytic cycle in response to super infection of the Raji cell line or immunoglobulin cross linking in the Akata cell line, in the presence of protein synthesis inhibitors, three leftward mRNAs are transcribed The BZLF1, BRLF1 and BL'LF4 encoded proteins are potent trans-activators of early EBV lytic gene expression (Takada and Ono 1989, Kieff E 1996) The functional and physical interaction of BZLF1 with NfkB is an important mediator of LMP1 effects in EBV latent infection BZLF1 can also down-regulate the EBNA Cp promoter possibly facilitating the transition from latent to lytic infection (Sinclair *et al*, 1992) BZLF1 has also been shown to inhibit both cellular

differentiation and cell cycle progression in epithelial cells The mechanism mediating this effect is not known however activation of cell cycle inhibitors p21 and p27b was not observed (Swenson *et al*, 1999) BRLF1 was also shown to bind Rb in vivo shortly after induction of the viral lytic cycle in EBV infected Akata cells This interaction may initiate cell cycle progression and facilitate viral DNA synthesis during lytic replication (Zacny *et al*, 1998)

1.9.2 Early Genes

The early genes are expressed when the lytic cycle is induced in the presence of inhibitors of DNA synthesis By this criteria at least 30 EBV mRNAs are early gene products (Hummel and Kieff 1982) Two very abundant early proteins have been mapped to specific DNA sequences The BALF2 protein is homologous to a HSV DNA binding protein and is important in DNA replication (Hummel and Kieff 1982, Kieff 1996) The BHRF1 protein, which is expressed in moderate abundance, has extensive collinear homology with bcl2 (Pearson *et al*, 1983, Austin *et al*, 1988) BHRF1 can protect EBV negative BL cells from apoptosis (McCarthy *et al*, 1996), however EBV recombinants lacking the BHRF1 ORF are fully capable of initiating and maintaining cell growth transformation and they can also enter the lytic cycle and produce virus (Lee and Yates 1992, Marchini *et al*, 1991) Several of the early genes are linked to DNA replication as indicated in Figure 1 15 above Transfection experiments indicate that some of these genes are activated in the process of cell differentiation in the absence of other viral gene products, suggesting a possible role for cellular factors in regulating the productive cycle at least in certain cell types

1.9.3 Late Genes.

The late genes code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress (IARC Monographs 1997) Among the non-glycoproteins, the major nucleocapsid protein is encoded by BCLF1 BNRF1 encodes the major external non-glycoprotein of the virion, and BXRF1 is likely to encode

a basic core protein The genes encoding the EBV glycoproteins are illustrated in bold in Figure 1 15 above The late BCRF1 gene, which is located in the middle of the EBNA regulatory domain between ori-P and Cp, is a close homologue of the human IL-10 gene with nearly 90% collinear identity in amino acid sequence (Moore *et al*, 1990, Vieira *et al*, 1991, Touitou *et al*, 1996) BCRF1 has most of the activity of human IL 10 including negative regulation of macrophages and NK cell functions and inhibition of IFNγ production Thus, virally expressed IL-10 may have a local effect on these responses to reactivate infection (IARC Monographs 1997)

1.10 Apoptosis

Apoptosis is a genetically pre programmed form of cell suicide involving dramatic morphological changes including cell shrinkage, nuclear re-organization, blebbing and eventual fragmentation of the cell into membrane bound apoptotic bodies (Allday 1996) Although apoptosis can occur to eliminate redundant cells during development, it is also an emergency active programme triggered in response to radiation damage, viral infection, or aberrant growth induced by the activation of oncogenes In order to maximize their replicative capacity many viruses deregulate normal cell cycle controls and some have also ensured selective advantage and biological success by evolving mechanisms which suppress either the triggers of apoptosis or the programme itself, EBV is one such virus (Shen *et al*, 1995) By preventing infected lymphocytes from undergoing apoptosis EBV ensures survival/persistence in the host system at least until such time as sufficient viral numbers have been produced to transmit infection to other individuals

The morphological features associated with apoptosis involve a cascade of proteolytic cleavage events resulting in the cleavage of genomic DNA in a pattern identified on agarose gels as a distinctive "ladder" effect The proteolytic events characteristic of apoptosis are effected by a family of related caspases, and their activation is regulated by another family of proteins the Bcl2 family The Bcl2 family of intracellular proteins is the central regulator of caspase activation, and includes both pro and anti-apoptotic proteins

It is generally agreed that the combined action of these proteins in the cell sets the threshold for activation of the apoptosis programme Anti-apoptotic members of the Bcl2 family include Bcl2, BclXl and Bfl-1, while the pro-apoptotic members of the family including proteins such as Bax, BclXs, Bik, Bak and Bad accelerate apoptosis (White *et al*, 1996) Other proteins such as Mcl-1 (a Bcl2 homologue), A1, A20 and the C elegans CED9 have also been identified as apoptosis inhibitors (Fries *et al*, 1996, Laherty *et al*, 1992, Wang *et al*, 1999) All members of the Bcl2 family possess 1 of 4 conserved regions of homology, known as Bcl2 homology domains (BH1 to BH4) (reviewed by Adams *et al*, 1998) Certain regions of homology namely BH1 and BH2 appear to be essential to the anti-apoptotic proteins while the BH3 domain is crucial to a subset of the pro-apoptotic proteins

1.10 1 EBV AND APOPTOSIS

EBV is interesting because it manipulates both pro and anti-apoptotic proteins to its advantage in the course of its life cycle This duality of purpose is facilitated by the ability of EBV to (i) regulate host cell apoptotic death machinery often by inducing the expression of anti-apoptotic proteins such as Bcl2, Bfl-1, Mcl-1 and A20 (Fries *et al*, 1996, Laherty *et al*, 1992, Wang *et al*, 1999, D'Souza *et al*, 2000), or by (ii) deregulating the function of oncogenes and tumor suppressor genes involved in the cell cycle such as c-myc, p53 and pRb and (iii) by encoding its own gene products which mimic anti-apoptotic proteins of the host cell such as BHRF1 and BZLF1 (Henderson *et al*, 1993, Tarodi *et al*, 1994) In terms of EBV infection, protection from apoptosis is desirable in the latent stage to ensure persistence in the host memory B cell compartment and in the lytic stage to delay cell death until virion numbers have been sufficiently amplified Although EBV infection is primarily associated with an asymptomatic latent disease state, infection in adolescence is associated with infectious mononucleosis, also unsurprisingly as a result of its subversion of proliferation and apoptotic pathways, EBV is also associated with several lymphoproliferative disorders in immunocompromised individuals such as Oral Hairy Leukoplakia (Thomas *et al*, 1991) and a number of human cancers, including undifferentiated Nasopharyngeal Carcinoma (NPC) (Zur

Hausen *et al*, 1970, De-The 1981), Hodgkin's disease (Pallesen *et al*, 1993, Brousset *et al*, 1993), rare Nasal T cell lymphomas (Pallesen *et al*, 1993), gastric carcinoma (Shibata and Weiss 1992, Selves *et al*, 1996), breast carcinoma (Labrecque *et al*, 1995) and B and T cell lymphomas in immunocompromised individuals (Miller *et al*, 1990, Pallesen *et al*, 1993, Young *et al*, 1989, Gratama *et al*, 1991, Shibata *et al*, 1991)

1 10.2 EBV Induces the Expression of Anti-Apoptotic Proteins.

1.10.2.1 Bcl2

Three EBV proteins -LMP1, EBNA2 and EBNA3B -have been shown to up-regulate expression of the antiapoptotic Bcl2 protein (Henderson *et al*, 1991, Finke *et al*, 1992, Silins and Sculley 1995) In addition to inducing proliferation, EBV latent proteins may also facilitate cell survival by suppressing the apoptotic programme The first evidence to support this theory comes from studies involving Type I and Type III BL cell lines These cell lines exhibit the latency I and latency III latent gene expression profiles respectively (Table 1 0), thus only EBNA1 is expressed in the type I BLs whereas the full complement of latency associated proteins are expressed in the type III BLs Type I cells are apoptosis sensitive while the type III BLs like the LCLs display an increased resistance to apoptosis induced by a number of triggers including, serum deprivation and Ca++ ionophores (Gregory *et al*, 1991) Further studies determined that type I BLs express little or no Bcl2, however the apoptosis-resistant type III BLs were found to express Bcl2 (Henderson *et al*, 1991) Transfections with EBV LMP1 also resulted in enhanced survival of these cells The ability of LMP1 to confer resistance to apoptosis to the type I BLs resulted from its ability to induce endogenous Bcl2 expression (Gregory *et al*, 1991, Henderson *et al*, 1991) EBNA2 was also found to enhance the *bcl2*-inducing effect of LMP1 in BJAB cell lines but not induce *bcl2* itself in the same experiment Other transfection studies however showed that EBNA2 could independently up-regulate Bcl2 expression in a range of EBV negative cell lines as could LMP1, with the strongest effect on *bcl2* conferred by transfection with LMP1 (Finke *et al*, 1992) Also Ectopic expression of EBNA2 has been shown to independently induce bcl2 expression in Bjab

cell lines, as has LMP1 however in this system no co-operative effect between LMP1 and EBNA2 in inducing Bcl2 expression was observed (Martin *et al*, 1993) Another EBV latent protein EBNA3B has also been identified as up-regulating Bcl2 expression in an EBV negative cell line (Silins and Sculley 1995)

Although in vitro LMP1 and EBNA2 appear to up-regulate Bcl2 expression, in vivo, the recirculating B cells of peripheral blood which are a normal target of EBV, already express relatively high levels of Bcl2 and EBV infection and thus LMP1or EBNA2 or combined expression of the two, does not significantly increase this, thus the role of LMP1/EBNA2 may be in maintaining the high constitutive level pf Bcl2 in uninfected cells rather than inducing it (Martin *et al*, 1993)

Over expression of Bcl2 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, with the inhibition of cell death being proportional to the amount of Bcl2 expressed (Henderson *et al*, 1991, Milner *et al*, 1992) However in a panel of group I cell lines, including group I, group I *bcl2*+, (*bcl2* Transfectants of the group I cell line) and group III cells from the same parental BL line, it was found that the degree of apoptosis inhibition imparted by the group III phenotype far outweighed that apparent from the levels of Bcl2 protein measured in the group III cells compared to the levels of Bcl2 in the group I Bcl2 transfectants Two explanations are obvious here, firstly in the group I cells, *bcl2* function is suppressed/antagonized or secondly other additional *bcl2* independent survival pathways are operational in group III cells With respect to the first possibility, *bcl2* homologues *bax* and *bclxs* which can suppress *bcl2* function have been identified Reviewed by (Allday 1996, Korsmeyer 1995) However no consistent differences in the expression of these proteins in group I and group III cells has been identified (Spender *et al*, 1999) In support of the second possibility, it is conceivable that EBV induced apoptosis suppressor proteins, such as the Bcl2 homologues Bclxl, Mcl1, or A20 act in concert with Bcl2 in group III cells to promote survival In this regard, LMP1 has been shown to up-regulate the expression of other anti-apoptotic proteins including Mcl-1, A20 and Bfl-1 (Wang *et al*, 1996, Laherty *et al*, 1992, D'Souza *et al*, 2000)

1.10.2.2 MCL-1

The maximum induction of Bcl-2 by LMP1 takes about 48-72 h (Rowe *et al*, 1994) However transient up-regulation of the anti-apoptotic Bcl2 homologue Mcl-1 occurred prior to LMP1-induced Bcl2 up-regulation and Mcl-1 levels decreased when Bcl-2 levels started to increase (Laherty *et al*, 1992) These findings support the hypothesis that Mcl-1 functions as a rapidly inducible, short-term effector of cell viability LMP1 also blocked the decline in the 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 Burkitt lymphoma 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)

1.10.2.3 A20

A20 was identified as a tumor necrosis factor α (TNF) –inducible gene, and encodes a 790 amino acid zinc finger protein A20 conferred resistance to apoptosis induced by TNFα Transcriptional activation of the A20 gene is mediated by binding NF-κB to two KB sequences within the A20 promoter (Beyaert *et al*, 2000) and A20 expression is induced in response to inflammatory mediators and cellular activators in several cell types including fibroblasts, lymphocytes and endothelial cells (Beyaert *et al*, 2000) The widespread distribution of A20 suggests that its anti-apoptotic function may also be widespread (Laherty *et al*, 1992) A20 expression has also been shown to be mediated by LMP1 LMP1 has been shown to up-regulate A20 expression in B-lymphocytes as well as epithelial cells and this effect is mediated by NF-κB, mapping to both CTAR1 and CTAR2 (Laherty *et al*, 1992, Fries *et al*, 1996, Miller *et al*, 1995, Miller *et al*, 1997) A20 is also constitutively expressed in EBV immortalized B cells (Spender *et al*, 1999) LMP1 mediated protection of epithelial cells from p53 induced apoptosis has been shown to involve A20, while A20 expression in BL cells has been shown to enhance LMP1 mediated protection from apoptosis (Fries *et al*, 1996, Laherty *et al*, 1992) A20 has also been identified as functioning in a negative feedback loop by inhibiting the activation of NF-κB from both CTAR1 and CTAR2 (Eliopoulos *et al*, 1999b) This effect was

mediated by the binding of A20 to TRAF2, this effect is also evident in CD40 signaling which also induces expression of A20 in BL cells (Sarma *et al*, 1995)

1.10 2.4 Bfl-1

Recent studies in our laboratory have also identified another anti-apoptotic protein, Bfl-1, that is up-regulated by LMP1 expression in BL cell lines (D'Souza *et al*, 2000) Bfl-1 is especially relevant to the subject matter of this thesis as, in this study, EBNA2 was identified as another EBV latent protein, which regulates the expression of Bfl-1 in BL cell lines This anti-apoptotic protein was initially identified as being over expressed in stomach cancers (Choi *et al*, 1995) Although bfl-1 exhibits ~72% homology with its murine homologue A1, their distribution appears to be quite different, while *A1* expression is restricted to haematopoetic tissues, *bfl-1* expression has been reported in endothelial cell lines, numerous cancer cell lines, and normal leukocytes as well as haematopoietic cells (Karsan *et al*, 1996, Kenny *et al*, 1997) A possible protective role for bfl-1 in the inflammatory response has been proposed as expression of the bfl-1 gene is up-regulated in cultured epithelial cells and leukocytes in response to phorbol ester, and inflammatory cytokines, TNFα and IL1 (Karsan *et al*, 1996, Moreb and Schweder 1997) *bfl-1* expression is also up-regulated during differentiation of leukemic cells to granulocytes and macrophages (Moreb and Schweder 1997) The protein product of the bfl-1 gene also called Bfl-1 is 175 ammo acids long and is considered a member of the Bcl2 family of proteins as Bcl2 homology domains, (BH1 BH2 and BH3) have been identified in the Bfl-1 protein (D'Sa-Eipper and Chinnadurai 1998) Analysis of the functions of the Bfl-1 protein have shown that it suppresses p53-mediated apoptosis, protects BL cells from apoptosis induced by growth factor withdrawal and exhibits cell proliferation and transforming activities in vitro, these studies also propose that Bfl-1 anti-apoptotic and transforming activities may be related (D'Sa-Eipper and Chinnadurai 1998, D'Sa-Eipper *et al*, 1996, D'Souza *et al*, 2000) *bfl-1* gene expression was originally identified in a proportion of colon and stomach cancers (Choi *et al*, 1995) as well as in cell lines derived from leukemias and lymphomas (Choi *et al*, 1995, Kenny *et al*, 1997) however, more recent studies have extended this list to include EBV positive BL cell lines exhibiting a group III phenotype (D'Souza *et al*, 2000) Type III EBV

positive BL cell lines and LCLs in which the full set of EBV latent proteins was expressed, exhibited elevated levels of bfl-1 mRNA relative to their type I counterparts Using a system in which the expression of LMP1 was inducibly regulated by tetracycline, D'Souza *et al* 2000, showed that LMP1 when expressed as the sole EBV protein, 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) These findings further substantiate the growing link between EBV and the regulation of apoptosis

1.10.3 EBV INTERACTS WITH COMPONENTS OF THE CELL CYCLE TO PROTECT AGAINST APOPTOSIS.

Apart from inducing the expression of anti-apoptotic proteins, EBV, particularly EBNA2 and LMP1 also control apoptosis by regulating the expression of tumor suppressor proteins involved in the cell cycle including pRb, and p53

1.10.3.1 CELL CYCLE EVENTS.

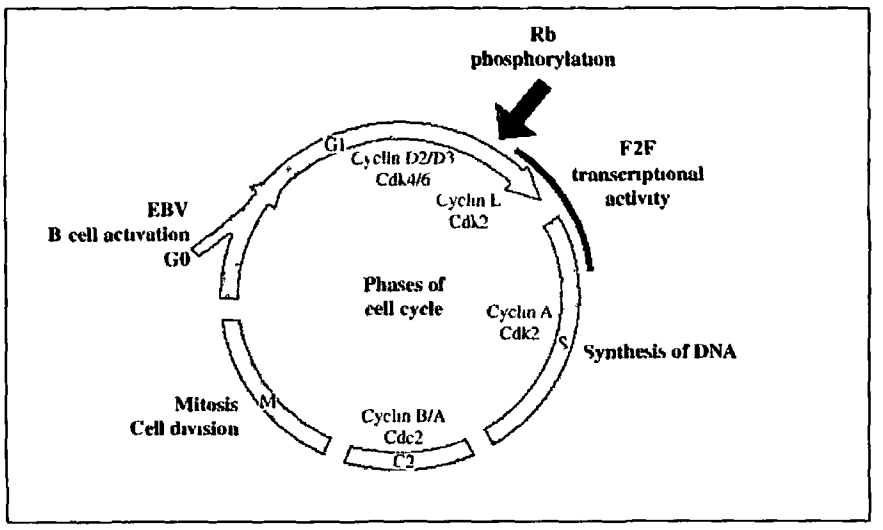


Figure 1 16 Phases of the Lymphocyte Cell Cycle and some of the factors involved therein

The cell cycle progresses through four distinct phases designated G1, S G2 and M The G1 phase is the longest and occurs prior to DNA synthesis DNA is synthesized in the S phase while cell division or mitosis occurs in the M phase (Brennan 2001) Certain EBV latent proteins notably LMP1, EBNA2, EBNALP and EBNA3C have been shown to regulate cell cycle related genes in order to induce lymphocyte proliferation and escape apoptosis, thereby facilitating viral persistence and survival

The first biochemical event observed in early G1 is the induction of D-type cyclins and their partners the cyclin dependant kinases (cdks) cdk4 and cdk6 The induction of D type cyclins is paralleled by a loss in cdk inhibitors such as p27kip1 (Brennan 2001) The most important substrate for the cyclinD/cdk4/6 complex is a family of pocket proteins typified by pRb (the retinoblastoma susceptibility gene product, (Nevins 1997) The mam

function of pRb and its familial proteins is to suppress a family of transcription factors termed E2F, which are important in driving cells onto the S phase of the cell cycle (Nevins 1997) The fact that cells in which the E2F transcription factors are deregulated, either apoptose or proliferate depending on the down or up regulation of target genes respectively, indicates the crucial link between these factors and cell fate choices Phosphorylation of pRb inactivates the protein during late G1 at a point in the cell cycle that coincides with the late G1 restriction point Phosphorylation of pRb by cdk4 releases E2F proteins bound to the dephosphorylated pRb, thereby relieving repression of these genes or activating their transcription (Taya 1997, Levine 1997) pRb is the principal target for inactivation by several tumor virus oncoproteins including Simian Virus 40, large T antigen, human papilloma virus E7 protein and adenoviral E1A suggesting that active pRb acts as a restraint to the mitogenic actions of these virus proteins (Taya 1997) and the same may be true for EBV

1 10 3.2 EBV, pRb and the Cell Cycle

Mostly, cell cycle events in B cell proliferation, have been studied in mouse models (Solvason *et al*, 2000) however a number of studies have examined the induction of cell cycle proteins in human B cells in response to EBV infection and it is these which provide the compelling evidence linking EBV to cell cycle control as a method of avoiding apoptosis and ensuring its own survival (Spender *et al*, 2001, Cannell *et al*, 1996, Spender *et al*, 1999, Kempkes *et al*, 1995a) The first cell cycle protein expressed after EBV infection of B-lymphocytes is cyclin D2, which is detected approximately 24 hours after EBV infection (Spender *et al*, 1999) There is a concomitant loss of the cyclin dependent kinase inhibitor p27kip1, and 6 hours later, phosphorylation/de-activation of the pRb protein occurs All these events occur during normal B cell proliferation as well as proliferation after EBV infection in B lymphocytes suggesting EBV exploits normal cell pathways to regulate pRb phosphorylation/inactivation during the cell cycle (Cannell *et al*, 1996, Kempkes *et al*, 1995a 1995b 1995c) By up-regulating cyclins involved in phosphorylation or by directly inactivating pRb itself, EBV could free E2F transcription factors to drive potentially infected cells through the cell cycle It has been shown

(Sinclair *et al*, 1994), that co expression of EBNA2 and EBNALP was sufficient to induce cyclin D2, and that this event closely followed the induction of the two latent EBV proteins The mechanism by which EBNA2 links to cyclin D2 remains to be characterized, as does the cooperative function of EBNA2 and EBNALP in regulating cyclin D2 expression However c-Myc which has been shown to be a direct target gene of EBNA2 has also been shown to trans-activate the D2 promoter (Spender *et al*, 2001, Kaiser *et al*, 1999, Bouchard *et al*, 1999) and c-Myc was therefore proposed as a link between EBNA2 and cyclin D2 Nevertheless, c-Myc was shown to trans-activate the cyclin D2 promoter using an E box located outside the region of the promoter trans-activated by EBNA2 thus the prospect of c-Myc as a link between EBNA2 and cyclin D2 is unlikely (Bouchard *et al*, 1999) Apart from up-regulating cyclins involved in the inactivation of pRB, EBV may also repress cyclin dependent kinase inhibitors again to facilitate the inactivation of pRB The induction of cyclm D2 is paralleled by the loss of the cyclm dependent kinase inhibitor p27kipl in response to EBV and in many other systems (Spender *et al*, 2001, Slingerland and Pagano 2000) Although the regulation of p27kipl is well studied in other systems, only one EBV protein has been shown to regulate its expression, namely EBNA3C (Parker *et al*, 2000) Because of the relationship between cyclinD2 and p27kipl, the link between EBNA3C and p27kipl suggests that EBNA3C along with EBNA2, EBNALP and LMP1 are important in driving cells through the G1 phase of the cell cycle

FIGURE 1.17. EBV PROTEINS INTERACT WITH A NUMBER OF ELEMENTS INVOLVED IN REGULATING CELL CYCLING AND PROLIFERATION

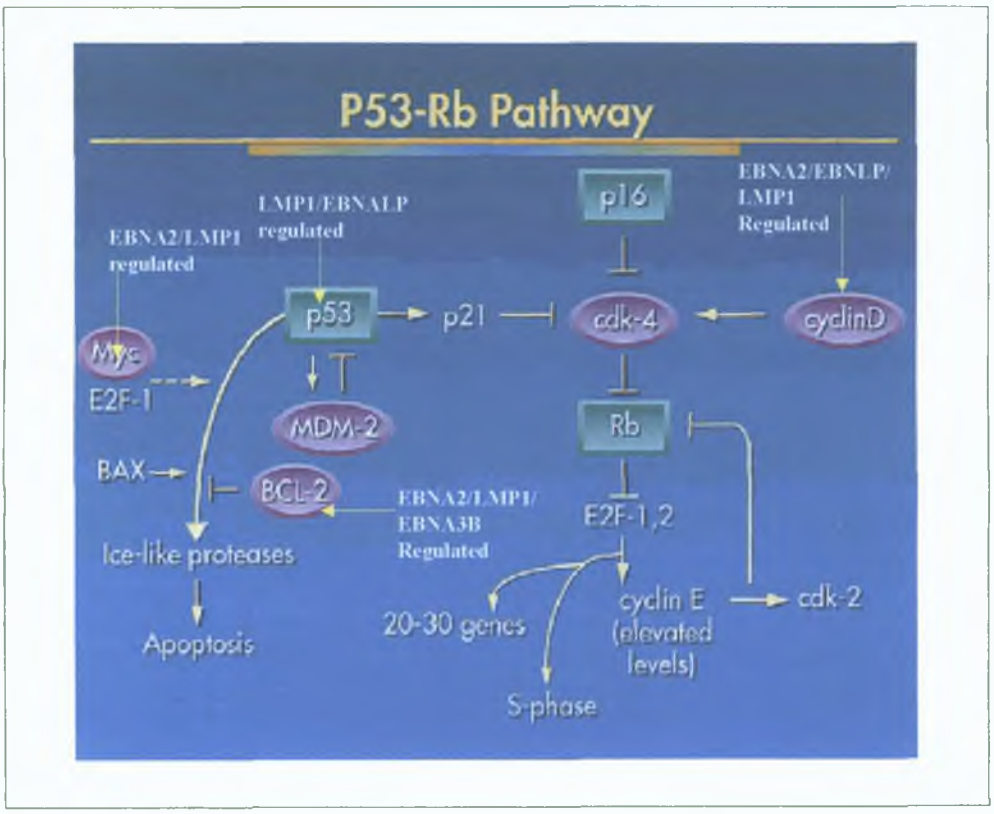


Figure 1.17. EBV interacts with a number of elements involved in regulating cell cycling, and proliferation, which ultimately may decide the apoptotic fate of the cell. The p53–Rb Pathway and the interrelationship between a number of oncogenes (purple circles) and tumor suppressor genes (green squares) that regulate the G1–S phase restriction point, its relation to a DNA damage checkpoint mediated by p53, and the choice by p53 whether to initiate a G1 arrest (via p21) or apoptosis. Shown are the p53–MDM2 autoregulatory loop that reverses this checkpoint control and the gene products that positively or negatively act on the probability of entering apoptosis. Those proteins which can also be regulated by EBV after infection are also indicated (adapted from Levine 1997)

1.10.3.3 C-MYC

c-Myc is a sequence specific DNA binding phosphoprotein that is a transcriptional activator and whose expression is required to drive cells through the G1 into the S phase of the cell cycle. A model explaining the role of c-Myc in the seemingly 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*, 1995) Thus c-Myc deregulation is regarded as priming normal proliferating cells for apoptosis

1.10.3.4 EBV AND C-MYC.

EBV-negative BL cells which display deregulated c-Myc expression due to the t(8,14) translocation proliferate rapidly in culture but are highly sensitive to apoptosis induced by growth factor withdrawal (Gregory *et al*, 1991) However, expression of either LMP1 or EBNA2 in these cell lines has been shown to have growth inhibitory and anti-apoptotic effects which may be in part due to down regulation of c-Myc in response to transcriptional down regulation of the translocated Ig-μ locas (Floettmann *et al*, 1996, Jochner *et al*, 1996) In the same way that deregulated c-Myc expression primes normal proliferating cells for apoptosis, EBV infection, primes LCLs for apoptosis by deregulating c-Myc expression Thus under favourable growth conditions LCLs immortalized by EBV infection proliferate rapidly, however under serum starvation conditions the cells die by apoptosis (reviewed by Cherney *et al*, 1994) C-Myc was specifically implicated in these effects since antisense oligonucleotides to c-myc specifically inhibited apoptosis in the growth factor deprived LCLs but suppressed growth of undeprived LCLs (Cherney *et al*, 1994) These studies showed that although deregulated c-Myc expression exerts these effects on cell growth and death, one or some of the EBV latent proteins expressed in LCLs must be responsible for the deregulation of c-Myc

Studies using an LCL in which the function of EBNA2 is dependent on the presence of estrogen designated ER/EB have revealed that EBNA2 is the EBV gene predominantly responsible for deregulating c-Myc in LCLs (Kempkes *et al*, 1995c) Later studies also revealed that cMyc is a direct target gene of EBNA2 (Kaiser *et al*, 1999) In the initial studies, primary B lymphocytes were infected with recombinant virus such that in the presence of estrogen, growth transformation of the infected B cells was achieved leading to the formation of estrogen dependent LCLs expressing EBNA2 (as a fusion protein

attached to the estrogen receptor hormone binding domain), in addition to the other EBV latent proteins Removal of estrogen, thus in the absence of EBNA2 activation, about 50% of the cells entered a quiescent non-growth phase while the remainder succumbed to apoptosis Growth arrest was recorded at both G1 and G2 phases of the cell cycle, which is indicative of a requirement for EBNA2 function at these two stages However only those arrested at G1 could be rescued (into continuing through the cell cycle), on induction of EBNA2 and this was shown to correlate with the induction of c-Myc and LMP1 expression, followed by the induction of cyclinD2 (G1 specific cyclin) and cdk4 and then the phosphorylated form of pRb In conjunction with the expression of the phosphorylated/inactive pRb, E2F also became detectable, thus EBNA2 sets a cascade into action which results in the activation of the E2F transcription factors allowing cells to cycle through to the S phase (Zimber-Strobl *et al*, 1996) These findings confirmed, that in LCLs functional EBNA2 is required not only for the induction and maintenance of deregulated c-Myc expression but also for cellular (B cells) proliferation

Since expression of EBNA2 leads to the up-regulation of LMP1, further studies were carried out to assess if the EBNA2 effect on proliferation was actually due to LMP1 expression In the absence of functional EBNA2 it was found that LMP1 could not sustain B cell proliferation (at levels of expression similar to that in LCLs), but did promote increased cell viability (Zimber-Strobl *et al*, 1996) Only in the presence of functioning EBNA2 was proliferation resumed These results, showing that LMP1 was not sufficient to maintain B cell proliferation in the absence of functional EBNA2 proved that EBNA2 acts independently of the up-regulation of LMP1 to maintain the proliferation of EBV immortalized B cells (Zimber-Strobl *et al*, 1996)

1.10.3.5 P53

Apart from pRb another transcriptional repressor p53, has a crucial function in regulating the passage of cells through the cell cycle P53 functions to limit the progression of cells containing damaged genomes through the cell cycle and it does this by either enforcing cell cycle arrest or triggering apoptosis (Levine 1997, Bates and Vousden 1996) The high incidence of p53 functional mutation in human tumours indicates its critical role in limiting neoplastic progression By inducing damaged cells to apoptose, p53 prevents the

progression of potentially mutant cells together with the tumourigenic risk they present Following DNA damage p53 levels increase swiftly as a result of stabilisation of the p53 protein which appears to be an important stage in the G1 arrest and apoptosis, which occurs in response to DNA damage Tumour cells that have lost functional p53 exhibit resistance to induction of apoptosis by a range of genotoxic agents (Allday 1996)

P53 can act as a transcription factor and its function in inducing growth arrest is facilitated by its ability to regulate specific target genes notably p21 (also known as WAF-1 or Cip-1), a cyclin dependent kinase inhibitor whose action arrests cells in late G1 High quantities of p21 inhibit kinase activity and thus cell cycle progression The link between p53 and apoptosis is still under investigation

1.10.3.6 EBV AND P53.

High incidences of p53 mutation have been identified in Burkitts lymphoma tumor biopsies and BL cell lines, occurring in 33% and 63% of cases respectively (Farrell, 1991) 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 1995) LMP1 however can protect against wild-type p53 mediated apoptosis, a phenomenon observed in both BL and epithelial cell backgrounds based on the use of cell lines expressing temperature sensitive p53 In BL cells, protection by LMP1 is probably a result of inducing Bcl2 expression (Okan *et al*, 1995) However in epithelial cells protection against p53 mediated apoptosis was as a result of the induction of the A20 protein (Fries *et al*, 1996) In both cases protection afforded by LMP1 did not involve an effect on the trans-activation function of p53 Another EBV latent gene EBNALP can also interact with p53 in vitro and also with pRb, however the functional significance of these interactions has yet to be identified as LP does not alter the role of these molecules when bound (Szekely *et al*, 1993)

Resting primary B cells are essentially p53 negative (Allday 1995) Some of these cells spontaneously undergo apoptosis when placed in culture through a p53 independent mechanism EBV infection of resting B cells rescues them from spontaneous cell death and also results in the accumulation of p53 (Allday 1995) This accumulation of p53 was

shown to be dependent on EBV gene expression as infection with a UV inactivated virus did not result in p53 accumulation (Chen 1996)

The observation that the presence of dephosphorylated pRb correlates with apoptosis in LCLs suggests that EBV must encode a function which enables the G1/S restriction point to be overcome and thus allow progression through the cell cycle, converting the p53 arrest response to apoptosis (Levine 1997) Candidate mechanisms by which EBV may overcome the G1/S restriction point include deregulation of c-myc or utility of a virally encoded E1A-like function, which generates high levels of transcriptionally active E2F

1.10.3.7 Cytokines

EBV induces the synthesis of various cytokines following B cell activation Cytokines are involved in lymphocyte proliferation and their expression suppresses apoptosis The most relevant cytokines in terms of EBV induced cell proliferation and apoptosis escape are IL-6 and IL-10 which both play an important role in the growth of EBV transformed cells (Haddad *et al*, 2001, Kitagawa *et al*, 2000) An IL-6 antibody has been shown to inhibit the growth of EBV immortalized cells in vivo in a recent clinical trial (Haddad *et al*, 2001) and IL-6 differentiated B cells die by apoptosis (Altmeyer *et al*, 1997) Both IL-10 and IL-6 have been shown to be regulated by LMP1 through NF-κB and the p38 stress activated protein kinase (Eliopoulos *et al*, 1997, Eliopoulos *et al*, 1999a, Nakagomi *et al*, 1994, Vockerodt *et al*, 2001)

New observations have also suggested that IL-10 may be regulated by EBV EBERs (Kitagawa *et al*, 2000) EBERs are expressed in EBV positive BL cells and in normal EBV transformed LCLs EBV positive Akata and Mutu BL cell lines express higher levels of IL-10 than their EBV negative subclones Transfection of EBV negative Akata BL cells with EBERs resulted in the induction of IL-10 while EBER1 or EBER2 was sufficient, the optimal induction of IL-10 was seen when both were introduced (Kitagawa *et al*, 2000) Increase in the levels of the cytokines as a result of EBER expression conferred tumourigenic and apoptotic resistance phenotypes in several BL cell lines (Akata, Mutu I and Bjab) (Kitagawa *et al*, 2000)

Another cytokine TGFβ is also targeted by EBV TGFβ peptides belong to a family of structurally related molecules with a variety of biological functions including differentiation and apoptosis on a variety of cell and tissue culture types including human B and T lymphocytes (Arvanitakis *et al*, 1995) EBV negative BL lines and EBV positive BL lines with a group I phenotype are generally sensitive to the growth inhibitory and apoptotic effects of TGFβ and this results from cells accumulating in G1 and undergoing apoptosis However EBV immortalized LCLs and EBV positive BLs that express the type III phenotype are resistant to the growth inhibitory effects of TGFβ (Altiok *et al*, 1993, MacDonald *et al*, 1996)

LMP1 can independently reproduce the ability of EBV to confer TGFβ resistance on infected B-lymphocytes and group III BLs (Arvanitakis *et al*, 1995) TGFβ is known to arrest cells in the mid to late G1 phase of the cell cycle in not only mitogen-activated human B cells but also in non-lymphoid cell systems (MacDonald *et al*, 1996, Chaouchi *et al*, 1995) This G1 arrest has been linked to an up-regulation of at least 3 inhibitors of cyclin dependent kinases (cdks) namely p15, p27, and p21 and in some cells to a down regulation of cdk4 (MacDonald *et al*, 1996) Binding of these inhibitors to cdk inhibits the formation of cdk-cyclin complexes that are required for progression through the cell cycle The inhibition of cyclin D function leads to a block in phosphorylation of pRb and this in turn represses the activity of the E2F transcription factors thus preventing cells from making the transition from G1 to S phase of the cell cycle The protection against TGFβ induced G1 arrest afforded by LMP1 was shown to be related to the induction of cyclin D 2 expression by LMP1 leading to the maintenance of pRb in a hyperphosphorylated state (Taya 1997) The maintenance of pRb in the hyperphosphorylated state by LMP1 thus leads to cell cycle progression regardless of the negative growth regulatory signal imposed by TGFβ

FIGURE 1.18. MOLECULAR INTERACTIONS BETWEEN EBV PROTEINS AND CELL SURVIVAL AND PROLIFERATION MACHINERY.

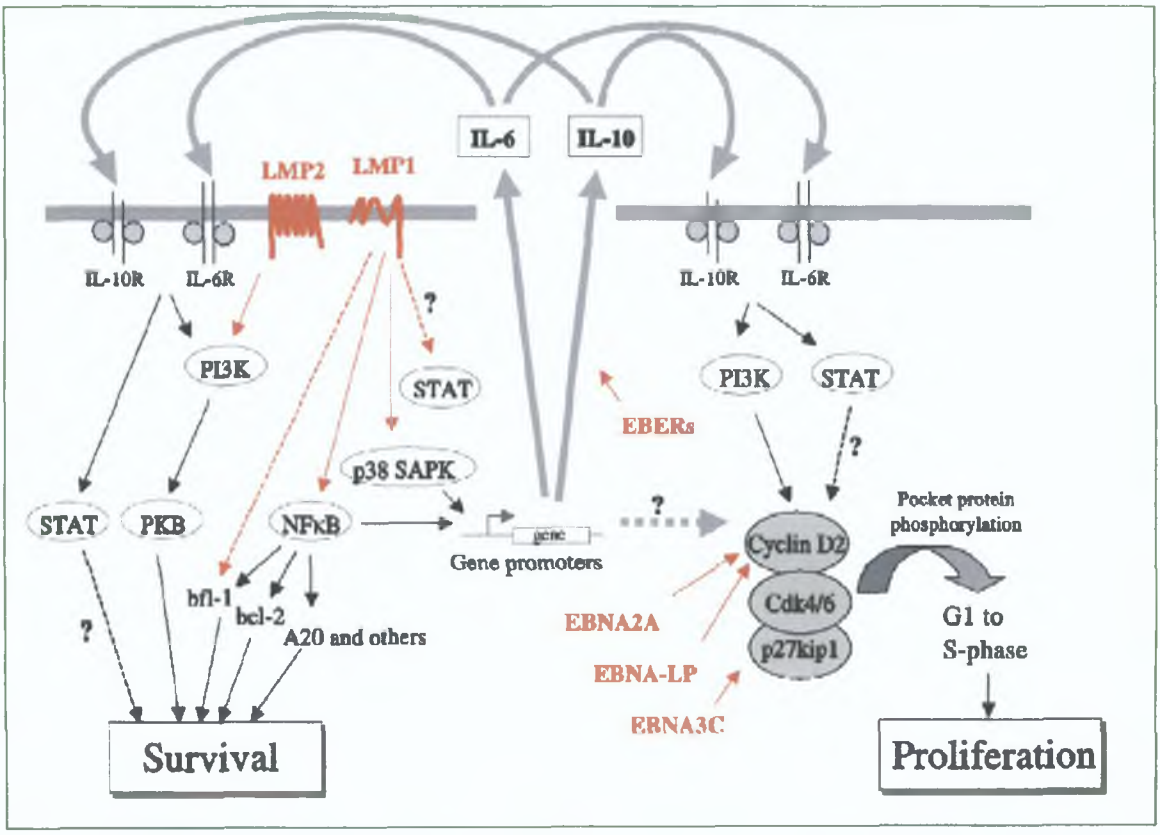


Figure 1.18. Overview of Molecular Interactions Between EBV Proteins and Cell Survival and Proliferation Machinery.

1.10.4 EBV LYTIC PROTEINS ENCODE ANTI-APOPTOTIC FUNCTIONS.

Unlike most viruses EBV maintains a very stable persistent infection in vivo. Another state in which EBV may be found is in the process of active replication and the production of progeny virions, the lytic cycle. If the cells are driven to proliferate by EBV then (as in type II latency), apoptosis will be the most likely outcome. In this situation additional repressors of apoptosis can provide a selective advantage. Consistent with this model, there are at least two known virally encoded proteins BHRF1 and BZLF that are synthesized during the lytic cycle that can provide a survival advantage to the infected cells (Allday 1996). These lytic gene products can therefore delay apoptosis in order to maximize viral production. Expression of BHRF1 in epithelial cells has been

shown to confer enhanced resistance to cisplatin induced apoptosis and can also inhibit differentiation (Dawson *et al*, 1995, Dawson *et al*, 1990) In lymphoid cells BZLF1 has been shown to interact with p53 and inhibit its trans-activating function, thereby providing a mechanism for preventing p53-mediated apoptosis However in epithelial cells expression of this EBV protein was shown to result in growth arrest without inhibiting the trans-activation function of p53 (Cayrol and Flemington 1996) EBV also encodes a viral homologue of IL-10 albeit expressed in the lytic virus productive cycle, which enhances the growth transformation of B cells infected with EBV (Stuart *et al*, 1995)

1.11.0 Relevance and Objectives of this Study.

Apoptosis can be induced in Type I BL cells and EBV negative cell lines with relative ease, compared to EBV positive, type III cell lines and LCLs which display a higher resistance to apoptosis induced by a variety of stimuli including growth factor withdrawal, and over expression of the p53 tumor suppressor protein (Henderson *et al*, 1991) and (Okan *et al*, 1995) This increase in resistance to apoptosis has been linked to the up-regulation of the anti-apoptotic *bcl-2* gene by EBV latent proteins including LMP1 and EBNA2 (Henderson *et al*, 1991, Rowe *et al*, 1994, Finke *et al*, 1992) and also the anti-apoptotic stress response proteins A20 and Mcl-1 (Fries *et al*, 1996, Laherty *et al*, 1992, Wang *et al*, 1996) More recently however, studies in our lab have shown that LMP1 also transcriptionally up-regulates another anti-apoptotic cellular gene, *bfl-1*, which similarly to *bcl2* also encodes an anti-apoptotic protein, namely Bfl-1 (D'Souza *et al*, 2000)

Bfl-1 suppresses apoptosis induced by p53, serum starvation and TNFα-induced cytotoxicity and exhibits proliferative and potent cooperative transforming properties in vitro (D'Souza *et al*, 2000, D'Sa-Eipper *et al*, 1996, Karsan *et al*, 1996, Zong *et al*, 1999) In the study by D'Souza *et al*, elevated *bfl-1* mRNA levels were shown to be a consistent feature of EBV immortalized B cell lines (LCL) and BL cell lines expressing the full spectrum of EBV latent proteins (Type III BL) In addition however, although LMP1 expression accounted for part of this up-regulation of *bfl-1* mRNA, other EBV

proteins were also likely to be involved as the steady state levels of *bfl-1* mRNA in LCLs and type III BL cell lines were dramatically higher than in cell lines in which LMP1 was being expressed as sole EBV protein In this study we pursued evidence for LMP1 independent mechanisms involved in the regulation of the *bfl-1* gene As the second major effector of phenotypic change in EBV infected cells, EBNA2 was a likely candidate for regulating the *bfl-1* expression and this study focuses on the role of EBNA2 in regulating the expression of this anti-apoptotic gene

CHAPTER 2

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIALS

2.1.1 CELL LINES

TABLE 2.1 CELL LINES USED IN THIS STUDY

Cell Line	EBV Status	Cell Classification	Description
DG75	-	EBV negative BL	Lymphoid B cell line derived from an Israeli Burkitt-like lymphoma case (Ben-Bassat *et al.*, 1977)
DG75 tTA EBNA2	-	BL (Stable transfectants)	Tetracycline-regulated system established in DG75 whereby the expression of EBNA2/LMP1 can be induced by the removal of tetracycline from the growth media (Floettmann *et al.*, 1996)
DG75 tTA LMP1	-		
DG75-SG5	-	BL (Stably Transfected cell pools)	EBV negative BL cell pools stably transfected to express EBNA2 and the CBF1 binding mutant EBNA2WW323SR. DG75 Cells were also stably transfected with the background pSG5 vector (Stratagene).
DG75-Sg5EBNA2	-		
DG75-SG5EBNA2WW323SR	-		
BL41-ER/EBNA2 (K3)	-	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.*, 1995a)
BL41P3HR1-ER/EBNA2 (9A)	+	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.*, 1995a]
BL41P3HR1-mNotchIC (cl31)	+	EBV positive BL (Stable transfectant).	EBV negative BL infected with the P3HR1 strain of EBV and subsequently stably transfected to express mouse NotchIC where Notch activation is dependent on the presence of estrogen (Strobl *et al.*, 2000)
Bjab	-	BL	
Mutu 1	+	Type I BL	Early passage BL cell line expressing EBNA1 as the sole viral latent gene (Gregory *et al.*, 1990)
Mutu 3 c95	+	Type III BL	A clone of Mutu I cells that have upon serial passage in culture, "drifted'' to express the entire complement of EBV latent genes (Gregory *et al.*, 1990).
Mutu 3 (LMP1-)	+	Type III BL	This is a partially characterized Mutu 3 clone which no longer expresses LMP-1
BL41,	-	EBV-negative BL	These cell lines are a matched set.
IARC 171	+	Type III phenotype LCL	BL41-an EBV negative BL cell line (Calender *et al.*, 1987). IARC 171 is a spontaneously transformed LCL established from the same patient from whom the BL41 cell line was derived (Andersson *et al.*, 1991)
IARC 290B	+	LCL	A spontaneously transformed LCL
X50-7	+	LCL	A spontaneously transformed LCL (Wilson *and* Miller 1979)
Ag876	+	Type III BL	Type III BL cell line expressing all of the EBV latent genes
Jurkat	-	T cell	Acute T-lymphocytic leukemic cell line (Brattsand et al., 1990)
C33A,	-	Epithelial cell	These are cervical epithelial cell lines. C33A is the parental cell line. (Miller,

			1995)
VSMC	-	Vascular Smooth Muscle Cell Line	Bovine Smooth muscle cell line (Professor Paul Cahill DCU).

All BL cell lines, LCLs, and the Jurkat cell lines were obtained from Professor Martin Rowe, University of Wales, Cardiff. The epithelial cell line C33A and its derivatives were a gift from Dr. Nancy Raab-Traub, University of North Carolina, USA. The estrogen responsive Cell Lines K3, 9A and Cl31 were gifts from Dr. Bettina Kempkes GSF Munich Germany. The vascular Smooth muscle Cell line was a gift from Professor Paul Cahill, Centre for Vascular Research DCU. Ireland.

2.1.2 Antibodies

The monoclonal antibodies against LMP-1, CS1-4, and the anti EBNA2 antibody PE2 were generous gifts from Professor Martin Rowe, University of Wales, Cardiff. The antibodies were supplied as cell culture supernatants and stored at 4°C or -20°C prior to dilution. The anti-EBNA2 antibody R3 was a gift from Dr. Bettina Kempkes GSF, Munich Germany. The anti A1/Bfl-1 antibody was purchased from Santa Cruz Biotechnology California. The Anti Notch Antibody Tan1 was obtained from Dr. Gerry Weinmaster. The alkaline-phosphatase (AP) conjugated anti-mouse antibodies were purchased from Promega and the alkaline-phosphatase (AP) conjugated anti-rabbit antibody was purchased from Boehringer Mannheim. The HRP conjugated anti-rabbit antibodies were purchased from Amersham Biosciences.

2.1.3 Bacterial Strains

E.COLI DH5α, GENOTYPE: F-, *END* A1, HSDR17 (R_K-,M_K=), *SUP*E44, *THI* –1,λ-, *REC* A1, *GYR* A96, *REL* A1, ϕ 80*LAC* ZδM15

E.COLI JM109, GENOTYPE: ENDA1, RECA1, GYRA96, THI, HSDR17 (RK -, MK +), RELA1, SUPE44, . λ-, Δ(LAC- PROAB), [F′, TRAD36,

PROA + B + , LACL Q □ΔM15]

E.COLI ES1301 MUTS, GENOTYPE: LACZ53, MUTS201::TN5, THYA36, RHA-5, METB1, DEOC, IN(RRND- RRNE)

E.COLI XL10 GOLD, GENOTYPE TET^{R} .Δ*(MCR*A)*183* . Δ *(MCRCB-HSDSMR-MRR)173 ENDA1 SUPE44 THI-1 RECA1 GYRA96 RELA1 LAC* HTE[F′ *PROAB LACIQZ* . Δ*M15* T*N10* (TET^{R}) AMY CAM^{R}]

2.1.4 EXPRESSION AND REPORTER CONSTRUCTS.

TABLE 2.2 PLASMIDS USED IN THIS STUDY

PLASMID	GIFT FROM	DESCRIPTION
pSG5 pSG5EBNA2 *pSG5EBNA2WW323SR	Lindsey Spender. Ludwig Institute for Cancer Research . Imperial college school of medicine London. *Proffessor Diane Hayward. Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA	PSG5EBNA2 (pPDL151) expresses the wild type B95-8 EBNA2 gene which has been cloned into pSG5 (Stratagene). The pSG5EBNA2WW323SR plasmid is identical to the pSG5EBNA2 vector except that EBNA2 has had two tryptophan residues mutated to a serine and arginine residues at positions 323 and 324 respectively. Thus pSG5EBNA2WW323SR does not bind CBF1. (Ling *et al.*, 1993)
pSG5 LMP-1	Professor Martin Rowe, University of Wales College of Medicine, Cardiff, U.K.	pSG5 LMP-1 is an LMP-1 expression plasmid in which the LMP-1 gene from the B95.8 virus was cloned in front of the SV40 promoter contained in pSG5 (Stratagene).
p-1374/+81Bfl-1 CAT p-1240/+81Bfl-1 CAT p-367/+81Bfl-1 CAT p-129/+81Bfl-1 CAT	Dr. Celine Gelinas, University of Medicine and Dentistry of New Jersey, New Jersey. U.S.A.	p-1374/+81Bfl-1 CAT contains the region –1374 to +81 of the human bfl-1 gene cloned in the promoter less vector, pCAT-basic, expressing a CAT reporter gene. p -1240/+81 Bfl-1 CAT, p-367/+81 Bfl-1 CAT and p-129/+81 Bfl-1 CAT contain the regions –1240 to +81, -367 to +81 and –129 to +81 of bfl-1 cloned into pCAT-basic. (Zong *et al.*, 1999)
p –1374/+81 Bfl-1 Luc	Dr. Brendan D'Souza, Laboratory for molecular and cellular biology. Dublin City University, Glasnevin, Dublin 9.	Consists of the BamH1-XbaI fragment of –1374/+81 Bfl-1 CAT inserted into the BamH1-HindIII portion of the pGl2 basic vector which contains the luciferase reporter (D'Souza *et al.*, 2000)

pcDNA3 HABfl-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 (D'Sa-Eipper *et al.*, 1996).
pUCCD21	Dr. Bettina Kempkes. Laboratrie fur Molekular biologie und Tumur genetik. GSF. Munich Germany.	Contains a 1.6Kb portion of the CD21 cDNA inserted into the multiple cloning site of pUC19 (Weiss *et al.*, 1986; Strobl *et al.*, 2000)
pGa50-7 pGA981-6	Dr. Bettina Kempkes. Laboratorie fur Molekular biologie und Tumur genetik. GSF. Munich Germany.	The pGa981-16 reporter construct was generated using a 50-bp oligonucleotide harboring both RBP-J binding sites of the EBV *TP1* promoter, which then was ligated as a hexamer into plasmid pGa50-7 (Minoguchi *et al.*, 1997).
pCMV pCMV EBNA3A pCMV EBNA3B pCMV EBNA3C	Dr Celine Gelinas University of Medicine and Dentistry of New Jersey, New Jersey. U.S.A.	Contains the EBNA3A/3B/3C cDNA inserted into the pCMV vector (Staratagene). (Le Roux *et al.*, 1994)
pcDNA3RPMS1	Dr. Paul J. Farrell. Ludwig Institute For Cancer Research, Imperial College School of Medicine, London.	Contains the full length RPMS1 cDNA ORF cloned into the pcDNA3HA vector (Invitrogen) (Smith *et al.*, 2000).
***pSG5Lp** (4x W repeats) pSG5LpΔY2 (4 x W repeats) **pRSP211** pSG5Lp (2x W repeats)**pJT125** pSG5Lp.RRR47/113AAA(2x W repeats) **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 expresses wild type EBNA-Lp containing 4W repeats. PRSP is a non functional mutant of pSG5Lp, which also contains 4 W repeats, however the Y2 region of Lp has been deleted. This expression plasmid is also known as pRSP2111. pSG5Lp containing only 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. All the EBNA-Lp expression plasmids are cloned into the pSG5 vector (Stratagene) (Harada and Kieff, 1997; Peng *et al.*, 2000).
pPHACS1 pED1 pED4	Dr. Bettina Kempkes. Laboratorie fur Molekular Biologie und Tumorgenetik GSF, Munich, Germany.	ED1 expresses NotchIC. PED4 expresses NotchIC without its RAM domain rendering it unable to interact with CBF1. pPHACS1 is the HA tagged vector into which NotchIC and NotchIC delta Ram were cloned

pJT111 pJT112 *pGK3puro	Dr. Paul Ling, Department of Molecular Virology and Microbiology, Baylor College of Medicine. Baylor Plaza, Houston, Texas.	(pJT111) Human Notch1IC cDNA with 3' terminal Flag epitope. EcoRI-BglII fragment cloned into EcoRI-BglII site of pSG5. PJT112Human Notch2IC cDNA with 3' Flag epitope. EcoRI-BamHI fragment cloned into EcoRI-BglII site of pSG5. (Gordadze *et al.*, 2001).
pCEP4 pCEP4-EtsDN	Dr. Hiroshi Sato, Department of Molecular Virology and Oncology, Cancer Research Institute, Kanazawa University Japan.	The cDNA sequence encoding Ets-DN, which lacks transcription activation domain and corresponds to amino acids residues 306 ± 441, was obtained by PCR amplification of human Ets-1 cDNA. The cDNa fragment thus obtained was inserted behind the ATG start codon introduced into pCEP4 expression plasmid, which contains hygromycin resistant gene (Invitrogen) (Kim *et al.*, 2000).

2.1.5 OLIGONUCLEOTIDES

MWG BIOTECH LTD.

Oligo for introducing the CBF1 Mutation in the bfl-1 promoter (For use with Altered sites mutagenesis kit: Promega)

-1374/+81MCBF1

5'-CCTTGTAGATTCGTGGTTCTAGAGTGAAATGGTACAACCC-3'

(PHOSPHORYLATED)

Oligos for introducing the Ets1/PU.1 Mutations in the bfl-1 promoter (For use with the XL Quickchange site Directed Mutagenesis kit: Stratagene)

-213 Fwd

5'-GGATTCTAATTTCTCCACCTGCAGCATTTAAGACTTGCAAAGCTG-3'

-213 REV

5'-CAGCTTTGCAAGTCTTAAATGCTGCAGGTGGAGAAATTAGAATCC-3'

-177Fwd

5'-GCAAAGCTGAATTAA TCACAGGCTGCAGAAGTGGCTTCTCTG-3'

-177REV

5'- CAGAGAAGCCACTTCTGCAGCCTGTGATTAATTCAGCTTTGC-3'

-146Fwd

5'-GGAAGTGGCTTCTCTGAAACATCTGCAGCTTTCACATTTT-3'

-146REV

5' –AAAATGTGAAAGCTGCAGATGTTTCAGAGAAGCCACTTCC-3'

Oligonucleotides used in the EMSA Analyses.

Cp [Ling, 1994]

5'-GATCTGGTGTAAACACGCCGTGGGAAAAAATTTATG-3' (TOP)

5'-GATCCATAAATTTTTTCCCACGGCGTGTTTACACCA-3' (BOTTOM)

Bflp

5'-GATCCCATTTCACATTCCCACCAGCAATCTACAAGG-3' (TOP)

5'-GATCCCTTGTAGATTGCTGGTGGGAATGTGAAATGG-3' (BOTTOM)

2.1.6 COMMERCIAL KITS AND RESTRICTION ENZYMES

Restriction enzymes	Roche Biochemicals and New England Biolabs
In Vitro Transcription kit	Pharmingen
Random Priming kit	Boehringer Mannheim
Sp6 DNA Polymerase	Boehringer Mannheim
BCA Protein assay kit	Pierce
Qiagen Tip-100	Qiagen
RPA kit	Pharmingen

Wizard PCR Preps DNA purification system	Promega
Altered sites II In Vitro Mutagenesis System	Promega
Quickchange XL, Site Directed Mutagenesis kit	Stratagene
Luciferase Reporter Reagents	Promega
ECL (Chemiluminescent reagent)	Pierce

2.2 CHEMICAL MATERIALS

Protein prestained markers	Sigma Colourburst
DNA Markers	
2-log DNA Ladder	NEB
1Kb Ladder	Invitrogen
α^{32}P labeled UTP (3000Ci/mol)	New England Nuclear
α^{32}P labeled dCTP (3000Ci/mol)	New England Nuclear
dNTPs	Pharmacia Biotech
Rnase A	Pharmacia Biotech
Poly (dI-dC)	Pharmacia Biotech
MicroSpin™ G-25 columns	Pharmacia Biotech
Marvel	Premier Beverages
Chloroform	BDH
Isopropanol	BDH

Sigma-Aldrich-Fluka Chemical Co..

Urea, Dithothreitol, Coomassie blue R, BCIP/NBT, Tetracycline, Liquid Phenol, Nitocellulose, Ampicillin, Potassium acetate, Tween-20, BSA, Sodium azide, Sigmacote, Ammonium phosphate, Mineral oil, α-Thiol-glycerol, PMSF, MOPS, BCS *E. coli* tRNA, Aprotinin, Formaldehyde, Micophenolic acid, BCS, DEAE-dextran, okadaic acid Rhubidium chloride

Merck:

Boric acid, Ammonium persulphate, Sodium acetate, Magnesium chloride, Glucose, Sodium chloride, Potassium chloride, Sodium hydroxide, Sodium dodecylsulphate, Calcium chloride, Glycine, Methanol

BDH·

TEMED, Bromophenol blue, Potassium dihydrogen phosphate, Potassium hydrogen phosphate, Sodium phosphate, Glycerol, Tris (hydroxymethyl) methylamine, EDTA, Magnesium sulphate, Ethidium bromide, Isoamyl alcohol, Hydrochloric acid, Acetic acid, Methanol, Isopropanol, Nondent P40, Sucrose, Paraformaldehyde

Boehringer Mannheim:

Agarose, Low melt agarose, IPTG, Hygromycin B, Geneticin (G418), Leupeptin

Oxoid·

Agar technical, Bacto-Tryptone, Yeast extract

KODAK:

X-ray film, X-ray film developer, X-ray film fixer

National diagnostics:

Acrylagel, Bis-acrylagel, Accugel

Gibco-BRL:

RPMI 1640, DMEM-H, Trypsin EDTA, Fetal calf serum, Penicillin, Streptomycin, L-Glutamine, Hepes, Sodium Pyruvate, 1Kb DNA ladder

2.3 DNA MANIPULATION

2.3.1 STORAGE OF DNA SAMPLES

DNA samples were stored in TE buffer pH 8 0 at 4^0C EDTA was used to chelate heavy metal ions that are needed for DNase activity while storage at pH 8 0 minimizes deamidation DNA was also stored in sterile distilled H_2O (dH_2O)

2 3.2 EQUILIBRATION OF PHENOL

Before use, phenol was equilibrated to pH 8 0 with TrisCI pH 8 0 as DNA partitions into the organic phase at <pH 7 8 Solid phenol was melted at 68^0C, hydroxyquinoline was added to a final concentration of 0 1% (w/v) (acts as an antioxidant, a chelator of metal ions, and an RNase inhibitor) An equal volume of buffer (0 5 M TrisCI pH 8) was added to the liquified phenol and stirred for 15 mins The two phases were then allowed to equilibrate and as much as possible of the upper aqueous phase was removed The extraction was repeated using equal volumes of 0 1 M TrisCl pH 8 until the pH of the phenol was > 7 8 An equal volume of TrisCl pH 7 and 0 2% (w/v) β-mercapthoethanol were added to the phenol, which was then stored at -20°C in the dark

2 3 3 PHENOL/CHLOROFORM EXTRACTION AND ETHANOL PRECIPITATION

Phenol/chloroform extraction and ethanol precipitation was carried out to concentrate nucleic acid samples or change the buffers in which a sample was dissolved An equal volume of phenol/chloroform/isoamyl alcohol (25 24 1) was added to the DNA solution, mixed by vortexing and centrifuged for 10 min at 13,000 x g The upper aqueous phase was removed, taking care not to take any material from the interphase, this was placed in a sterile microfuge tube An equal volume of chloroform/isoamyl alcohol (24 1) was added to the aqueous phase, vortexed as before and centrifuged for 5 min at 13,000 x g Again the upper aqueous phase was removed to a fresh tube One tenth volume of 3 M sodium acetate (pH 5 2) was added to the solution of DNA, mixed and then 2 volumes of 100% (v/v) ethanol This mixture was vortexed and incubated at room temperatures for 5

min The DNA samples were then centrifuged for 30 min at 12,000 x g at 4°C, the supernatant was removed and pellets were washed with 1 ml 70% (v/v) ethanol to remove excess salts The tube was centrifuged for 5 min at 10,000 x g, the supernatant was removed and pellets were air dried for approximately 10 min Pellets were resuspended in an appropriate volume of sterile Tris-EDTA (TE) (pH 8 0) or dH_2O

2 3.4 RESTRICTION DIGESTION OF DNA

Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition site The restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X) DNA was digested with restriction endonucleases for identification purposes or to linearise or cut fragments from a plasmid DNA digests were performed by adding

200 ng – 1 µg of DNA (Final concentration of <300 ng/µl)
1µl of enzyme/µg of DNA solution (10 U)
10 X buffer to a final concentration of 1X
dH_2O to the final volume required

The reaction was gently mixed, centrifuged, then incubated for 2 hr at the optimum enzyme temperature (between 37°C and 50°C, usually 37°C)

2.3.5 REPAIR OF DNA TERMINI

The majority of restriction endonucleases digest DNA leaving either a 5' or a 3' overhang When DNA is ligated together these ends must be compatible, if they are not the ends must be repaired and a blunt ended ligation carried out This was achieved using the Klenow fragment of *Ecoli* DNA polymerase I The DNA was resuspended at a concentration of 50 µg/ml in 1X Eco Pol buffer (supplied with the Klenow), dNTPs were added to a final concentration of 33 µM each, 1 µl of Klenow was added and the reaction was placed at 25°C for 15 min The enzyme was inactivated by heating to 70°C for 10

min This DNA was then purified by phenol/chloroform extraction and ethanol precipitation (see Section 2 3 3)

2.3 6 PREPARATION OF COMPETENT CELLS

A modified Rhubidium chloride ($RbCl_2$) method was employed to prepare competent cells This rubidium chloride protocol gives better transformation efficiencies than the CaCl2 procedure for most strains The procedure is an adaptation of one described by Hanahan et al 1985 (Altered Sites Mutagenesis Kit Promega) An *E coli* strain was streaked from a glycerol stock on to an LB agar plate and incubated at 37°C overnight An isolated colony was then picked using a sterile inoculating loop and used to inoculate 5 ml of LB (appendix A) broth This culture was incubated in a shaking incubator at 200 rpm overnight 37°C The resulting culture (2 5 ml) was then used to inoculate 250 ml of sterile Lb supplemented with 20mM MgSO4 and incubated in a 1L flask at 37°C until the O D of the culture at 640 mm was between 0 4 and 0 8 (approximately 4-5 hours) The cells were then transferred to two sterile 250 ml centrifuge tubes and pelleted by centrifugation at 4,500 x g, 4°C for 5 min The resulting pellets were resuspended in 0 4 original volume ice cold TFB1 (Appendix) (100ml for 250ml culture- 50ml/centrifuge tube) and the two pellets combined Cells were kept on ice for all subsequent steps and pipettes tubes and flasks were chilled The resuspendend cells were then incubated on ice at 4°C for 5 minutes and pelleted by centrifugation at 4500x g , 4°C for 5 minutes Cells were then gently resuspended in 1/25 of the original volume of ice-cold TFB2 (For 250ml subculture use 10ml) Cells were then incubated on ice for 1hr, aliquoted at 100ul/tube for storage at -70°C Prior to storage the aliquoted cells were snap frozen in a dry ice/isopropanol bath JM109 and DH5α competent cells prepared by this method are stable for 1 year ES1301 mutS competent cells generally are stable for 3–6 months

2 3.7 TRANSFORMATIONS

Two hundred microliters of competent cells were placed in a pre-chilled microcentifuge tube containing 10µl The contents were mixed gently and incubated on ice for 30 min,

during which time an aliquot of SOC (appendix A) was pre-heated at 42°C After 30 min on ice the cells were heat-pulsed at 42°C for 90 s followed by incubation on ice for a future 2 min One milliliter of preheated SOC was then added to the cells and incubated at 37°C in a shaking incubator for 1 hr 10 min The cells were concentrated by centrifugation following which ~800 µl of supernatant was removed and discarded The cells were resuspended in the remaining supernatant and plated out with the appropriate controls on LB plates containing ampicillin (125ug/ml) or tetracycline (12 5ug/ml) and incubated overnight at 37°C If the cells are transformed they become ampicillin/tetracycline resistant thus only transformed cells will yield colonies These were used to prepare broth cultures by inoculating 5 ml of LB containing ampicillin (125ug/ml) or tetracycline(10ug/ml), and incubated overnight at 37°C and DNA minipreparations were carried out as described in section 2 3 10

2.3.7.1 TRANSFORMING XL-10-GOLD ULTRACOMPETENT CELLS

XL-10-Gold ultracompetent cells were provided with the QuickChange XL Site Directed Mutagenesis kit™(Stratagene) The XL-10-Gold cells were thawed on ice and 45ul of ultracompetent cells per transformation were aliquoted into pre-chilled eppendorfs Two microlitres of β-ME mix provided with the kit was added to each of the 45ul aliquots of cells and the mixture was incubated on ice for 10 minutes with gentle mixing every 2 minutes Two microlitres of DpnI-treated DNA from each sample reaction was then added to the eppendorfs containing the cell-β-ME mixture and incubated on ice for 30 minutes after gentle mixing The transformation mixes were then heat pulsed at 42°C for 30 seconds in a water bath and incubated on ice for 2 minutes Next, 500ul of preheated (42°C) NZY^+ broth (Appendix) was added to each eppendorf and incubated at 37°C for 1 hour with constant agitation at 225 rpm Two hundred and fifty microlitres of the transformation reaction was then plated on two plates containing the appropriate antibiotic as well as X-gal (80ug/ml) and IPTG(20mM)

2.3.8 SMALL SCALE PREPARATION OF PLASMID DNA (MINIPREP)

This is a modification of the method of Birnboim and Doly, (1979) and Ish-Horowicz and Burke (1981) A single bacterial colony was used to inoculate 5 ml of LB medium (with appropriate antibiotic) and incubated overnight at 37°C An aliquot (1 5 ml) of this culture was added to a sterile microfuge tube and centrifuged for 30 s at room temperature, the remainder was stored at 4°C The medium was removed from the tube, leaving the pellet as dry as possible The pellet was resuspended thoroughly in 100 µl of solution I by vigorous vortexing To this 200 µl of freshly prepared solution II was added, the tube contents were mixed by inverting the tube rapidly a number of times Ice-cold solution III (150 µl) was added and the tubes were vortexed gently for 10 s

The lysate was centrifuged for 5 min at 12,000 x g, the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate An equal volume of phenol/chloroform/isoamyl alcohol (25 24 1) was added, mixed by vortexing and centrifuged for 5 min at 12,000 x g The upper aqueous phase was removed to a fresh table, to which 2 volumes of 100% (v/v) ethanol were added, the solution was vortexed and centrifuged for 5 min at 12,000 x g The supernatant was discarded, the pellet was washed with 1 ml 70% (v/v) ethanol, centrifuged as before and the supernatant was removed The pellet was air-dried, then resuspended in 50 µl of TE (pH 8 0), 1 µl of Dnase-free Rnase A (20 µg/ml) was also added, vortexed, incubated at 37°C for 1 hr then stored at 4°C Glycerol stocks of all bacterial cultures were prepared at this stage by the addition of 0 5 ml of a 50% (v/v) glycerol solution to 0 5 ml of the overnight bacterial culture of interest and storing at –80°C

2.3.9 QIAGEN™ PLASMID DNA PURIFICATION PROTOCOL

Plasmid DNA was purified using the QIAGEN-tip 100 solution system from Promega All buffers used are described in appendix A A glycerol stock of the bacteria of interest was streaked out on LB ampicillin/tetracycline agar and incubated overnight at 37°C, an isolated colony from this plate was used to inoculate a 5 ml LB ampicillin/tetracycline

starter culture and incubated in a shaking incubator at (-300rpm) 37°C for 8 hrs One millilitre of the starter culture was used to inoculated 25 ml of LB (containing the appropriate antibiotic) in a 250 ml sterile flask and incubated overnight in a shaking incubator at 37°C The O D of the culture must read 1-1 5 at 600 m m The following centrifugation steps were carried out using a JA-20 rotor in a Beckman centrifuge The bacteria culture was transferred to a centrifuge tube and centrifuged by spinning at 6,000 x g for 15 min at 4°C The supernatant was removed and the pellet was dried by inverting the tube on tissue paper and allowing the supernatant to drain off The bacterial pellet was resuspended completely in 4 ml of Buffer PI containing RnaseA (100μg/ml), 4 ml of freshly prepared Buffer P2 was added and incubated at room temperature for 5 min Following incubation, 10 ml of prechilled Buffer P3 was added, mixed gently by inverting the tube 5-6 times then incubated on ice for 20 min The mixture was then centrifuged for 1 hr at 20,000 x g at 4°C

The Qiagen-tip 100 was equilibrated by applying 4 ml 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 After the centrifugation step the supernatant was removed immediately from the tube without disturbing the pelleted material and applied to the column by filtering through 1MM filter paper The QIAGEN-tip was washed with 2 x 10 ml of Buffer QC DNA was then eluted with 5 ml of Buffer QF DNA as precipitated by adding 0 7 volumes of room-temperature isopropanol and centrifuged immediately at 15,000 x g for 30 min at 4°C and the supernatant was carefully removed The resulting pellet was washed with 70% (v/v) ethanol, allowed to air dry for 5 min and re-dissolved in a suitable volume of TE or dH_2O DNA was then quantified by spectrophotometric analysis as described in section 2 3 13

2.3.10 AGAROSE GEL ELECTROPHORESIS OF DNA

Electrophoresis through agarose gel is the standard method used to separate, identify, and purify DNA fragments The technique is simple, rapid to perform and can be used for the isolation of DNA fragments

An appropriate quantity of agarose or low melt agarose was added to 100 ml 1X TBE/TAE buffer The amount of agarose depends on the percentage agarose required This was decided in relation to the size of the DNA fragments being separated The mixture was boiled to dissolve, when cooled sufficiently (~60^{0}C) the gel was cast into the Hybaid horizontal gel electrophoresis system, the comb was inserted The gel was allowed to set before filling the chamber with 1X TBE/TAE, the comb was then removed To a 20 µl sample, 4 µl of DNA sample buffer was added and loaded into the wells made by the comb DNA sample buffer was also added at 1X concentration to 500 ng of a 1Kb DNA ladder which was loaded as a size marker The gel was run at constant voltage (5 V/cm) for 1-2 hr When complete, the gel was stained in ethidium bromide (0 5 mg/ml) for 30 min, placed in distilled water to destain for 15 min and viewed under UV illumination

2 3.11 ISOLATION OF DNA FROM AGAROSE GELS

Low melting point agarose were prepared in 1X TAE buffer (gel isolation is not carried out in TBE buffer as borate ions are difficult to remove from the resultant DNA solution) Ethidium bromide was added to the samples before electrophoresis so as to liquefy manipulations with the fragile low melting point agarose gels After electrophoresis, the gels were viewed under 70% UV illumination The time of exposure to UB light was kept to a minimum, as overexposure to UV would cause damage to the DNA The DNA band of interest was excised from the gel using a clean scalpel, excess agarose was cut away to liquefy the size of the gel which was then placed in a sterile microfuge tube

2.3.12 PURIFICATION OF DNA FROM LOW MELT AGAROSE

All DNA fragments of interest were purified from agarose using the Wizard PCR Preps DNA Purification System from Promega as follows, the agarose gel slice (300 mg) was placed in a sterile microcentifuge tube at 70^{0}C until the agarose had melted completely

One millilitre of the resin provided was added to the liquefied solution and mixed gently but thoroughly for 20 s (not vortexed)

A 2 ml syringe (plunger removed) was attached to the Wizard minicolumn the DNA/agarose/resin mix was added to the syringe, the plunger was replaced and the mix was pushed gently through the column The syringe was detached and the plunger removed again, then it was reattached and the column was washed with 2 ml of 80% (v/v) isopropanol The column was then spun at 10,000 x g for 2 min to dry the resin The DNA was eluted by adding 50 µl of TE or dH_2O to the minicolumn and allowed to stand for 1 min at room temperature and centrifuged again at 10,000 x g for 20 s The purified DNA was stored at 4^0C or at -20^0C

2.3.13 SPECTROPHOTOMETRIC ANALYSIS OF NUCLEIC ACIDS

DNA and RNA concentration was determined by measuring the absorbance at 260 nm, which is the wavelength at which nucleic acids absorb maximally ($^{\lambda}_{max}$) A 50 µg/ml preparation of pure DNA has an absorbance of 1 unit at 260 nm while 40 µg/ml of pure RNA also has an absorbance reading of 1 at this wavelength The purity of an RNA or DNA preparation was determined by reading absorbance at 260 nm, the $^{\lambda}_{max}$ for nucleic acids and at 280 nm, the $^{\lambda}_{max}$ for proteins and obtaining the ratio for these absorbances Pure DNA and RNA have A_{260}/A_{280} ratios of 1 8 and 2 0 respectively Lower ratios indicate the presence of protein while higher ratios often indicate residues of organic reagents

2.3 14 Site Directed Mutagenesis Reactions.

2.3.14.1 Altered Sites II In Vitro Mutagenesis™ Reactions

This kit was used to mutate the putative CBF1 binding site at position –243 to –249 on the bfl-1 promoter Initially, the –1374/+81 portion of the bfl-1 promoter was excised from the –1374/+81bfl-1 CAT vector by XbaI-HindIII restriction with the appropriate fragment excised from an agarose gel (Section X above) and purified in the usual manner The pAlter1 vector was similarly digested and the bfl-1 promoter was ligated into pAlter Recombinant clones were selected as white colonies on tetracycline (125ug/ml), X-gal IPTG plates (See Results chapter page X and Figure3 24i) The double stranded DNA from the recombinant plasmids was purified using Quiagen Tip 100 kit (Quiagen) and denatured by alkali denaturation, heat-denaturation does not work for this application because the two strands reanneal too quickly The mutagenesis reaction was then carried out, this reaction involved annealing the mutagenic oligonucleotide to the denatured DNA template while also annealing an ampicillin repair and a tetracycline knockout oligonucleotide The mutant strand incorporating these oligonucleotides was synthesized with T4 DNA polymerase and the DNA was transformed into the mismatch repair-minus E coli strain ES1301mutS, which were selected in liquid medium containing ampicillin(100ug/ml) DNA was prepared from this strain using miniprep procedure and transformed into JM109cells Mutants were screened on ampicillin plates and again DNA was prepared via the miniprep method and analysed by restriction analysis with the XbaI restriction endonuclease The mutated promoter was then excised by BamHI-HindIII restriction and ligated between BglII and SmaI in the multiple cloning site of pGL2-Basic (Figure 3 24i) Again the presence of the mutation was confirmed by endonuclease restriction with the XbaI enzyme The same procedures and sub cloning were undertaken without mutagenising the bfl-1 CBF1 site thus a pair of promoter constructs were generated, identical except that one no longer contained a CBF1 consensus sequence and instead contained an extra XbaI site

2.13.14.2 Alkaline Denaturation Reaction.

This generates enough DNA for 10 mutagenesis reactions

dsDNA template 0 5pmol	Xμl (approx 2μg)
2M NaOH, 2mM EDTA	2μl
sterile, deionized water to a final volume of	20μl

This mixture was incubated for 5 minutes at room temperature and 2μl of 2M ammonium acetate (pH 4 6) and 75μl of 100% ethanol were added This Reaction mixture was then incubated –70°C for 30 minutes The DNA was precipitated by centrifugation at top speed in a microcentrifuge for 15 minutes The pellet was washed with 200μl of 70% ethanol and pelleted by centrifugation again as before The pellet was then dissolved in 100μl of TE buffer (pH 8 0)

2.13 14.3 Annealing Reaction and Mutant Strand Synthesis

Mutagenesis Reaction (to synthesize –1374/+81 mCBF1bfl-1 Luc)

Alkaline-denatured dsDNA (bfl-1pAlter1)	10μl (0 05pmol)
Repair Oligonucleotide (2 2ng/μl), phosphorylated	1μl (0 25pmol)
Knockout Oligonucleotide (2 2ng/μl), phosphorylated	1μl (0 25pmol)
mutagenic oligonucleotide, phosphorylated (-1374/+81 mCBF1)	Xul (1 25pmol)
Annealing 10X Buffer	2μl
Sterile, deionized water to a final volume of	20μl

2.13.14.4 Control Reaction (to synthesize –1374/+81 wtbfl-1 Luc)

Alkaline-denatured dsDNA (bfl-1pAlter1)	10μl (0 05pmol)
Repair Oligonucleotide (2 2ng/μl), phosphorylated	1μl (0 25pmol)
Knockout Oligonucleotide (2 2ng/μl), phosphorylated	1μl (0 25pmol)
Annealing 10X Buffer	2μl
Sterile, deionized water to a final volume of	20μl

2.13 14.5 Control Reaction

Alkaline-denatured nonrecombinant pALTER ® dsDNA	10μl (0 05pmol)

Repair Oligonucleotide (2 2ng/µl), phosphorylated	1µl (0 25pmol)
Knockout Oligonucleotide (2 2ng/µl), phosphorylated	5µl (1 25pmol)
Annealing 10X Buffer	2µl
sterile, deionized water to a final volume of	20µl

The annealing reactions were heated to 75°Cfor 5 minutes and allowed to cool slowly to room temperature to minimize nonspecific annealing of the oligonucleotides The annealing reactions were then placed on ice and the following components were added in the order listed The final volume was 30µl

Sterile, deionized water	5µl
Synthesis 10X Buffer	3µl
T4 DNA Polymerase	1µl (5–10u)
T4 DNA Ligase	1µl (1–3u)

The reactions were then incubated at 37°C for 90 minutes to perform mutant strand synthesis and ligation The mutant plasmid DNA was then transformed (1 5ul of the above mixture) into competent ES1301mutS cells, miniprepped and JM109 competent cells were then transformed with the mutant/non mutant plasmid DNA After restriction analysis, the mutated/non-mutated promoter was sub cloned back into the pGL2Basic luciferase reporter vector

2 3.14.6 QUIKCHANGE XL SITE DIRECTED MUTAGENESIS™ REACTIONS

This kit was used to introduce mutations at putative Ets1 and PU 1 binding sites on the bfl-1 promoter (Positions of these sites are shown Chapter3, Figure 3 22A) A control reaction was set up as indicated below to test for the efficiency of transformation and mutant plasmid generation using blue white screening for the pWhitescript plasmid The pWhitescript contains a stop codon (TAA) at the position where a glutamine codon (CAA) would normally appear in the β-galactosidase gene of the pBluescriptIISK(-) phagemid XL-10-Gold cells transformed with this plasmid appear white on LB Amp

plates, containing IPTG and X-gal because β-galactosidase, activity has been destroyed, however the oligonucleotide control primers create a point mutation that reverts the T residue in the stop codon to the C residue of the glutamine codon thus reinstating β-galactosidase activity, therefore colonies which have bacteria expressing the functional β-galactosidase gene appear blue on the IPTG, X-gal plates

2.3.1.4.7 Control Reaction.

10X reaction buffer	5ul
pWhitescript 4 5Kb control plasmid(5ng/ul)	2ul (10ng)
Oligonucleotide control primer #1 (34-mer 100ng/ul)	1 25ul (125ng) of
Oigonucleotide control primer #2 (34-mer 100ng/ul)	1 25ul (125ng)
dNTP mix	1ul
QuickSolution	3ul
Double-distilled water to a final volume of 50ul	36 5ul

Then 1ul of *PfuTurbo* DNA polymerase (2 5U/ul) was added

2.3 14.8 Sample Reaction

10X reaction buffer	5ul
dsDNA template (-367/+81 bfl-1 Luc)	Xul (10ng)
oligonucleotide primer #1 (-213 Fwd/-177Fwd/-146Fwd)	Xul (125ng)
oligonucleotide primer #2 (-213Rev/-177Rev/-146Rev)	Xul (125ng)
dNTP mix	1ul
QuickSolution	3ul

ddH2O to a final volume of 50ul

Then 1ul of PfuTurbo DNA polymerase(2 5U/ul) was added

The control reaction and sample reactions were then placed in a Hybaid Omnigene thermocycler and cycled according to the table below in order to denature the plasmid, anneal the oligonucleotide primers containing the desired mutation and extend and incorporate the mutagenic primers to produce mutated plasmids containing nicked circular strands

Table 2.3. Thermocycling Conditions for generating Bfl-1 Mutation

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	2 minutes/kb of plasmid length*
3	1	68°C	7 minutes

*Since the template DNA (-367/+81 bfl-1 Luc) is 5.8Kb, the time allowed here was 11 minutes.

Following the temperature cycling, the reaction tubes were placed on ice for 2 minutes to allow the temperature to cool to 37°C. One microlitre of the restriction enzyme DpnI (10U/ul) was added to each reaction to digest non-mutated parental supercoiled DNA. The reactions were then gently mixed and spun down by quick centrifugation for 1 minute. The reactions were then placed in a water bath at 37°C for 1 hour. XL-10-Gold ultracompetent cells were then transformed with the various reaction mixtures. The number of blue colonies on the control plates were then used to assess a suitable number of colonies to pick for analysis from the sample reaction plates which contained 100ug/ml ampicillin. Since the XL-10-Gold cells are Tetracycline resistant but ampicillin sensitive, in theory the only colonies on the ampicillin plates should contain the mutated plasmid DNA. The presence of the mutated plasmid DNA was then verified by restriction digestion of purified DNA using the PstI endonuclease, as an additional PstI site had been introduced in each of the mutated plasmids. (See Results Figure 3.42).

2.4 Cell Culture Methods

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

2.4.1 Culture of Cells in Suspension

All media compositions and media supplements are given in Appendix A. The cell lines DG75, DG75 tTA EBNA2, DG75 tTA LMP1, Mutu 1, Mutu 3 c95, Mutu 3 LMP1-, X50-

7, BL41, IARC 171, IARC 290B, Ag876 III, Jurkat, BL41ER/E2 (K3), BL41P3HR1-ER/E2 (9A) and BL41P3HR1-ER/mNotchIC were maintained in supplemented RPMI 1640 Additional supplements were added to some culture media (see section 2 4 3) Cultures were seeded at a density of 2 x 10^5 to 5 x 10^5 cells per ml in 25 cm^2 flasks and expanded in 75 cm^2 flasks Cells were sub-cultured two or three times per week by harvesting into a sterile centrifuge tube and centrifuge at 1000 x g for 5 min at room temperature The cell pellet was resuspended gently in an appropriate volume of fresh media and replaced into the tissue culture flask All cell lines were incubated in a humid 5% CO_2 atmosphere at 37^{0}C in a Heraeus cell culture incubator

2.4.2 Culture of adherent cells

C33A VSMC were maintained in high glucose DMEM supplemented with 10% (v/v) FCS, penicillin (100 units/ml) and streptomycin (1 μg/ml) VSMC were maintained in Cultures were seeded into 25 cm^2 and 75 cm^2 tissue culture flasks As the cells were strongly adherent trypsinisation was required for harvesting prior to sub-culturing For trypinisation the medium was decanted and the cells were washed with 2 ml of sterile 1X PBS to remove any residual FCS which contains a trypsin-inhibitor activity (α_2 – macroglobulin) Two millilitres of 1X trypsin EDTA solution was placed in each flask and the flasks incubated at 37^{0}C for 5 min or until all the cells could be visualized as having detached form the flask surface The cell suspension was then decanted into a sterile centrifuge tube containing 5 ml of sterile supplemented media (FCS inhibits trypsin) and centrifuged at 1000 x g for 5 min Cells were resuspended in supplemented medium at 2 to 5 x 10^5 cells/ml, using 5 ml per 25 cm^2 flask and 15 ml per 75 cm^2 flask Cells were then incubated as in section 2 4 1

2 4.3 Media Supplements

Supplements were added to the growth media of certain cell lines to (a) improve cellular proliferation or (b) to select cells containing transfected plasmids L-cysteine is required for the survival and proliferation of most group 1 BL cell lines However, L-cysteine is

rapidly oxidated under normal culture conditions To improve proliferation of the group 1 Burkitt lymphoma cell line Mutu 1 α-thioglycerol was added to growth media as a stable substitute for L-cysteine The α-thioglycerol was dissolved in bathocuprine disulfonic acid (BCS) which effectively prevents autoxindation of thiols in liquid solutions Sodium pyruvate was also added to protect against H_2O_2, which may be generated HEPES was added to maintain an alkaline pH of 7 4 The cell lines DG75 tTA EBNA2 and DG75 tTA LMP1 are tetracycline responsive cell lines in which the gene of interest is cloned downstream if a promoter containing a binding site for a hybrid tetracycline regulated trans-activator (tTA) which is constitutively expressed from a second co-transfected plasmid Tetracycline binds the tTA and prevents it binding to the promoter, which remains silent, but upon removal of tetracycline from the growth medium the tTA binds the promoter sequence and activates transcription Theses cell lines were maintained in supplemented RPMI containing 1 μg/ml of tetracycline Every three weeks the transfected cells were reselected by the addition of 500 μg/ml of hygromycin to DG75 tTA 500 μg/ml of hygromycin and 1,000 μg/ml of geneticin (G418) to DG75 tTA EBNA 2 and 800 μg/ml of hygromycin and 2,000 μg/ml of genetiem (G418) to DG75 tTA LMPI

The stably transfected cell lines BL41ER/E2 (K3), BL41P3HR1ER/E2 (9A) and BL41P3HR1-ER/mNotchIC (Cl31) were maintained in supplemented RPMI 1640 these cell lines were maintained under permanent selection with 800ug/ml and 1200ug/ml of G418 in the K3 and 9A media respectively Hygromycin at a final concentration of 150ug/ml was added to the Cl31 media

2.4.4 Cell Counts

Cell counts were performed using an improved Neubauer haemocytometer slide Trypan blue exclusion dye was routinely used to determine cell viability Ten microlitres of trypan blue was added to 90 μl of a cell suspension and mixed A sample of this mixture was added to the counting chamber of the haemocytometer and cells were visualized by light microscopy Viable cells excluded the dye and remained clear while dead cells

stained blue Cell numbers were ascertained by multiplying the average cell count by the dilution factor and again by the volume of the haemocytometer

2 4.5 Cell Storage and Recovery

Cell stocks were prepared for long term storage as follows Suspension cells 1 x 10^7 cells in exponential phase were pelleted and resuspended in 800 µl of supplemented RPMI to which 100 µl of FCS was added, then placed on ice for 10 min DMSO was added to a final concentration of 10% (v/v), mixed gently and transferred to a sterile cryotube Adherent cells one confluent 75 cm^2 flask of adherent cells was used per cell stock Adherent cells were washed with 1X PBS followed by trypsinization and resuspension in 900 µl of FCS and 100 µl 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 immersed in liquid nitrogen in a cryofreezer (Cooper Cryoservices Ltd) Cells were recovered form liquid nitrogen by thawing rapidly at 37^{0}C and transferring to a sterile centrifuge tube containing 5 ml of prewarmed supplemented media The cells were centrifuged at 1000 x g for 5 min, the pellet was resuspended in 5-10 ml of fresh supplemented medium, transferred to a culture flask and incubated at 30^{0}C in 5% CO_2

2.4.6 Induction of Gene Expression using the Tetracycline-Regulated System

After counting cells, an appropriate volume was pelleted at 1,000 x g for 5 min and washed three times in sterile PBS Cells were then incubated in supplemented RPMI medium for 1 h at 37°C Cells were then washed again once in sterile PBS and seeded at approximately 2 x 10^5 cells per ml in the presence or absence of tetracycline Uninduced cells (Tet+) were washed as per induced cells (Tet -) but were constantly maintained in the presence of tetracycline

2.4.7 Activation of EBNA2 or mNotchIC in the K3, 9A and Cl31 Cell Lines

In the case of the Estrogen responsive K3, 9A and Cl31 cell lines, the EBNA2 or Notch activation domains are fused to the estrogen receptor binding domain such that the activity of EBNA2 or NotchIC is dependent on the presence of estrogen In experiments where EBNA2 or Notch activation was required, cells were washed twice in PBS and placed in fresh supplemented RPMI with the appropriate antibiotics for selection, estrogen (β estradiol) was then added to a final concentration of 1uM

2.4.8 Transient Transfections

Transient transfection of cells was performed by the DEAE-dextran protocol, by electroporation or by the Lipofectamine method In all cases, cells were seeded at a density of 5 x 10^5 per ml of medium 24 h prior to transfection Total DNA for transfection was normally co-precipitated in 100% ethanol, then washed in 70% ethanol and resuspended in a final volume of 30 µl T E pH 7 4 (this pH is very important), using the same total quantity of DNA per transfection

2.4.8.1 Electroporation of B Lymphocytes

On the day of transfection, cells were centrifuged at 1,000 x g for 5 min, washed once in a small (one-fifth) volume of cold complete RPMI medium and resuspended in cold complete RPMI medium at a density of 2 x 10^7 cells per ml For each transfection, a 60 mm culture dish with 5 ml complete medium (RPMI 1640/glutamine/10 %FCS) was preincubated at 37°C for at least 1 h prior to transfection The DNA to be transfected (dissolved in 30 µl T E pH 7 4) was added to the bottom of labeled cuvettes (Biorad, 0 4 mm) placed on ice 500µl of the cell suspension (= 1 x 10^7 cells) was then dispensed into the cuvettes and mixed with the DNA Each cell/DNA mix was then pulsed at either 270 V in the case of Mutu I cells or 300 V in the case of IARC 290B and IB4 cells with a capacitance of 960µF (with capacitance extender) in a Biorad electroporator, and the cuvettes returned to ice immediately after electroporation Cells must not stay longer than 10 mm on ice before being transferred to media Contents of cuvettes were

transferred to culture dishes using a micropipette and yellow tip, treating the cells gently Cuvettes were washed with media from the culture dish, and placed at 37°C m a 5% CO_2 incubator for the required amount of time

2.4.8 2 DEAE-DEXTRAN-MEDIATED TRANSFECTION

Details of all solutions required for this protocol are given in the Appendix section The day before transfection, cells were seeded at 5 x 10^5 cells/ml After 24 h in culture, cells were counted again – it was essential for cell numbers to have almost doubled before beginning the transfection, thus ensuring that cell growth is in logarithmic phase and that cells are at their optimum for the uptake of DNA during transfection 1 4 x 10^7 cells were used for each transfection On the day of transfection, cells were centrifuged at 1,000 x g for 5 min, resuspended in sterile TBS and 1 4 x 10^7 cells dispensed into 25 ml sterilin tubes After centrifugation, all of the supernatant was removed with any traces of TBS carefully (without disturbing the cell pellet) removed using a pipette tip The DNA-DEAE dextran mixes were prepared in microfuge tubes as follows A total of between 11 and 16 0 µg of DNA made up to a volume of 30 µl with TE buffer pH 7 4 was used per transfection and 570 µl TBS was then added to the DNA in a microfuge tube For any transfection set the total amount of DNA was kept constant with any deficiencies made up with the corresponding empty vector Then, 600 µl of 1 mg/ml DEAE dextran solution made up in TBS was mixed with the DNA solution and the cell pellet gently resuspended in the DNA-DEAE dextran mix The transfection cocktails were incubated at room temperature for 30 min (40 min in the case of Jurkat T cells) with gentle swirling every 5-10 min to allow homogenisation Transfections were terminated by adding 10 ml of warm (37°C) complete RPMI medium and the cells pelleted by centrifugation at 1,000g for 5 min The cells were then washed once more with 10 ml complete medium and then transferred in 10 ml of medium to 25 cm^2 cell culture flasks for incubation

2.4.8 3 LIPOFECTAMINEPLUS™ MEDIATED TRANSFECTION

The day before transfection, cells were trypsinized and counted so that they were 50-90% confluent on the day of transfection At the time of plating and during transfection, no antibiotics were added - this helps cell growth and allows transfection without rinsing the cells DNA for transfection was diluted into serum free DMEM, mixed, and the PLUS Reagent added This pre-complexed DNA mixture was then incubated at room temperature (RT) for 15 mm It was important to dilute the DNA into medium and mix before adding PLUS Reagent as DNA may precipitate LIPOFECTAMINE Reagent was then diluted into serum free medium in a second tube and mixed and this diluted LIPOFECTAMINE Reagent was combined with the DNA-plus reagent mixture and incubated at room temperature for 15 minutes to allow complexes to form At this point, fresh serum free media was placed on the cells The DNA-Plus- LIPOFECTAMINE reagent complexes were then added to the cells, mixed gently and incubated at 37°C at 5% CO2 for 3 h After 3hr incubation, the volume of medium was increased to the normal volume (2ml) and serum was added to each well to a final concentration of 10% Luciferase reporter activity was then measured after 48 hours using the luciferase assay

2.4.9 LUCIFERASE ASSAY

Cells for luciferase assay were normally harvested 24 or 48 h post-transfection Cells were pelleted by centrifugation at 1,000 x g for 5 min at room temperature, washed once in sterile PBS and then transferred to a microfuge tube in 1 ml of sterile PBS After centrifugation at 5,000 x g for 5 min at room temperature, the supernatant was completely removed and the cell pellet resuspended in 100 µl of Reporter lysis buffer (1 X, Luciferase Assay System, Promega) The tubes were vortexed for 10-15 s and lysis allowed to proceed for 15 min on ice The lysates were clarified by centrifugation at 12,000 x g for 5 min and the supernatant saved in a fresh tube Samples were stored at – 80^{0}C until required, when 20 µl was taken for assay Samples are stable in lysis buffer over several freeze-thaw cycles At the time of assay, it was important to allow sufficient time for the detection reagent (stored at - 80^{0}C) to equilibrate to room temperature

Subsequently, 100 µl of detection reagent was added to the sample, mixed by repetitive pipetting (3 times) and light emission integrated over a period of 60 s after lag period of 10 s Luciferase activity levels were adjusted for transfection efficiencies, estimated using β-galactosidase activities expressed from pCMVlacZ construct (1µg) included in the transfections

2.4.10 β-GALACTOSIDASE ASSAY

The β-galactosidase assay was performed using the same lysates as that used for measuring the luciferase activity Mock-transfected (lacking DNA) cells were included as a control to account for endogenous enzyme activity and these values used to calculate the activity due to transfection of the lac Z construct Cell extract (30 µl) was added to 3 µl 100X Mg solution, 66 µl ONPG and 201 µl 0 1 M sodium phosphate (see Appendix A) and incubated at 37^0C for 30 min or until a faint yellow color developed A reaction tube was included in which ONPG substrate was omitted in order to obtain a background reading Reactions were terminated by adding 500 µl 1 M Na_2CO_3 Optical densities were read at 420nm over a linear range of 0 2-0 8

2.4.11 STABLE TRANSFECTIONS

2.4.11.1 ESTABLISHMENT OF SENSITIVITY OF CELLS TO PUROMYCIN

DG75 cells, in logarithmic phase of growth, were seeded at a density of 1 x 10^5 cells per ml in media containing, 0, 0 25, 0 5, 1 0, 2 0, 5 0, and 10 0 ug of active puromycin per ml and grown for 10 days under standard conditions Media was changed every 3 days during this time and cell viability measured every 3 days by trypan blue exclusion The minimum concentration of puromycin which killed > 95% of the cells after the 10 day period was determined and this concentration then used in subsequent selection procedures for the generation of stable transfectants (section 2 4 11 2)

2.4.11.2 Generation of a pool of transfectants OF DG75 cells Expressing EBNA2 and EBNA2WW323SR

Stable transfectants of DG75 expressing EBNA2 or EBNA2WW323SR were established by electroporation of the cells with 10 μg of pSG5EBNA2 or pSG5EBNA2WW323SR as well as 3ug of the antibiotic resistant pGK3puro vector which expresses the *pac* gene The expression of the *pac* gene confers puromycin resistance to transfected mammalian cells expressing it and was useful for the selection of cells harboring the EBNA2 and pGK3puro plasmids carrying puromycin resistance genes, it is also useful in that it quickly kills eukaryotic cells that do not contain the *pac* gene A mock transfection (without DNA) was also set up and the cells maintained under similar experimental conditions as the *pSG5EBNA2 or pSG5EBNA2WW323SR* transfected cells Also a transfection with pGK3Puro and pSG5, the vector into which EBNA2/EBNA2WW323SR had been cloned was undertaken At 48 h post-electroporation, the media was replaced with selection medium i e normal growth medium but containing 1μg of active puromycin per ml Media containing antibiotic was changed every 3 – 4 days for the next 12 days by which time all of the mock-transfected cells were killed The transfected population of cells was then examined for the expression of EBNA2 or EBNA2WW323SR and their functional consequences on apoptosis and cell cycling within the next 14 days

2 4.12 Apoptosis Assays

2.4 12.1 Acridine Orange Staining

A sample of the cell culture was mixed with acridine orange at a final concentration of 5μg/ml, placed on a glass slide and immediately visualised under a fluorescence microscope Non-apoptotic cells display a diffuse chromatin staining pattern while apoptotic cells display a characteristic condensed chromatin-staining pattern (Gregory et al , 1991) Acridine orange is a metachromatic dye which differentially stains double stranded(ds) and single stranded(ss) nucleic acids When acridine orange intercalates into the dsDNA of healthy cells it emits green fluorescence upon excitation at 480-490nm On

the contrary it emits orange fluorescence when it interacts with the ssDNA of apoptotic cells or RNA The percentage of apoptotic cells was determined from counts on at least 150 cells per individual culture Images were captured using Kodak 1D image analysis software

2 4.12 2 Light Scatter and Cell Cycle 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 some information on the *relative size* of individual cells, whereas side light scatter provides some information on the *relative granularity* of individual cells In this case these two are combined to identify the characteristic light scatter pattern emitted by condensed, shrunken apoptotic cells, presented as dot plots

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 in the process Propidium Iodide is a fluorescent dye that binds to nucleotides Propidium iodide (PI) penetrates only damaged cellular membranes Intercalation complexes are formed by PI with double-stranded DNA, which effect an amplification of the fluorescence The resulting data is displayed as a histogram which depicts the relative numbers of cells in the various phases of the cell cycle

In order to stain cells with propidium iodide, cells were washed twice with ice cold PBS Cell pellets were then resuspended in ice-cold Ethanol PBS 70 30, and fixed at 4°C for at least 40 minutes Cells were then pelleted by centrifugation at 2000xg for 5 minutes in a microfuge and resuspended in 40ug/ml Propidium iodide made up in PBS and incubated on ice for 15 minutes Prior to analysis, RnaseA was added to a final concentration of

50ug/ml Light scatter and cell cycling were then analysed using a Flow Cytometer (FacsCalibur, Beckton Dickinson) at 488nm, measuring forward and side (orthogonal) light scatter CellQuest software deconvaluted the data to create dot plots depicting cell density and granularity and histograms depicting cell cycle distribution

2.5 RNA ANALYSIS

2.5 1 RNASE-FREE ENVIRONMENT

RNA is easily degraded by ubiquitous RNases thus measures were employed to avoid this potential hazard All glassware and metal spatulas were baked prior to use at 180°C for 16 - 24 h in order to inactivate any RNases Sterile disposable plasticware is generally considered RNase-free and thus did not require treatment RNases are resistant to autoclaving but they can be deactivated by the chemical diethylpyro-carbonate (DEPC) when it is added to solutions at a final concentration of 0 1 % (v/v), incubated at room temperature for 18 hr and autoclaved Solutions, which contain amines such as Tris, cannot be DEPC-treated as the DEPC is inactivated by these chemicals Solutions containing these chemicals were prepared using DEPC-treated upH_2O followed by autoclaving Hands are a major source of RNase contamination, thus, gloves were used at all times and changed frequently

2.5.1 RNA EXTRACTION FROM CULTURED CELLS

Prior to RNA isolation the cells were examined by phase contrast microscopy to determine the condition of the cells A cell count was performed as described in section 2 4 4 RNA was extracted from cultured cells using the commercial reagent RNA ISOLATOR™ Cells grown in suspension were pelleted and then lysed in RNA ISOLATOR™ repetitive pipetting One millilitre of RNA ISOLATOR™ was used per 1 x 10^7 cultured cells Cells grown in monolayers were lysed directly in the cell culture flasks as trypsin can lead to the introduction of Rnases, cells were removed from the flask by a sterile cell scraper and homogenized as above The homogenised sample was

incubated at room temperature for 5 min to allow complete dissociation of nuclear protein complex, (the procedure may be stopped at this point by storing samples at – 70°C) Phase separation was achieved by adding 0 2 ml of chloroform per 1 ml of RNA ISOLATOR The samples were covered and shaken gently but thoroughly for 15 s or until completely emulsified Samples were incubated at room temperature for 15 m The resulting mixture was centrifuged at 12,000 x g for 15 min at 4°C Following centrifugation the mixture separates into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase The aqueous phase, which contains the RNA, was removed to a fresh tube and RNA was precipitated by adding 0 5 ml of isopropanol per ml of RNA ISOLATOR used initially The samples were stored for 10 mm at room temperature, then centrifuged at 12,000 x g for 10 min at 4°C The resulting RNA pellet was washed using 1 ml of 75% (v/v) ethanol by inverting the tube 5 times The pellets were then recentrifuged at 10,000 x g for 5 min at 4°C and the 75% (v/v) ethanol was removed 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 and gel electrophoretic analysis

2.5.2 RNA Analysis by Gel Electrophoresis.

In order to check the integrity of RNA, isolated samples were run on 1% (v/v) agarose gels These gels were prepared as outlined in section 2 3 12 The RNA samples (5 µl) were prepared for electrophoresis by adding 15 µl of RNA sample buffer and 3 µl of RNA loading buffer The samples were heated to 65°C for 10 min prior to loading on the gel The gel was run in 1X TAE as described in section 2 3 12 As ethidium bromide is included in the RNA loading buffer the gels did not require further staining and could be visualized directly on a UV transilluminator The presence of two strongly staining bands represent the 28 S and the 18 S ribosomal RNAs, which indicated intact RNA Degradation is observed by a smear running down the length of the gel

2.5.3 NORTHERN BLOTTING

Northern blotting was carried out according to Sambrook *et al*, (1989) RNA of interest was first separated on a formaldehyde gel

2.5.3.1 TREATMENT OF ELECTROPHORESIS APPARATUS

Prior to running an RNA gel, the electrophoresis apparatus was treated to remove any RNase The tank, gel tray, comb and lid were washed in detergent and rinsed well in DEPC-treated H_2O then air dried in 100% (v/v) ethanol The tank, gel tray and comb were immersed in a 3% (v/v) solution of Hydrogen peroxide for 15 minutes The apparatus was then rinsed thoroughly in DEPC-treated upH_2O and allowed to dry

2.5.3.2 ELECTROPHORESIS OF RNA/MRNA THROUGH GELS CONTAINING FORMALDEHYDE

As formaldehyde vapours are toxic these gels were prepared in a fume hood The gel was prepared by melting the appropriate amount of agarose in water cooling to 60°C and adding 5X formaldehyde gel running buffer and formaldehyde to give a final concentration of 1 X and 2 2 M respectively The gel was cast in a fume hood and allowed to set for at least 1 h The samples were prepared by mixing the following in a microcentrifuge tube

RNA (up to 30 µg)	1 5µl
5X formaldehyde gel running buffer	2 0µl
Formaldehyde	3 5µl
Formamide	10µl

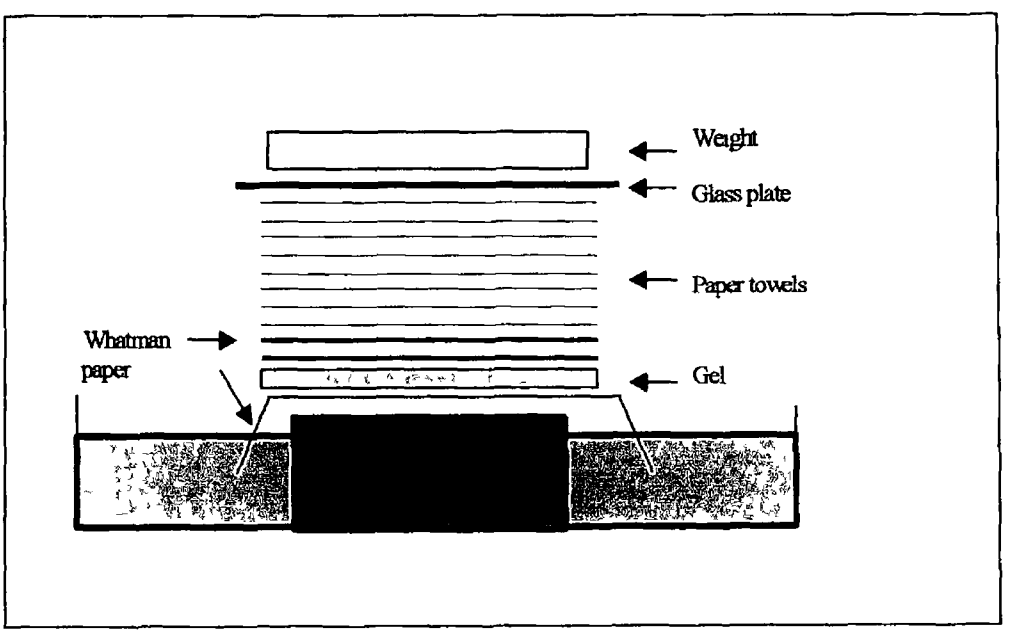
Ethidium bromide was added to samples (0 01 µg/sample), which were to be used for northern blots in order to examine the RNA briefly under UV (70% strength) for equal loading prior to blotting The samples were incubated at 65°C for 15 min, chilled on ice

then centrifuged briefly to collect the sample Sterile DEPC-treated gel loading buffer (2 µl) was added to each sample and applied to the gel immediately after prerunning the gel for five minutes at 5V/cm Gels were run while submerged in IX formaldehyde gel running buffer for approximately 3 hr or until the dye front had migrated two thirds of the way down the gel The gel was removed from the buffer and viewed under UV light Samples to be blotted were treated as described in the next section.

2.5.3.3 TRANSFER OF DENATURED RNA TO NITROCELLULOSE FILTERS

Gels which contained formaldehyde were first washed in several changes of DEPC-treated H_2O As the percentage agarose used to prepare the gel was greater than 1% (w/v), the gel was soaked in 0 05 M NaOH for 20 min this treatment partially hydrolyses the RNA and improves the efficiency of transfer The gel was then rinsed in DEPC treated H_2) and soaked in 20X SSC for 45 min (Sambrook et al , 1982) Unused areas of the gel were trimmed away and the top left hand corner was cut for orientation purposes in this and succeeding operations Capillary transfer was used to transfer the RNA onto the filter transfer was set up as illustrated in Fig 2 1

FIGURE 2.1. CAPILLARY TRANSFER IN NORTHERN BLOTTING



A solid support was placed in a bath of 20X SSC A sheet of 3MM Whatman paper was cut to cover the support and dip down either side into the buffer The washed gel is then placed (wells facing down) on to the Whatman and covered by a piece of nitrocellulose membrane cut to the size of the gel This membrane had been floated on a bath of deionized water and wetted completely from below then soaked in 20X SSC for at least 5 min prior to placing it on the gel The buffer chamber was then covered with cling film Two pieces of 3MM Whatman paper which had been soaked in 2X SSC were then placed on top of the filter paper, care was taken at all times to ensure that no bubbles were trapped when preparing the transfer A stack of paper towels were then placed on top of the gel and held in place by a weight Transfer was allowed to continue overnight After transfer was completed the saturated paper towels were removed, as was the Whatman paper The gel and the filter paper were removed together and turned upside down on a clean piece of towel The position of the wells were marked using a ballpoint pen, the gel was then discarded The filter was washed briefly in 6X SSC to remove any remains of the gel then placed on a fresh sheet of Whatman paper and allowed to dry for at least 30 mm The dried filter was then placed between two pieces of 3MM Whatman paper and baked in an oven at 80°C for 2 hr The filter was then used directly for prehybridisation or stored at room temperature wrapped in aluminium foil

2.5.3.4 GENERATION OF THE BFL-1 AND GAPDH RIBOPROBES.

GAPDH template DNA was purchased from Pharmingen and this was used in a T7 RNA polymerase driven in-vitro transcription reaction as per probe synthesis for the RPA assay (Section 2 5 4 below) The bfl-1 riboprobe was generated by linearising the pcDNA3Habfl-1 construct by BamHI restriction and subsequent in vitro transcription of the linearised product with Sp6 RNA polymerase (Boehringer Mannheim) again the same reaction components employed in the RPA in vitro transcription reaction were employed here As per RPA the reactions were stopped by addition of Rnase free Dnase and EDTA, the riboprobes were precipitated by the addition of 2µg of yeast tRNA and 2 volumes of ice-cold ethanol and incubated at –20°C for at least 2 hours After centrifugation at 13,000 x g for 30 min at room temperature, the pellet was washed with 75% ethanol, air dried and dissolved in 50µl of DEPC-treated upH_2O A 1µl sample of the purified

riboprobe was used to measure the level of incorporation of radiolabel using a scintillation counter

2.5.3.5 Generating the CD21 DNA Probe.

A 1 6Kb portion of the CD21 cDNA was excised from pUC19CD21 by EcoR1 restriction and excision from an agarose gel The CD21 cDNA was then purified using a Promega Wizard spin column and resuspended in sterile nuclease free H_2O A Random Priming DNA labeling kit (Boehringer Mannheim) was used to synthesize a uniformly labeled DNA probe Twenty five nanogramms of linearised CD21cDNA was added to a final volume of 9ul of distilled water and denatured by heating at 95°C for 10 minutes using a water bath The reaction mixture was then placed on ice for 5 minutes and 3ul of dNTP stock mix was added as well as 2ul of reaction mixture containing reaction buffer and hexanucleotides (both supplied, Boehringer Mannheim) Five microlitres of α-^{32}PdCTP (3000uCi/mMol) and 1ul of Klenow enzyme The reaction was incubated at 37°C for 30 minutes and stopped after this point by addition of 2ul of 0 2M EDTA and heating at 65°C for 10 minutes Unincorporated deoxyribonucleoside triphosphates were removed using Quickspin microspin columns (Pharmacia) The level of incorporation of radiolabel was verified as before using a scintillation counter (Beckman)

2.5.3.6 Prehybridization and Hybridization Protocol

The dried membrane was placed in a baked hybridization bottle, 10 ml of hybridization buffer (Appendix) was added and incubated in a Hybaid roller oven for 2 to 3 hours at 55°C The prehybridization buffer was then removed and fresh hybridisation buffer (preheated to 55°C) containing the riboprobe/DNA-probe (1-2 x 10^6cpm/ml) was promptly added Hybridization was allowed to proceed for 16-24 hours at 55°C

2.5.3.7 MEMBRANE WASHING

Following the overnight incubation the hybridization buffer was removed and the membrane was washed successively as follows:

TABLE 2.4 MEMBRANE WASHING CONDITIONS

WASH SOLUTION	TEMPERATURE	TIME
1X SSC, 0.1% (w/v) SDS	Room temperature	30 s
1X SSC, 0.1% (w/v) SDS	22°C	30 min
1X SSC, 0.1% (w/v) SDS	22°C	30 min
0.1X SSC, 0.1% (w/v) SDS	65°C	30 min
0.1X SSC, 0.1% (w/v) SDS	65°C	30 min

After washing the membrane was removed gently from the hybridization bottle and placed in a stomacher bag in a cassette. The blot was kept moist at all times with 0.1X SSC, 0.1% (w/v) SDS. The membrane was exposed to X-ray film for 24-96 hr at –70°C. Film was developed as described below.

2.5.4 RNase Protection Assay.

The ribonuclease protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species The RiboQuant® RNase protection assay system (PharMingen) was employed during this study The procedure is outlined below

Figure 2.2. Outline of Procedure for Ribonuclease Protection Assay

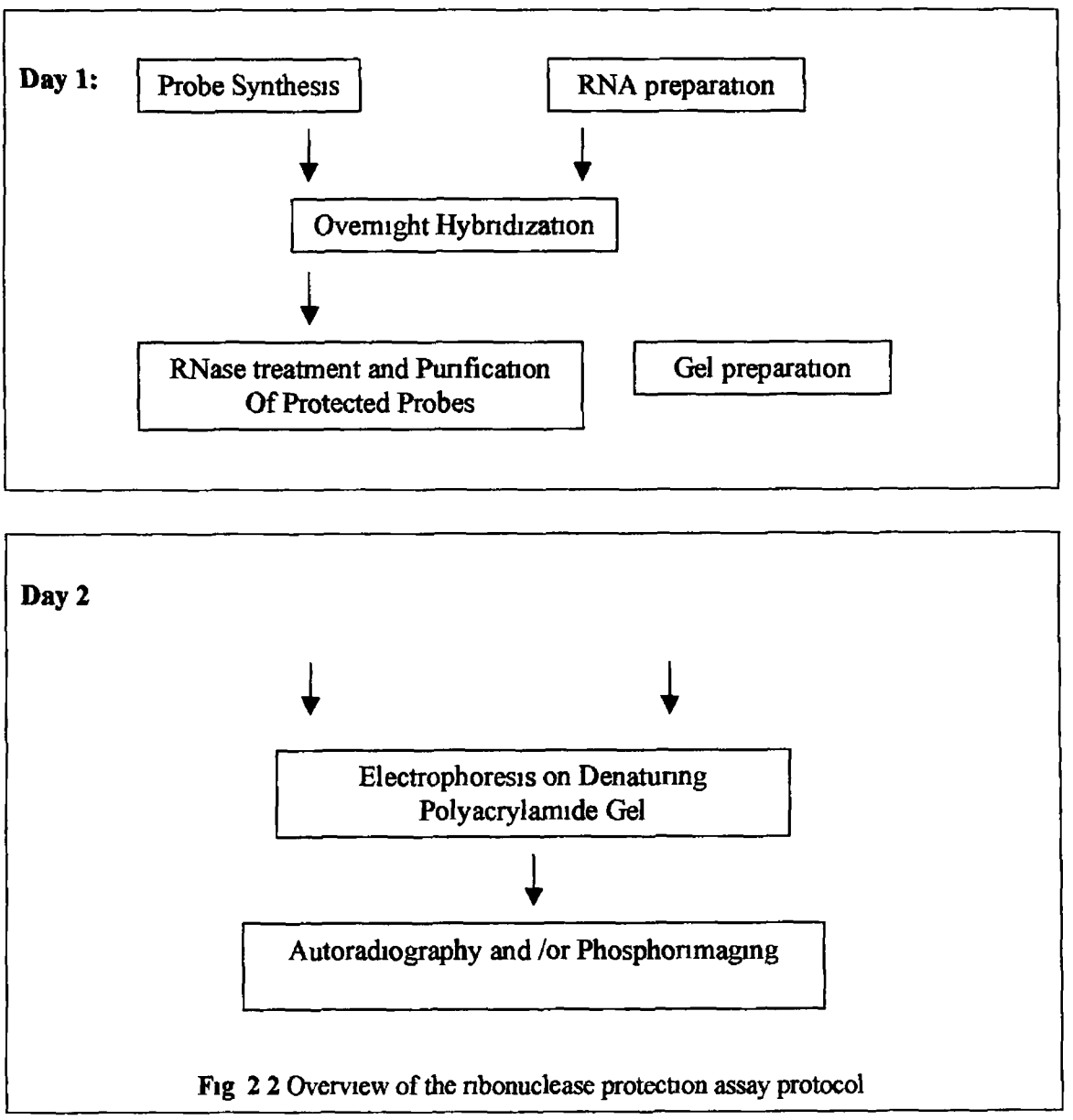


Fig 2 2 Overview of the ribonuclease protection assay protocol

2.5.4.1 PROBE SYNTHESIS

The [α-^{32}P]UTP, GACU nucleotide pool, DTT, 5X transcription buffer and the template DNA set was brought to room temperature prior to setting up the reactions The following were added to a 1 5 ml microcentifuge tube for each probe synthesis

Rnasin	1µl
GACU pool	1µl
DTT	2µl
5X transcription buffer	4µl
Template DNA	1µl
[α-^{32}P]UTP	10µl
T7 RNA polymerase	1µl

The contents of the tube were mixed by gentle pipetting and centrifuged quickly followed by incubation at 37°C for 1 hour The reaction was terminated by adding 2 µl of RNase - free Dnase mixing gently and incubating at 37°C for 30 min The following reagents were then added to the reactions

EDTA 20 mM	26µl
Tris saturated phenol	26µl
Chloroform isoamyl alcohol (50 1)	25µl
Yeast tRNA	2µl

The contents were vortexed into an emulsion and centrifuged for 5 min at room temperature The upper phase was transferred to a fresh tube containing 50µl of chloroform isoamyl alcohol (50 1), the tube was vortexed and microcentrifuged (top speed) for 2 min at room temperature The upper aqueous phase was transferred to a sterile 1 5 ml tube to which 50µl of 4M-ammonium acetate and 250µl of ice cold 100% (v/v) ethanol was added The tube was inverted to mix and incubated at -70°C for 30 min

followed by centrifugation at 4°C for 15 min The supernatant was removed and the pellet was washed with 100µl of ice cold 90% (v/v) ethanol after which the supernatant was removed and the pellet was air-dried for 5-10 min The pellet was solubilized by the addition of 50 µl of hybridization buffer and gentle vortexing, contents were collected by a brief centrifugation Duplicate 1 µl samples of the labeled probe were quantified in a scintillation counter A maximum yield of –3 x 10^6 Cherenkov counts/µl with an acceptable lower limit of –3 x 10^5 Cherenkov counts/µl was expected The probe was stored at -20°C until required Generally probe can only be used for two overnight hybridizations when labeled with [α-^{32}P]UTP

2.5.4.2 RNA PREPARATION AND HYBRIDIZATION

RNA was prepared using the RNA isolation method outlined in section 2 6 2 above, 20 µg of total RNA was used for each probe hybridization Each RNA sample was made up to 50 µl with DEPC-treated upH_2) to which 50 µl of 4 M ammonium acetate and 250 µl of ice cold 100% (v/v) ethanol were added The samples were mixed by inverting and stored at -70°C for one hr or -20°C overnight The precipitated RNA was collected by centrifugation at 12,000 x g for 30 min at 4°C, the pellet was washed with 90% (v/v) ice-cold ethanol After removal of the supernatant and subsequent air-drying, the pellet was resuspended in 8 µl of hybridization buffer by gently vortexing for 3-4 mm followed by a brief centrifugation Two microlitres of the probe was then added to each RNA sample and mixed by pipetting A drop of mineral oil was added to each sample and the tubes were centrifuged briefly in the microfuge Samples were then placed in a heating block for 3 mm which had been preheated to 90°C, and then immediately turned down to 56°C, allowing the temperature to ramp down slowly, and incubated for 12-16 hr The heating block was then turned down to 37°C prior to RNase treatment and again the temperature was allowed to ramp down slowly and then was maintained at 37°C for 15 min

2.5.4 3 RNASE TREATMENTS

An RNase mixture was prepared by adding 2 5 ml of RNase buffer to 6 µl of RNase A + T1 mix, per 20 RNA samples (RNase A 80ng/µl, RNase T1 250 U/µl) The RNA samples were removed from the heating block and 100 /µl of the RNase cocktail was added underneath the oil into the aqueous layer (bubble) The tubes were microcentrifuged for 10 s and incubated for 45 min at 30°C Before the RNase treatment was completed a Proteinase K mixture was prepared (per 20 samples),

Proteinase K buffer 1 X	390 µl
Proteinsase K (10 mg/ml)	30 µl
Yeast tRNA (2 mg/ml)	30 µl

The mixture was mixed together and 18 µl aliquots were added to a sterile 1 5 ml microcentifuge tubes The RNase digests were extracted from underneath the oil and transferred to the tube containing the proteinsase K mixture (avoiding transfer of oil) The RNase/Proteinase K mixture was vortexed briefly, microfuged quickly and incubated for 15 min at 37°C Tris saturated phenol (65 µl) and 65 µl of chloroform isoamyl alcohol (50 1) were added to the samples, vortexed into an emulsion then centrifuged for 5 min at room temperature The upper aqueous phase was extracted, avoiding the interphase, and transferred to a fresh tube to which 120 µl of 4 M ammonium acetate and 650 µl of ice cold 100% (v/v) ethanol was then added The tubes were mixed by inverting and were subsequently incubated at -70°C for 30 min Samples were centrifuged for 5 min at 4°C, the pellet was then washed with ice cold 90% (v/v) ethanol, the supernatant was removed and the pellet was allowed to air-dry for 5-10 min Pellet was resuspended in 5 µl of 1 X loading buffer (provided in kit) Prior to loading onto the gel the samples were heated to 90°C for 3 min and placed immediately in an ice bath

Five percent (w/v) polyacrylamide-urea gels were prepared according to the formula given in appendix A Biorad gel electrophoresis system was used m this study The

plates were washed with detergent, rinsed first with tap water and then with dH_2O and finally wiped in one direction with tissue soaked with 100% (v/v) ethanol One plate was siliconsied using Sigmacote (Sigma) The apparatus was set up, the gel cast and the comb inserted as per manufacturer's instructions Prior to loading of the samples, the gel was pre-run in 1 x TBE at a constant power of 50 W for 45 mm – 1h The wells were washed several times with electrophoresis buffer (1 x TBE) to remove unpolymerised acrylamide and urea that would otherwise interfere with the electrophoresis of the sample After loading the samples, the gel was electrophoresed at 50 W for approximately 3 h After electrophoresis the gel was carefully removed, placed on two sheets of 3MM Whatmann paper cut to the size of the gel, covered with Saran Wrap and vacuum dried at 80°C for 1-2h The dried gel was then exposed to X-ray film in a cassette with two intensifying screens was exposed at -70°C for an appropriate length of time

2.6 Western Blot Analysis

2 6.1 Preparation of Cellular Protein

Prior to protein isolation the viability of the cells was examined and the viable cell count determined by trypan blue exclusion as before This method was employed to isolate total cellular protein (i e cytoplasmic and nuclear proteins) and was used for the extraction of EBNA2, Bfl-1, NotchIC and LMP-1 proteins Cells were pelleted at 1,000 x g for 5 min and washed with 5 ml of ice-cold PBS The cells were then transferred to a microfuge tube in 1 ml of ice-cold PBS, pelleted at 5,000 x g for 5 min and all of the supernatant removed Adherent cells were washed twice with 5ml of ice-cold PBS, scraped into 1 ml of PBS and then pelleted by centrifugation at 5,000 x g for 5 min The cell pellet was dispersed in ice-cold suspension buffer (0 1M Nacl, 0 01M Tris-Hcl pH 7 6, 0 001M EDTA pH 8 0, 1µg/ml aprotinin, 1µg/ml leupeptin and 100µg/ml PMSF) using 200µl of suspension buffer for every 5 x 10^6 cells An equal volume of 2 x SDS gel loading buffer [100mM Tris-Hcl pH 7 6, 4% (w/v) SDS, 20% (v/v) glycerol, 10%(v/v) 2-mercaptoethanol, 0 2% (w/v) bromophenol blue] was immediately added to the cell suspension, after which the sample becomes extremely viscous The sample was then placed in a boiling water bath for 10 min and then subjected to sonication for 1 min on

full power to shear the DNA The lysate was clarified by centrifugation at 12,000 x g for 10 min at room temperature The supernatant was aliquoted and stored at –20°C Samples were analyzed by SDS-PAGE, loading approximately 5 x 10^5 cells per lane

2.6.2 Estimation of Protein Concentration

In order to standardize the amount of protein used in SDS-PAGE or EMSA, the concentration of protein in the cell extracts was determined using the BCA (bicinchonnic acid) protein assay kit essentially according to manufacturer's instructions The 'microwell plate protocol' was used A standard curve was constructed using BSA, with concentrations ranging from 0 to 2000 µg/ml using PBS as the diluent The sample concentrations were determined within this range with test solutions diluted 1 5 and 1 10 in PBS (diluent) As a control for interference in the readings by substances in the extraction buffers, similar dilutions of extraction buffer as with the test solutions were included Also, all samples were prepared and assayed in duplicate The BCA assay reagent was prepared on the day of use and incubated with the samples in a microwell plate at 37°C for 30 min in the dark Absorbance was measured at 560nm on a plate reader and the readings subtracted from the appropriate controls and averaged accordingly A standard curve was plotted for the BSA standards (concentration versus absorbance) from which the protein concentration for each unknown sample could be determined

2.6.3 PROTEIN ELECTROPHORESIS, PREPARATION OF SDS-PAGE GELS

A two phase SDS-PAGE system was used to analyze proteins with a 5% stacking gel and either a 10% resolving gel as outlined below

Resolving Gel

10 ml	10% (ml)	15% (ml)
acrylagel	3 33	5 0
bis-acrylagel	1 35	2 025
1 5 M Tris (pH8 8)	2 5	2 5

dH_2O	2.61	0.265
10% (v/v) SDS	0.10	0.10
10% (v/v) APS	0.10	0.10
TEMED	0.01	0.01

Stacking Gel

2.5 ml	5% (ml)
acrylagel	0.42
bis-acrylagel	0.168
1 M Tris (pH6.8)	0.312
dH_2O	1.5475
10% (v/v) SDS	0.025
10% (v/v) APS	0.025
TEMED	0.0025

2.6.4 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

An ATTO protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water and then with dH_2O and finally wiped in one direction with tissue soaked with 100 (v/v) ethanol. The gasket was placed about the ridged plate, the plates were put together and secured with clamps. The depth of the resolving gel was marked on the outer plate. The resolving gel was then poured to within 2 cm 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 was inserted and the gel was allowed to polymerise for 45 min-1 hr. The electrophoresis tank was filled with 1X Tris-glycine running buffer to the level of the horizontal rubber gasket. After polymerisation the gaskets clamp stands and comb were removed. Unpolymerised gel was removed by gently rinsing the wells with dH_2O, the wells were then straightened using a loading tip. The prepoured gels were lowered into the buffer at an angle to exclude air bubbles from the gel buffer interface. The gel plates were fixed firmly in

place with the notched plate innermost. The chamber formed by the inner plates was filled with 1X running Buffer, the samples were loaded and the electrodes were attached. The gels were electrophoresed at a constant current of 30 mA per gel for approximately 1 hr in the case of the 10% resolving gel or 1h 30 min in the case of the 15% resolving gel. When complete the plates were removed, separated and the gel was either placed in transfer buffer prior to western blotting or stained in Coomassie blue. Staining took place for 30 min, agitating constantly. The gel was then placed in destain (see Appendix) with constant agitation, until all background staining was removed. The destain was changed as it became saturated with stain.

2.6.5 WESTERN BLOT ANALYSIS

An SDS-PAGE gel was run as described, with pre-stained markers (New England Biolabs). Two pieces of 3MM filter paper were cut to size of the gel as was the nitrocellulose membrane. The sponges from the transfer apparatus along with 4 pieces of 3 MM filter paper cut to size and the SDS gel were soaked in transfer buffer. Two sponges were placed on each side of the transfer apparatus and 2 pieces of filter paper in turn, on each of these. The gel was placed on one side of the filter paper. The nitrocellulose membrane which had been pre-wet in distilled water and then soaked in transfer buffer for 5 min was placed on top of the gel, ensuring that no bubbles were trapped between any of the layers. The second stack of filter paper and sponges were placed on top of the membrane, the transfer apparatus was assembled and placed in the blotting apparatus with the gel on the side of the negative (black) electrode and the nitocellulose on the side of the positive (red) electrode. Protein transfer was allowed to take place at a constant voltage of 80 V for 1 h with the buffer kept chilled by an ice pack (placed in the transfer apparatus) and recirculated. After transfer, the apparatus was disassembled and the membrane was washed briefly in TBS to remove any traces of gel. The membrane was then incubated in Blocking buffer for 1 - 2 h at room temperature followed by incubation with the appropriate antibody (diluted in Blocking buffer) at 4°C overnight. Sodium azide was added to each antibody solution to a final concentration of 0.02% (w/v) as a preservative thus permitting reuse of the antibody. In cases where the

secondary antibody was conjugated to HRP, sodium azide was not added to the primary antibody as it interferes with HRP.

Table 2.5 Incubation Conditions for Antibodies Used in Western Blotting

Primary Antibody	Name	Dilution In Blotto	Secondary Antibody	Dilution In Blotto
Anti EBNA2	PE2	1/50	Ap-Conjugated Anti Mouse IgG (Promega)	1/5000
Anti -LMP1	CS1-4	1/100	Ap-Conjugated Anti Mouse IgG (Promega)	1/5000
Anti bfl-1	Fl-175	1/200	HRP-Conjugated Anti Rabbit IgG (Amersham Biosciences)	1/2000
Anti NotchIC	Tan1	1/10	AP-Conjugated Anti-Rabbit IgG (Boehringer Mannheim)	1/1000 1/1000

After overnight incubation, the membrane was washed three times in TBST (0.1%(v/v) Tween-20 in TBS) for 10 min and then incubated for 10 min in blocking buffer. The filter was then incubated with the appropriate secondary antibody for 1 hr at room temperature, followed by washing three times with TBST for 10 min each. All of the above incubations were carried out with agitation. Membranes were then placed in a clean container and covered with BCIP/NBT(Sigma) substrate in the case of the Ap-conjugated complexes or in ECL-Supersignal reagent (Pierce) in the case of the HRP-conjugated complexes. The BCIP/NBT container was placed in the dark at room temperature without shaking for 30 min or longer if required. The membranes were then rinsed in distilled water to stop the reaction, photographed and then wrapped in cling film and stored in the dark. After addition of the ECL reagent, membranes were left in darkroom conditions for 5 min. The ECL reagent was then removed and membranes were wrapped in cling film and exposed to Kodak X-ray film. Autoradiographs were developed using the Xomat Developer.

2.7 Electrophoretic Mobility Shift Assay (EMSA) or Bandshift Assay

2.7.1 Preparation of Nuclear Extracts

Nuclear extracts were prepared essentially according to the method of [Dignam, 1983]. The composition of all buffers used is described in Appendix. Briefly, 50ml of cells in suspension (~2x 10^7 cells) were pelleted at 1,000 x g for 5 min. and washed with 5 ml. of ice-cold PBS (without $MgCl_2$ or $CaCl_2$). The pellet of cells was then resuspended in3-4 volumes (400ul) of Buffer A and kept on ice for 60 minutes. In this time the cells swell and after the 60 minutes the cell suspension was transferred to a 1ml douncer. The cell suspension was then homogenized by douncing up and down twenty times. (To permeabilize the cell membranes). Samples were then pelleted by centrifugation at maximum speed in a Heraeus microfuge at 4 °C for 10 seconds. Next 300ul of Buffer A was added to the sample pellets and the samples were vortexed (not resuspended) and again centrifuged at 4 °C for 10 seconds as before. The supernatant was discarded and the pellets were then resuspended in 300ul of Buffer B and incubated on ice for 30minutes. Samples were then mixed by vortex and clarified by centrifugation for 20 minutes at 4 °C at maximum speed in a Heraeus microfuge. The supernatant contains the nuclear extracts. The concentration of protein in the extracts was determined by using the BCA assay and the extracts were aliquoted frozen on dry ice and stored at -80^0 C until use.

2.7.2 Nature Of The oligonucleotide probes and Their labeling for use in EMSA

Unlabeled double stranded oligonucleotide probes for use in EMSA were purchased as single stranded oligonucleotides and then annealed in solution. Pairs of oligos were purchased from MWG Biotech and designed such that annealing would leave a 5' GATC overhang. Two pairs of oligonucleotides were used (See Section 2.15 above). The Cp oligos represented sequences from -359 to –388 of the Cp promoter that contain the Cp CBF1 binding site (5'-GTGGGAA-3') (Ling et al 1994), while the 36-mer Bflp oligo corresponds to –259 to –223 on the bfl-1 promoter which contains the putative CBF1 binding site from –243 to-249 on the reverse strand (Figure 3.22A). Labeling was based

on the usage of Klenow DNA polymerase. The enzyme catalyzes the addition of mononu-cleotides from deoxynucleoside-5′-triphosphates to the 3′-hydroxyl terminus of a primer/template DNA. Thus Klenow was used to repair the 5' overhangs generated after annealing, while in the process integrating the α^{32}P labeled dCTP to the 3' terminus of the template oligo. For preparation of the double stranded –Cp and bfLp oligonucleotides, equimolar amounts of the complimentary Cp (TOP and BOTTOM) and BfLp (TOP and BOTTOM) oligonucleotides were added to TE pH8.5 and placed in a heating block at 90^{0}C for 10 minutes to denature the oligonucleotides. The heating block was then removed from the apparatus and allowed to cool to room temperature slowly (60 – 75 min.) to allow annealing of the oligonucleotides to ensue. The annealed oligonucleotides were either used immediately in the labeling reaction or stored at –20°C.

The labeling reaction was performed by adding :

Template DNA (annealed) containing 5' GATC overhangs	2ul(50ng)
NEB Buffer 2 (Reaction buffer for Klenow Enzyme)	2ul
dATP, dTTP, dGTP(500uM)	2ul(final conc.50uM)
α-^{32}P dCTP (3000Cimol)	5ul
Distilled H_2O	7ul
Klenow Enzyme	2ul

The reaction was incubated at 37^{0}C for 1 hour after which labeled oligonucleotides were purified away from unincorporated labeled nucleotides by spin-column chromatography through MicroSpin™ G-25 columns essentially according to manufacturer's specifications (Amersham Pharmacia Biotech). The level of incorporation of radiolabeled α-^{32}P dCTP was assayed using a Beckman scintillation counter.

2.7.3 Binding Reaction

The composition of all buffers used in the binding reaction are given in the Appendix
The binding of nuclear proteins to labeled DNA probe was performed in a reaction comprising:

Binding reaction buffer (4X)	5µl
Non-specific competitor DNA poly.dI-dC (1µg/µl)	2µl
BSA (1mg/ml)	2ul
Binding Mix	9ul

Binding Mix	9ul
Antibody (R3)(For supershift)	5ul
Nuclear Extract (6ug)	Xul
Incubate for 5 minutes at room temperature	
^{32}P-labeled DNA probe (20,000 cpm/µl)	2µl
Distilled H_2O	20ul

A 'control reaction' lacking nuclear extract but containing all other components of the binding reaction was also set up. The reaction components were mixed gently and incubated at room temperature for 30 minutes. Samples were then loaded onto a 5% polyacrylamide gel in TBE. One extra lane with Bromophenol blue was also added so that the leading edge of the gel could be visualized.

2.7.4 Supershift Studies

The identity of a protein in a bandshift complex can be verified by incubating the nuclear extract with an antibody that recognizes the protein prior to performing the binding reaction with the labeled probe. The antibody (R3) binds to the EBNA2-CBF1 complex. Depending on whether the antibody recognizes an epitope within the DNA-binding domain or elsewhere, the complex formation will either be prevented *(observed as only a decrease in intensity of the shifted complex)* or further retarded in mobility in the gel (termed a 'supershift'). 'Supershift' studies were conducted by incubating the nuclear extract (See the binding reaction above) with 5.0µl of an EBNA2 specific antibody (R3). Reactions were then analysed by PAGE and visualized by radiography as described below.

2.7.5 Nondenaturing Polyacrylamide Gel Electrophoresis

Resolution of the bandshift complexes were conducted by nondenaturing polyacrylamide gel electrophoresis using the Protean II cell system (Biorad). A 5% polyacrylamide gel was used.

2.7.6 Preparation of the Protean II Cell Electrophoresis System

The glass plates, spacers and combs were cleaned with detergent, rinsed thoroughly with tap water followed by upH_2O, and wiped dry with clean dry tissue paper. 100% (v/v) ethanol was poured onto the glass plates and the plates wiped dry with tissue paper. The spacers and combs were wiped with tissue paper soaked in 100% (v/v) ethanol. The apparatus was then assembled and clamped together according to manufacturer's instructions (Biorad).

2.7.7 Casting the Gel

A 5% gel was used for the resolution of the bandshift complexes. The composition of the gels is outlined in the Appendix. After addition of TEMED to the gel mix, the gel solution was poured immediately into the gel mould and the comb inserted. The gel was allowed to set for at least an hour, after which the gel mould was removed and placed in the electrophoresis tank. The lower chamber of the tank was filled with 1.2 litres of electrophoresis buffer (1 X TBE) whilst the upper chamber was filled with approximately 350 ml of 1 X TBE. The comb was carefully removed and the wells were washed thoroughly with electrophoresis buffer to remove any unpolymerised acrylamide that would otherwise impair the migration of the sample. The gel was pre-run for 30–40 min at a constant voltage of 130V before the samples were loaded and electrophoresis was carried out at the same voltage for approximately 4 h at room temperature. It should be pointed out that no loading dye was added to the samples as bromophenol blue has been reported to disrupt protein-DNA interactions in certain cases (Maniatis et al 1989). It was therefore necessary to run just loading buffer containing bromophenol blue into one

well of the gel to locate the leading edge of the gel After electrophoresis, the gel was vacuum dried for approximately 2hrs and exposed to X-ray film to visualise bandshifts

2.7.8 Gel Drying and Autoradiography

After electrophoresis, the buffer was poured out of the electrophoresis tank and the plates disassembled A piece of Whatmann 3MM filter paper (cut to size) was placed on top of the gel, avoiding air bubbles and the paper lifted gently with the gel attached to it This was then covered with cling film and placed in a vacuum gel dryer, with the gel facing up The gel was dried at 80°C for 2h Once dry, the gel was placed in a cassette and exposed to X-ray film in the dark for at least 12 hours at -80 °C The film was developed using an Xomat developing machine

CHAPTER 3

RESULTS

Results

Introduction

3.0. LMP1-Independent Mechanism(s) Regulate *bfl-1*-mRNA Levels in an EBV-Positive Burkitt's Lymphoma Type III Cell Line.

EBV positive Group I BL cell lines and many EBV negative BL cell lines can easily be induced to undergo apoptosis, (Gregory *et al*, 1991) however, group III EBV positive BL cell lines such as LCLs which express the complete set of EBV latent proteins, display increased resistance to apoptosis induced by a variety of triggers (Gregory *et al*, 1991) Although this increased resistance to apoptosis is a result of numerous interactions between EBV and members of the cellular apoptosis programme (See Chapter 1), it has partly been attributed to an increase in the expression of Bcl2 an anti-apoptotic protein which is up-regulated by EBV LMP1, EBNA2 and EBNA3B after EBV infection (Henderson *et al*, 1991, Liu *et al*, 1991, Rowe *et al*, 1994, Finke *et al*, 1992, Silins and Sculley 1995) Recent studies however, have shown that another bcl2-related anti-apoptotic gene, bfl-1, has consistently elevated mRNA levels in EBV positive group III relative to group I BL cell lines (D'Souza *et al*, 2000) Thus bfl-1 may also be an important target for EBV latent genes in mediating the increased anti-apoptotic threshold of infected group III BLs and LCLs It has been shown that LMP1 transcriptionally up-regulated the bfl-1 gene when expressed independently of the other EBV latent proteins (D'Souza *et al*, 2000) Nonetheless, since the relative levels of bfl-1 mRNA are much higher in type III cell lines relative to bfl-1 mRNA levels in cells expressing LMP1 only, thus LMP1 is not the only EBV latent protein involved in regulating the cellular bfl-1 gene (Figure 3 1)

Initially a preliminary Northern Blotting experiment was undertaken using total RNA samples from three related cell lines, Mutu I an EBV positive BL cell line expressing only EBNA1 (the group I phenotype), Mutu III, a clone of Mutu I cells which have drifted to express the entire compliment of EBV latent genes (group III phenotype), and a Mutu III mutant cell line which no longer expresses LMP1 Steady state levels of bfl-1 mRNA in these cell lines were detected using an antisense bfl-1 riboprobe complimentary to a portion of the transcript from this gene (Figure3 4) Induction levels were

normalized by analyzing mRNA levels of the housekeeping gene GAPDH in each of the cell lines. GAPDH mRNA was detected using a commercially available antisense riboprobe. (Pharmingen). It can be seen below (Figure3.1), that significantly elevated steady-state levels of *bfl-1* mRNA were present in MUTU-III relative to MUTU-I cells. However, since the MUTU III clone, which does not express LMP1, still shows significant induction of *bfl-1* mRNA levels, other EBV latent proteins may also regulate *bfl-1* expression.

As EBNA2 is another key effector of phenotypic change in EBV-infected cells, it was investigated if the expression of this viral protein alone could contribute to an up-regulation of bcl2 family apoptosis-related gene products, including *bfl-1*.

Figure 3.1. An LMP1-Independent Mechanism Up-Regulates *bfl-1*-mRNA Levels in an EBV-Positive Burkitt's Lymphoma Cell Line

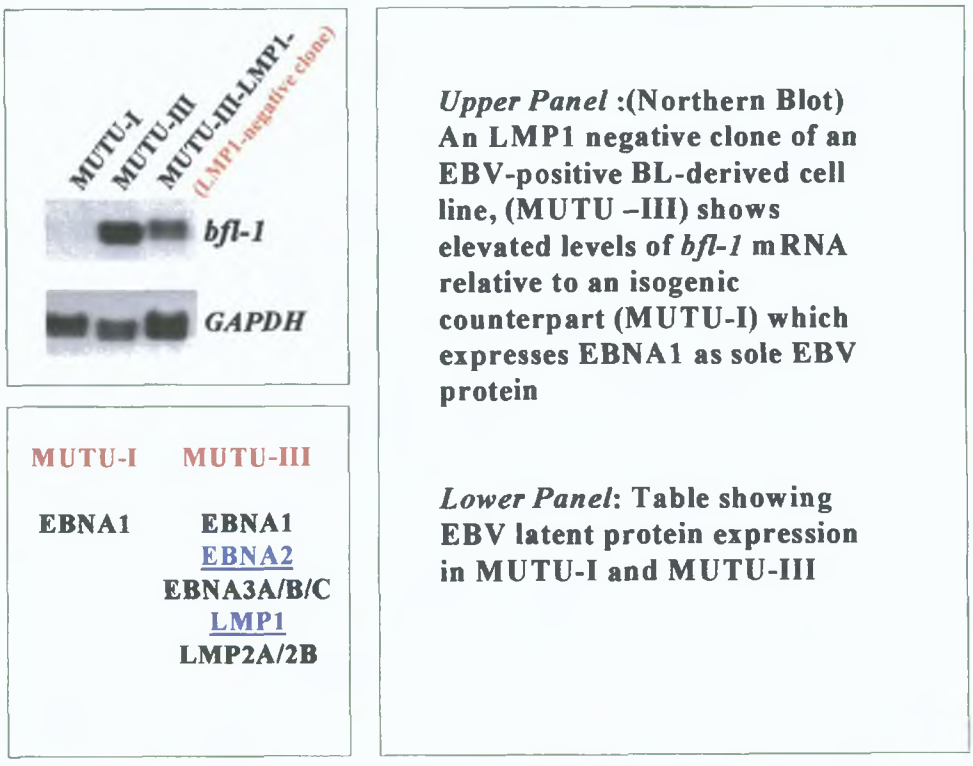


Figure 3.1. An LMP1-*independent* mechanism up-regulates *bfl-1*-mRNA levels in an EBV-positive Burkitt's lymphoma cell line. Left panel. Elevated levels of bfl-1 mRNA are evident in the group III relative to the group I BL cells thus EBV latent proteins appear to regulate transcription from the bfl-1 gene. Although LMP1 independently up-regulates transcription from the bfl-1 gene, (D'Souza *et al.*, 2000),

it appears other EBV latent proteins expressed in the group III phenotype also regulate bfl-1 activity The main candidate here is EBNA2

3.1. Regulation of Expression of *bcl-2* Family Members by EBNA2

This study set out to investigate if EBNA2 was capable of directly regulating the expression of *bcl-2* family members, in particular *bfl-1*, at the transcriptional level To this end, *bcl-2* gene family profiling was performed by ribonuclease protection assay using a custom designed set of riboprobes that were antisense to mRNAs of members of this group of genes (henceforth referred to as multiprobe RPA) Several established cell lines were then used in which EBNA2 could be either induced or activated in the absence of LMP1

3.1.1 Inducible Expression of EBNA2, Using the Tetracycline-Regulated System in Stably-Transfected B Cell Lines.

In order to ensure expression of EBNA2 as sole EBV protein, the DG75tTA-EBNA2 cell line was employed In this cell line EBNA2 expression is tightly regulated by the presence or absence of tetracycline (Figure 3 2) The inducible promoter driving EBNA2 expression contains binding sites for a hybrid tetracycline-regulated trans-activator (tTA) that is constitutively expressed in the parental clone DG75tTA Removal of tetracycline from the growth medium leads to the binding of tTA to the promoter and the induction of EBNA2 expression (Floettmann *et al*, 1996) The tetracycline-regulated trans-activator (tTA) is derived from a fusion of two genes (tetR and VP16 trans-activator) and is constitutively expressed from a CMV/IE promoter on the pJEF-3 plasmid Expression of EBNA2 from the responder plasmid pJEF-31 requires binding of the tTA to a 5' regulatory region containing a minimal promoter The cell line DG75tTA-EBNA2 had been generated by stable transfection of the EBV negative cell line DG75 with pJEF-3 and pJEF-31 and stable integration was selected by growth in medium containing hygromycin-B, G418, and tetracycline (Floettmann *et al*, 1996)

Figure 3.2. EBNA2 Expression is Tightly Controlled by the Presence of Tetracycline in the DG75-tTA-EBNA2 Cell Line.

The host cell line DG75 is stably transfected with two plasmids pJEF-3 and pJEF31which are selected during cell culture using the drugs Hygromycin and G418 respectively

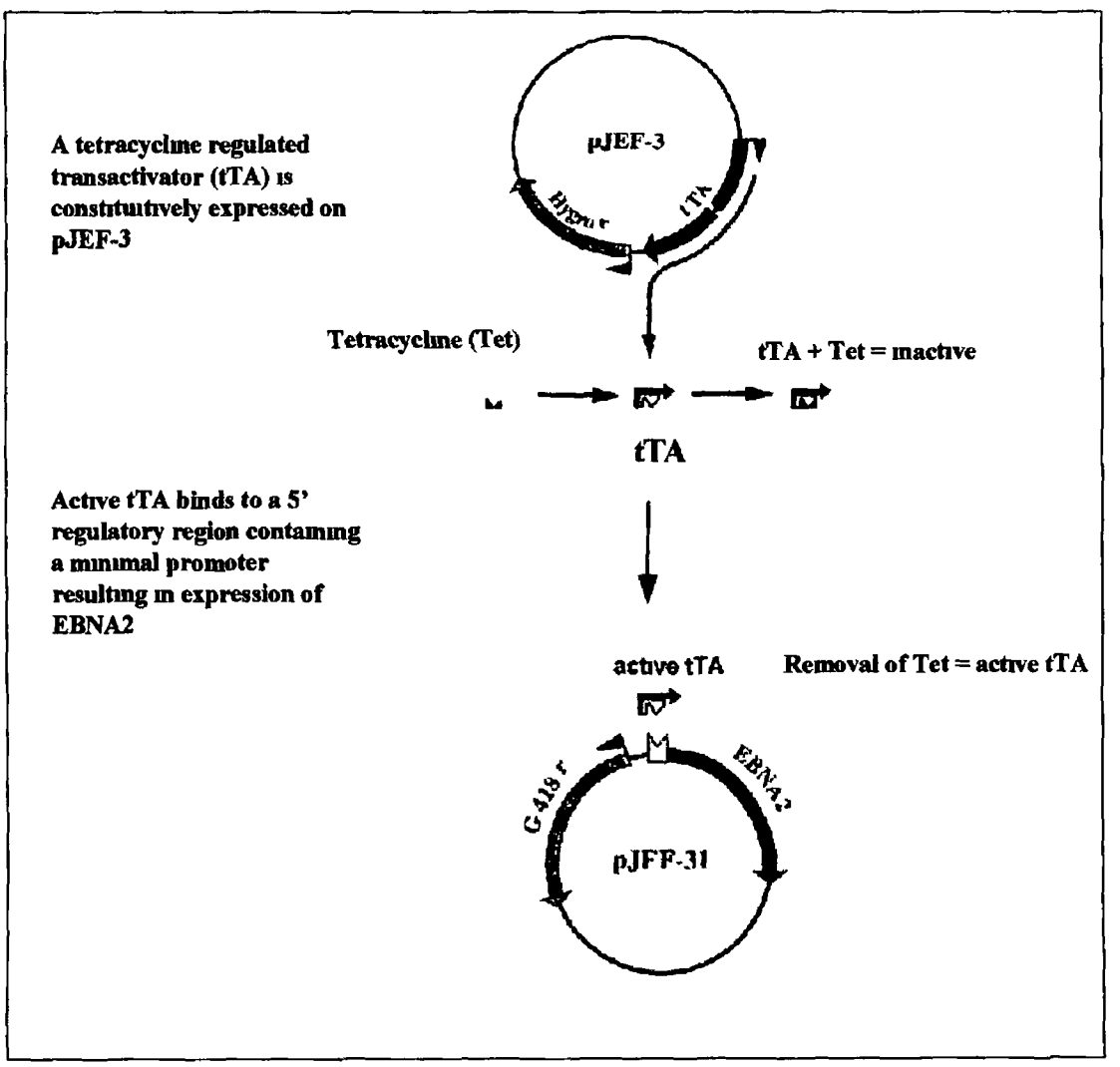
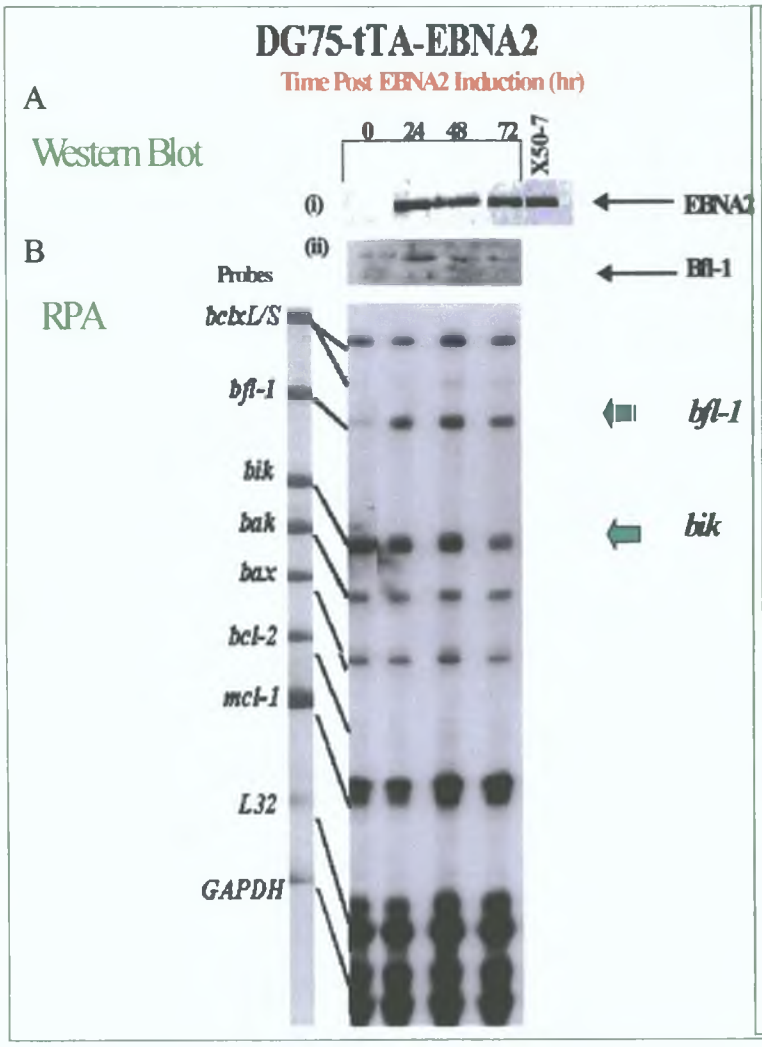


Figure 3.2 Schematic representation of the tetracycline-regulated gene expression system in the DG75 tTA EBNA2 cell line. In the presence of tetracycline the trans-activator cannot bind to the minimal promoter driving EBNA2 expression. Conversely, in the absence of tetracycline the tTA trans-activation domain binds to the promoter thereby facilitating induction of EBNA2 expression (Floettmann et al ,1996, Gossen and Bujard 1992)

EBNA2 Alone Up-Regulates *bfl-1* mRNA and Protein Levels in EBV Negative DG75 BL Cells.

EBNA2 expression was induced over a 72-hour time course in the DG75-tTA-EBNA2 cell line, by the removal of tetracycline from cell culture media Total protein and RNA were prepared simultaneously from cells harvested at the indicated time-points post-EBNA2 induction, (Figures 3 3 A and 3 3 B) and used in Western blot and multiprobe RPA analysis respectively The benefit of using this cell line was that EBNA2 is expressed as the sole EBV protein and that its expression is tightly controlled Western blotting was carried out to demonstrate the induction of EBNA2 over the course of the experiment and to measure Bfl-1 protein levels in response to EBNA2 induction (Figure 3 3A(i and ii) RPA permitted an investigation of the effects of EBNA2 induction on the steady-state levels of mRNA transcribed from the *bcl-2* family of apoptosis-related genes mRNA levels from the *bclx-L, bclx-S, bfl-1, bik, bak, bax, bcl-2,* and *mcl-1* genes were thus compared and the inclusion of probes for transcripts from the house-keeping genes *L32* and *GAPDH* allowed quantitative comparisons of mRNA levels to be made for each time-point The multiprobe hAPO2 template set (Pharmingen) and T7 RNA polymerase were used to synthesize ^{32}P labeled antisense riboprobes complementary to portions of transcripts from the apoptosis related genes and the house-keeping genes The labeled probe set was hybridized to 20μg of total RNA sample in solution, and RNase protected probe fragments were then resolved on 6% denaturing polyacrylamide gels and detected by autoradiography Band intensities were quantified by densitometric scanning of autoradiograms using Kodak imaging software

Figure 3.3. Induction of EBNA2 as the Sole EBV Protein Leads to an Increase in *bfl-1* mRNA and Protein Levels in the EBV-Negative BL Cell Line DG75-tTA-EBNA2.



UPPER PANEL (A):

(i) Western Blot showing EBNA2 induction in the DG75-tTA-EBNA2 cell line over 72 hours.

(ii) Western blot showing Bfl-1 is induced in the Dg75-tTA-EBNA2 cell line in Response to EBNA2 induction.

LOWER PANEL (B):

RPA shows effect of EBNA2 induction on mRNA levels in Bcl-2 related genes.

bfl-1mRNA is up-regulated in response to EBNA2 induction. Bik mRNA is down regulated in response to EBNA2 induction.

(A) (i) Western blot of DG75tTa-EBNA2 cells induced to express EBNA2 by reculturing cells in the absence of tetracycline. EBNA2 induction was carried over 72 hours. Cells were harvested and analysed for EBNA2 expression at the times indicated above each lane. **(ii) Bfl-1 stimulation in response to EBNA2 induction was also measured in this cell line by Western Blotting with the FL175 Anti Bfl-1 Antibody (Santa Cruz Biotechnology). (B). RPA** performed with RNA harvested simultaneously from the samples in panel A. mRNA levels from the apoptosis-related genes *bclx-L, bclx-S, bfl-1, bik, bak, bax, bcl-2,* and *mcl-1* were analysed over the 72 hour time-course. Unprotected ^{32}P labelled antisense riboprobes (5000cpm per lane) were loaded alongside RPA-processed samples and are shown linked to their smaller RNase-protected fragments, which correspond to the level of mRNA in the sample. Exposure to film was for 24 hours. An increase in *bfl-1* mRNA is seen upon EBNA2 induction. Densitometric scanning of relevant bands from RPA using GAPDH-corrected induction levels showed a 5-fold induction of *bfl-1* mRNA after 24 hours and a ~2 fold reduction in bik-mRNA after 72 hours.

Induction of EBNA2 was monitored by Western blot analysis using the anti-EBNA2 murine monoclonal antibody PE2 In these experiments, EBNA2 was usually detected in induced DG75tTA-EBNA2 cells at 24 hours and remained detectable up to at least 96 hours (Figure 3 3A(i)) At 24 hours the level of EBNA2 was comparable to that detected in the reference LCL, X50-7 (Figure 3 3A (i), lane 4) The levels of mRNA transcripts from the set of apoptosis-related genes were compared over the 72-hour time course following induction of EBNA2 in the Dg75tTA-EBNA2 cell line (figure3 3B) A significant increase in the level of *bfl-1* mRNA can be seen to coincide with EBNA2 expression The lane for 48hr is overloaded thus although there is an apparent peak in the *bfl-1* mRNA level after 48 hours of EBNA2 induction, after GAPDH correction, the real peak in *bfl-1* mRNA transcription occurs after 24hours Also, significant induction of *bfl-1*mRNA occurs at a physiologically relevant level of EBNA2, comparable to the level expressed in a reference LCL X50-7 Western blotting was then carried out with the same extracts and the levels of Bfl-1 protein stimulated in response to EBNA2 activation over the time course were measured It can be seen from Figure 3 3A (ii) that Bfl-1 levels are increased in conjunction with EBNA2 expression at 24 hours Thus Bfl-1 activation is a direct and early response to EBNA2 induction in this cell line Another observation was that mRNA levels for the *bik* gene were down regulated over 72 hours in response to EBNA2 expression Transcription from the other apoptosis related genes does not appear to be affected by EBNA2 expression in this system In this experiment, the Kodak software-imaging system was used to densitometrically scan all relevant bands from the RPA shown Results showed a 5-fold increase in *bfl-1* mRNA after 24 hours of EBNA2 induction Levels of *bik* mRNA decreased just under 2 fold after 72 hours of EBNA2 induction In all cases results were normalised using GAPDH densitometry values No significant changes in mRNA levels from any of the other genes were recorded

3.1.2. Northern Blot Analysis of EBNA2 Induction of *bfl-1* mRNA in DG75-tTA-EBNA2

Since RPA gives no indication about the size of a particular mRNA species, Northern blots were carried out to determine the size of the *bfl-1* transcript induced by EBNA2

3.1.2 1. Generating the *bfl-1* and GAPDH Riboprobes.

A vector, pcDNA3-HA-Bfl1 containing the *bfl-1 cDNA* was used to generate the ^{32}P-labelled bfl-1 riboprobe used in the Northern blots below (Also Figure 3 1) (Materials and Methods) Figure 3 4A shows the map of the vector pcDNA3 and the restriction sites in the polylinker region into which HA-bfl-1 was cloned (D'Sa-Eipper *et al*, 1996) Figure 3 4B shows the results of the restriction enzyme analysis used to confirm the organization of the DNA fragment encoding HA-Bfl-1 in the vector pcDNA3-HA-Bfl-1 After purification and precipitation, the remainder of the BamHI restriction product (lane 3) was used in an SP6 (Boehringer Mannheim) driven *in vitro* transcription reaction, and labeled with ^{32}P- to synthesize the antisense *bfl-1* riboprobe (Figure 3 5B) With respect to the GAPDH probe, a commercially available template was purchased (Pharmingen) and the RPA In Vitro transcription kit (Pharmingen) was used to synthesize the GAPDH riboprobe, which was also labeled with ^{32}P

Figure 3.4 Characterizing pcDNA3-HA-Bfl-1

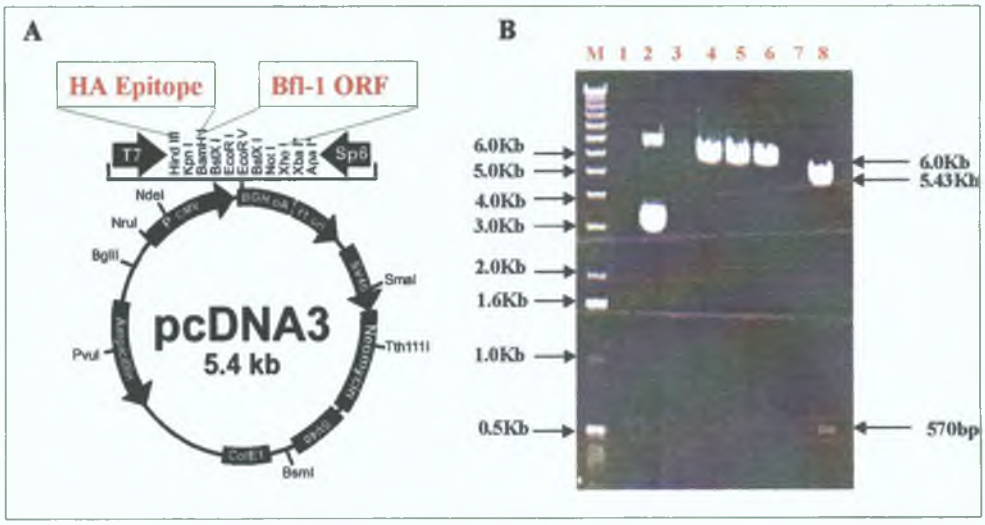


Figure 3.4. A. Map of the expression vector pcDNA3-HA-Bfl-1. The polylinker containing multiple cloning restriction sites is flanked by the T7 and Sp6 promoters. The HA epitope was cloned between the HindIII and BamHI sites and the bfl-1 ORF was cloned between the BamHI and XhoI sites in the polylinker region. B. Restriction enzyme analysis of pcDNA3-HA-Bfl-1. Lane 1 (M) = DNA molecular weight markers (sizes in kb indicated on the left hand side of the photograph.). Lane 2 is the undigested plasmid. Digestion with BamHI (lane 3), or XhoI (lane 4) linearises the plasmid (Approximately 6.0kb). Digestion of the plasmid with BamHI and XhoI excises the 570bp BFL-1 ORF encoding DNA fragment. (Lane 5).

A second independent experiment in which EBNA2 was induced in the DG75-tTA-EBNA2 cell line by omission of tetracycline from the culture media was performed as before. Total protein was harvested in the usual manner and again RNA was harvested from the same cells at the same time. Western blot analysis was used to detect the expression of EBNA2 over a 72 hour-time course and the effects of EBNA2 induction on bfl-1 mRNA levels were analyzed by Northern blotting. Western blotting (Figure 3.5A) as before showed EBNA2 expression after 24 hours of induction, these levels remain detectable over the 72-hour time-course.

Thirty microgram samples of total RNA from the DG75-tTA-EBNA2 time course in 3.5B and from control cell lines (in which *bfl-1* is known to be expressed, see below) were size fractionated on 1.3% formaldehyde-agarose gel and then transferred to

nitrocellulose membrane (BDH) The labeled antisense bfl-1 riboprobe was hybridized to the membrane–bound RNA in 6X SSC, 50% formamide, 1% SDS, 0 1% Tween, 100μg of *Escherichia coli* tRNA for 24 hours at 55°C Membranes were washed (according to materials and methods) prior to exposure to X-ray film at -70°C Blots were then stripped (Materials and Methods), and reprobed with the GAPDH riboprobe (Figure3 5B)

bfl-1 mRNA is up-regulated in response to EBNA2 expression (Figure 3 5B) As before, the intensities of relevant bands were estimated after densitometric scanning and corrected for the amount of RNA being analyzed in each lane using GAPDH densitometry data The exact level of up-regulation was difficult to establish accurately due to the very low levels of *bfl-1* mRNA transcript prior to induction of EBNA2, however densitometry analysis showed a 6 2 fold up-regulation of bfl-1 mRNA at 24 hours post EBNA2 induction

RNA from the control cell lines IARC 290B, IARC171, X50-7 (LCLs which express all the EBV latent genes), AG876 (a type III BL expressing all the latent EBV genes) and DG75-tTA-LMP1 (in which LMP1 had been induced for 24 hours) were analyzed in the same blot Only one *bfl-1* mRNA species of 0 8-0 85 kb was detected in all cases (controls and DG75 tTA EBNA2) albeit at very low levels in the DG75tTA EBNA2 cell line prior to EBNA2 induction

The size of the observed transcript was in agreement with the previously reported size of the transcript ~ 0 8Kb from this gene (Kenny *et al*, 1997) These experiments confirm the RPA results in Figure3 3 and show that expression of EBNA2 as sole EBV protein in the EBV-negative BL cell line DG75, leads to an increase in the level of *bfl-1* mRNA

3.5 Northern Blot Analysis of EBNA2 Activation of *bfl-1* mRNA

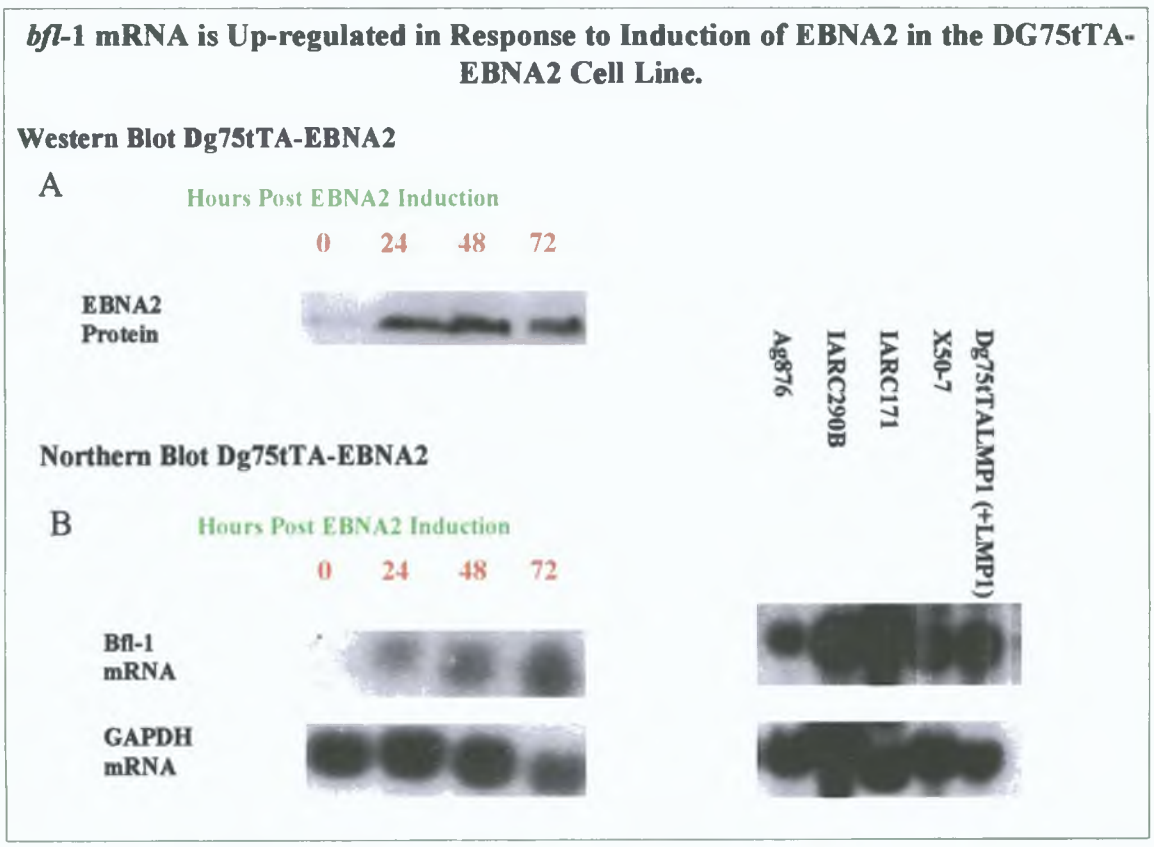


Figure 3.5. *bfl*-1 mRNA is Up-regulated in Response to Induction of EBNA2 in the DG75tTA-EBNA2 Cell Line. Protein and RNA from DG75tTA-EBNA2 cells induced to express EBNA2, were harvested simultaneously and assayed in panels A and B respectively. **(A) Western blot analysis of EBNA2 expression in the Dg75tTA-EBNA2 cell line over the 48 hour EBNA2 induction period. (B) Northern blot analysis of *bfl-1* mRNA levels in DG75-tTA-EBNA2 (upon induction of EBNA2) and in LCLS and type III cell lines** (upper panel). Total RNA was prepared at various time-points after EBNA2 induction (indicated in hours above each lane in all blots in this figure). RNA samples (30ug) were loaded onto the gel, which was then blotted and probed with antisense bfl-1 riboprobe as described in the text. The blot was exposed to film for 24h. The lower panel of (B) above shows the same blot reprobed with a GAPDH antisense riboprobe. Simultaneously, (same blot) Northern blot analysis of bfl-1 expression in IARC 290B, AG876, Bl72 and DG75tTA-LMP1 (in which LMP1 has been induced for 24 hours) was undertaken. A single ~850kb bfl-1mRNA transcript was apparent in all cases. This transcript matched the previously reported size for the transcript from this gene.

It was important to establish if bfl-1 regulation by EBNA2 was a general phenomenon in BL cells and to this end experiments with a similar objective were performed using established transfectants of a second well-studied EBV-negative BL cell line, BL41 These transfectants also used a conditional system, in this system, the function of EBNA2 was dependent on the presence of estrogen

3.1.3. Estrogen-Regulatable Expression of Functional EBNA2 in BL41-ER/E2- (K3) and BL41P3HR1-ER/E2- (9A) Cell Lines.

Fusion of a given protein with the hormone-binding domain of a steroid receptor can render protein function dependent on the presence of that hormone (Eilers *et al*, 1989, Picard *et al*, 1988) Kempkes *et al*, 1995a generated EBNA2-estrogen receptor fusion constructs by fusing the hormone-binding domain of the estrogen receptor to the N terminus of EBNA2, thus rendering EBNA2 functionality and its ability to interact with CBF1, dependent on the presence of estrogen They infected the EBV-negative BL-derived cell line BL41 with a mini-EBV plasmid carrying the EBNA2 gene that had the hormone-binding domain of the estrogen receptor fused to its N terminus giving rise to the BL41-ER/EBNA2-K3 cell line in which EBNA2 function is dependent on the presence of estrogen Because of the specific way in which EBNA2 activity can be controlled, this cell line, as with DG75-tTA-EBNA2, was useful for this study in analysing the individual effects of EBNA2 expression in an EBV and therefore LMP1 negative background Another similar cell line was also used in this study, namely BL41P3HR1-ER/EBNA2-9A This cell line was generated by infection of the BL41 cell line with two virions, the EBV-P3HR1 virus genome and the mini-EBV plasmid carrying the estrogen fused EBNA2 gene P3HR1 is an EBNA2-deficient strain of EBV (Kempkes *et al*, 1995a) To activate EBNA2 to its functional form, (in both BL41-ER/E2-K3 and BL41P3HR1-ER/E2-9A) estrogen (β-estradiol) was added to the culturing media to a final concentration of 1uM, in accordance with previously published studies (Kempkes *et al*, 1995a)

3.1.4. Functional EBNA2 is Expressed in the BL41-ER/E2- (K3) and BL41/P3HR1-ER/E2- (9A) Cell Lines in Response to Estrogen Addition.

Western blotting was first carried out (Figures 3 7A/B 3 8A/B) to show the presence of EBNA2 in the BL41-ER/E2- (K3) and BL41/P3HR1-ER/E2- (9A) cell lines EBNA2 was activated by adding estrogen to the cells during culturing, and both protein and RNA was harvested from the cells at the indicated time-points over a 24 hour-time period Activation of EBNA2 in response to the addition of EBNA2 was then analysed by examining transcription from CD21, an EBNA2 target gene, by Northern blotting (Figure 3 7C/D, 3 8C/D) It can be seen from the Western blot that there is an increase in the level of EBNA2 protein upon addition of estrogen A 2 fold and 2 4 fold increase in EBNA2 levels in BL41-ER/2-K3 and BL41P3HR1-ER/E2-9A respectively, was calculated from the Western blots after protein loading was corrected for by densitometric scanning of the blot after Ponceaux staining (3 7A/B and 3 8A/B) This increase in EBNA2 expression is most likely due to the fact that the estrogen responsive (ER)-EBNA2 fusion protein is being expressed from an EBNA2-regulated promoter (BamH1C) Thus activation of EBNA2 induces it to trans-activate the BamH1C promoter controlling its own expression This positive autoregulation of the BamH1C promoter by EBNA2 has been reported elsewhere (Jin and Speck, 1992, Rooney *et al*, 1992, Sung *et al*, 1991, Woisetschlaeger *et al*, 1991, Kempkes *et al*, 1995c) and is in itself evidence that addition of estrogen does indeed activate EBNA2 in this experiment

Analysed on the same Western blots was a protein extract from the reference LCL X50-7 (Lane 9, Figure 3 7A/B, 3 8A/B) This lane was included in the blots as a positive control for EBNA2 expression It can be seen from the blots that the EBNA2 detected from the fusion protein lysates is a different (larger) size, as expected, to the wild-type EBNA2 Differences in the size and thus the electrophoretic mobility of these fusion proteins compared to their wild-type counterparts, have been noted elsewhere (Kempkes *et al*, 1995a)

Although it is evident from the Western blot that EBNA2 is expressed, it is only possible to determine the functionality of that EBNA2 indirectly, by Northern blotting for CD21

mRNA, since CD21 is one of the known target genes of EBNA2 in this cell background (Cordier *et al*, 1990, Kempkes *et al*, 1995a) (Figure 3 7C/D, 3 8C/D)

3.1.5. Generating the CD21 DNA Probe.

A vector containing a 1 6Kb portion of the CD21 cDNA was obtained from Dr Bettina Kempkes (Table 2 2) Figure 3 6A shows the map of pUC19 and the restriction sites in the polylinker region into which a 1 6Kb fragment of CD21 cDNA was cloned (Weiss *et al*, 1986, Strobl *et al*, 2000) Figure 3 6B shows the results of the restriction enzyme analysis used to confirm the organisation of the DNA fragment encoding CD21 cDNA in the vector pUC19-CD21 The DNA probe used in the CD21 Northern blots was made by excision of the CD21 cDNA from pUC19-CD21 by restriction digestion with EcoR1 (1 6kb, in lane 3) (an extra EcoR1 site had been introduced in the sub cloning of the CD21 cDNA into pUC19) This 1 6kb fragment was then excised from the agarose gel and purified using a microspin column (Promega Wizard system) The probe was then labelled with ^{32}P by random priming using a commercially available kit (Boehringer Mannheim) and purified over a Sephadex column (Pharmacia) (As for Strobl *et al*, 2000) RNA size fractionation was performed in a denaturing formaldehyde-agarose gel followed by blotting and probing with the appropriate radiolabeled probes, as described in the materials and methods section 2 5 3

Figure 3.6. Characterizing PucCD21.

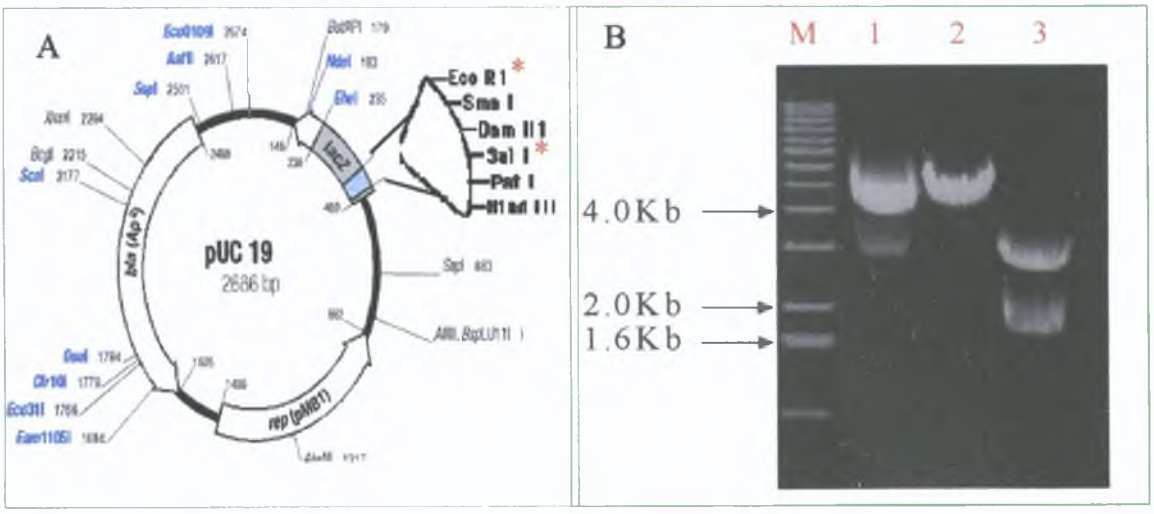


Figure 3.6. Characterizing PucCD21. (A) Restriction Map of the pUC 19 vector into which the CD21 cDNA was cloned. The 1.6Kb CD21 cDNA insert was cloned between the two restriction sites indicated in the multiple cloning site of pUC19, with the insertion of an extra EcoR1 site. **(B). Restriction digestion analysis of pUCCD21.** Lane M contains the Invitrogen 1Kb DNA ladder. Lane (i) contains undigested plasmid DNA. Lane 2 contains HindIII digested plasmid DNA resulting in the linearization of the plasmid with an expected size of 4.2Kb. Lane 3 contains EcoR1 digested pUCCD21. The 1.6Kb CD21 insert is clearly visible and this band was excised and used as the template for the synthesis of the CD21 probe (Figures 3.7 and 3.8).

Figure 3.7 EBNA2 is Present in BL41-ER/EBNA2-K3 and its Function is Activated in Response to Estrogen Addition.

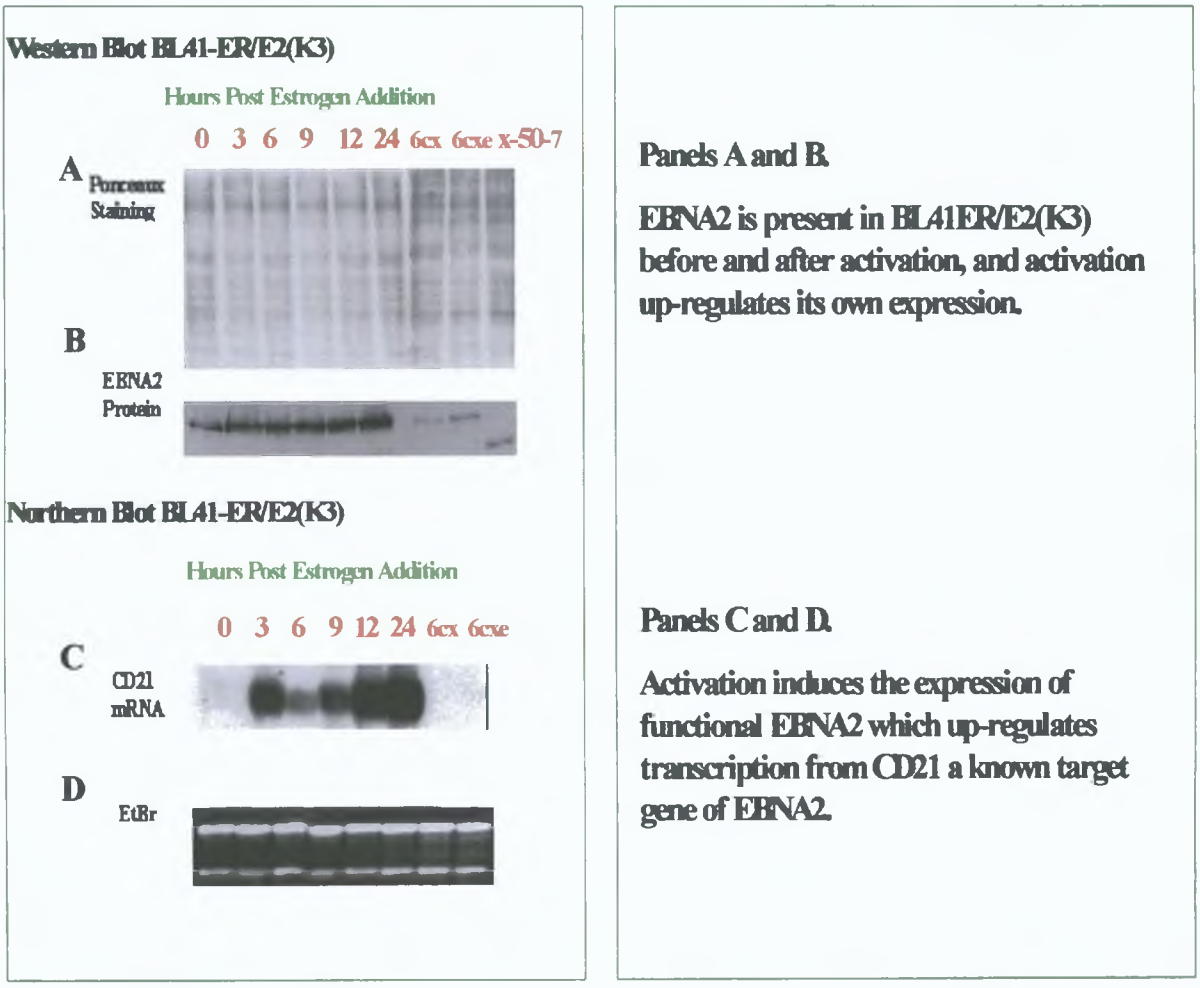
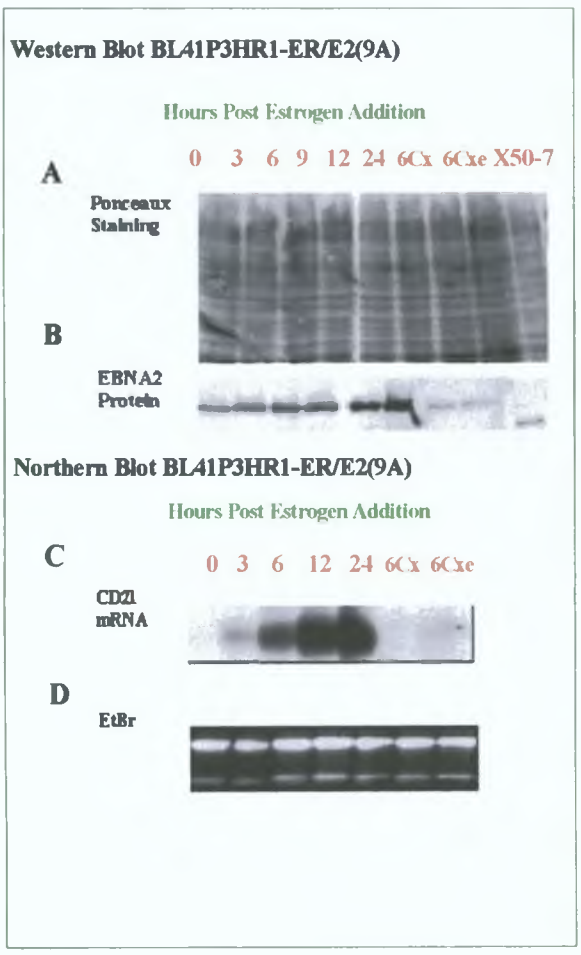


Figure 3.7. Functional EBNA2 is present in the BL41-ER/E2 (K3) cell line. EBNA2 function was activated in the BL41-ER/E2 (K3) cell line by addition of estrogen to the culture media. Total RNA and protein was harvested at 0, 3, 6, 9 12 and 24 hours post estrogen addition (EBNA2 activation) and after six hours in the presence of cyclohexamide (Cx) before and after EBNA2 activation. Panel B Western blot showing EBNA2 expression before and after induction over the experimental time course. EBNA2 activation induces its own expression as it up-regulates the BamH1C promoter thus EBNA2 expression is up-regulated over the 24-hour period post activation. Lane 9 of the Western blot shows wild-type EBNA2 expression in an LCL X50-7, the Estrogen fusion protein as expected is about 20Kb larger than its wild-type counterpart. Panel A shows the ponceaux staining of the Blot in panel B. In order to verify that the (EBNA2) protein expressed in the cells was functional, transcription from a known target gene of EBNA2 namely CD21 was analysed by Northern blotting. Panel C. Northern blot showing the up-regulation of CD21 in response to EBNA2 activation over the 24hr time course. Panel D shows the ethidium bromide staining of the gel used for blotting in panel C.

Figure 3.8 EBNA2 is Present in BL41P3HR1-ER/EBNA2-9A and its Function is Activated in Response to Estrogen Addition.



Panels A and B.

EBNA2 is present in BL41P3HR1ER/E2(9A) before and after activation, and activation up-regulates its own expression.

Panels C and D.

Activation induces the expression of functional EBNA2 which up-regulates transcription from CD21 a known target gene of EBNA2.

Figure 3.8. Functional EBNA2 is present in the BL41P3HR1-ER/E2 (9A) cell line. EBNA2 function was activated in the BL41P3HR1-ER/E2 (9A) cell line by addition of estrogen to the culture media. Total RNA and protein was harvested at 0, 3, 6, 9 12 and 24 hours post estrogen addition (EBNA2 activation) and after six hours in the presence of cyclohexamide (Cx) before and after EBNA2 activation. Panel B Western blot showing EBNA2 expression before and after induction over the experimental time course. EBNA2 activation induces its own expression as it up-regulates the BamH1C promoter thus EBNA2 expression is up-regulated over the 24-hour period post activation. Lane 9 of the Western blot shows wild-type EBNA2 expression in an LCL X50-7, the Estrogen fusion protein as expected is about 20Kb larger than its wild-type counterpart. Panel A shows the ponceaux staining of the Blot in panel B. In order to verify that the (EBNA2) protein expressed in the cells was functional, transcription from a known target gene of EBNA2 namely CD21 was analysed by Northern blotting. Panel C. Northern blot showing the up-regulation of CD21 in response to EBNA2 activation over the 24-time course. Panel D shows the ethidium bromide staining of the gel used for blotting in panel C.

The results show a significant elevation of CD21 mRNA levels in response to EBNA2 activation With respect to the BL41-ERE2(K3) time-course, a biphasic change in Cd21mRNA levels over the 24-hour period of EBNA2 activation was observed Levels start off high at 3 hours post induction but appear to fall after six hours post EBNA2 activation Levels then steadily rise again over the time-period analysed CD21 mRNA levels, interestingly do not show the same biphasic pattern for the BL41P3HR1-ERE2(9A) samples CD21 mRNA levels rise over the 14-hour time-period, in response to EBNA2 activation (figure3 8D) The lower panel shows the rRNA bands from the same ethidium bromide-stained gels used prior to blotting These results demonstrate that (i) ER-EBNA2 is present in both BL41-EREBNA2-K3 and BL41P3HR1-EREB9A and that (ii) EBNA2 function is activated in response to oestrogen in that one of its known target genes (namely CD21) is activated in response to the addition of oestrogen

3.1.6. *bfl-1* is a Transcriptional Target of EBNA2-Estrogen Fusion Proteins in the BL41/ER-EBNA2(K3) and BL41P3HR1/ER-EBNA2 (9A) Cell Lines.

Estrogen-regulatable EBNA2 was functional in the two cell lines (BL41-EREBNA2-K3 and BL41P3HR1-EREBNA2-9A) since CD21 mRNA levels were up-regulated in response to estrogen addition Northern blotting (using the probe from Figure3 5) was then used m order to ascertain if *bfl-1* was a transcriptional target of EBNA2 in this cell background The same RNA used in the CD21 blots above (Figures 3 7C/D and 3 8C/D) was used to measure *bfl-1* mRNA levels in response to EBNA2 activation over the same 24 hour-time period Protein extracts were also prepared over the time course and Western blotting was carried out to measure Bfl-1 levels in each of the cell lines before and after the activation of EBNA2 It can be seen in Figure 3 9 (Panel A) that Bfl-1 protein was detected in response to EBNA2 activation in both the K3 (Column I) and 9A (Column II) cell lines Similarly to the bfl-1 mRNA, the Bfl-1 protein was detected in the K3 cell line 3 hours after EBNA2 activation and after 6 hours in the 9A cell line Figure 3 9 also shows up-regulation of *bfl-1* mRNA in response to EBNA2 activation in both BL41-ER/E2 (K3) and BL41P3HR1-ER/E2 (9A) cell lines (Panels BI and BII) The fold induction was calculated at 6 2 and 5 8 fold respectively, m the two cell lines The lower

panel (PanelC) shows GAPDH mRNA levels obtained when the *bfl-1* blots were stripped and reprobed using the GAPDH probe as before. The *bfl-1* mRNA induction values recorded, were again measured after correction for loading was accounted for using the densitometry data for the GAPDH blots. Once again, RNA from cell lines known to express *bfl-1* were included in the Northern blots, (Column III) not only as a positive control for the bfl-1 riboprobe itself but also as an aid to showing the transcript obtained was the size of the *bfl-1* transcript recorded in the literature (Figure 3.9 III). A single mRNA transcript of the expected size of 0.8kb was observed.

Figure 3.9. *bfl-1* is a Target Gene of EBNA2 in EREBNA2-BL41-K3 and EREBNA2-BL41-P3HR1-9A Cell Lines.

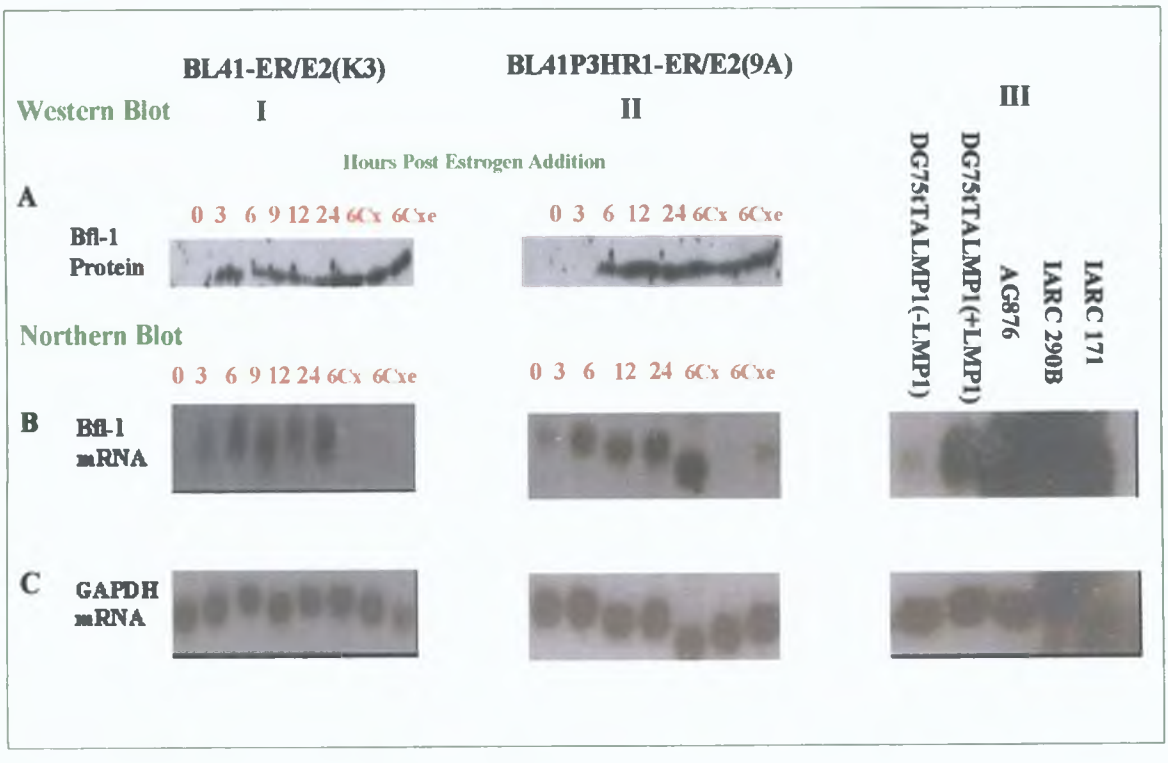


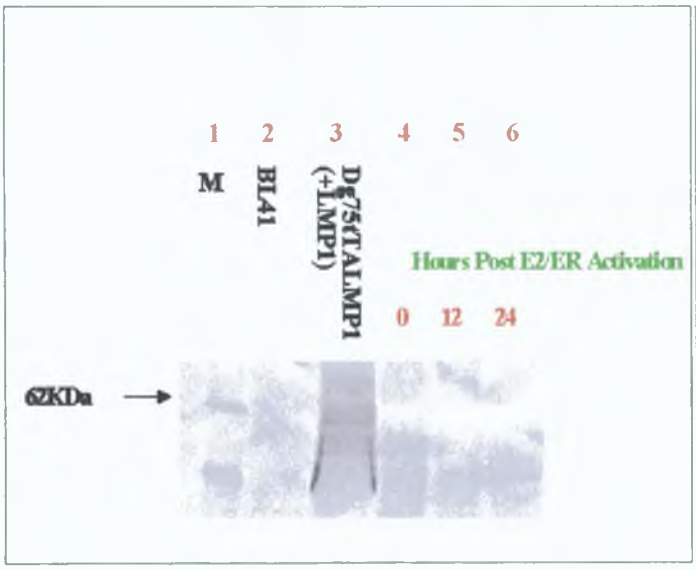
Figure 3.9. Bfl-1 is transcriptionally up-regulated by EBNA2 in BL41-ER/E2 (K3) and BL41P3HR1-ER/E2 (9A). Panel A shows the levels of Bfl-1 protein detected in the K3 (Column I) and 9A (Column II) cell lines, in response to EBNA2 activation. Panel B (Columns I and II) shows bfl-1 mRNA levels in BL41-ER/E2 (k3) and BL41P3HR1-ER/E2 (9A) respectively in response to EBNA2 activation over a 24-hour time-course. Panel B column III shows the steady state level of bfl-1 mRNA in a range of cell lines known to express bfl-1, including IARC 171 and IARC 290B which are LCLs and AG876 which is a type III BL cell line, these cell lines act as a positive control for *bfl-1* expression. Also shown in this panel are

the steady state levels of bfl-1 mRNA in the EBV negative DG75tTA-LMP1 before and after the induction of LMP1, these represent negative and positive controls respectively for the expression of *bfl-1* The Bfl-1 protein was detected in both the K3 and 9A cell lines within 3 and 6 hours respectively, of EBNA2 activation (Panel A columns I and II) In the case of BL41-ER/E2 (K3)(BI), and BL41P3HR1-ER/E2 (9A)(BII), bfl-1 mRNA is up-regulated in response to EBNA2 activation over the 24-hour time course In the control cell lines bfl-1 mRNA is present in relatively high levels in the 2 LCLs and the typeIII BL, it is also detected in the LMP1 expressing DG75tTA LMP1, but only at very low levels in the DG75tTALMP1 which has not been induced to express LMP1 In all cases the transcript detected is the same size ~0 8kb However a "smile" in columns I and II is apparent Panel C shows the GAPDH mRNA levels detected in the various cell lines after the blots in panel A above were stripped and reprobed Using densitometry data to normalise for loading, bfl-1 mRNA increased 6 2 and 5 8 fold in response to EBNA2 activation in BL41-ER/E2 (K3) and BL41P3HR1-ER/E2 (9A) respectively

3.1.7. EBNA2 Specifically Up-Regulates the *bfl-1* Gene in the EBV Positive BL41P3HR1-ER/E2 (9A) in an LMP1-Independent Manner.

Since BL41P3HR1-EREBNA2 9A is actually an EBV-positive (but EBNA2 and LMP1 negative) cell line and therefore expresses other EBV latent proteins, it was important to establish that LMP1 was not induced in response to EBNA2 activation Western blotting using the anti-LMP1 murine-monoclonal antibody cocktail CS1-4, demonstrated that LMP1 was not detected in BL41P3HR1-EREBNA2 9A pre- or post- addition of estrogen/EBNA2 activation (Figure 3 10), but was detectable in the control DG75tTA-LMP1 cell extract (24 hours post LMP1 induction) Thus, EBNA2 specifically up-regulates the bfl-1 gene in the EBV positive BL41-P3HR1-ER/E2 (9A) cell line in an LMP1-independent manner

Figure 3.10. EBNA2 Specifically Up-Regulates the *bfl-1* Gene in the EBV Positive BL41P3HR1-ER/E2 (9A) Cell Line, in an LMP1-Independent Manner.



Activation of EBNA2 in the BL41P3HR1-ER/E2 (9A) Cell Line Does Not Induce Expression of LMP1

Figure 3.10 EBNA2 specifically up-regulates the bfl-1 Gene in the EBV positive BL41P3HR1-ER/E2 (9A) in an LMP1-independent manner. Since BL41P3HR1-ER/E2 is EBV infected, Western blotting was undertaken to determine if LMP1 was up-regulated in response to EBNA2 activation in this cell line. Protein samples used in the Western in Figure 3.8B were also analysed for the presence of LMP1 using the CS1-4 anti LMP1 Antibody cocktail. LMP1 was not detected in BL41 an EBV negative Cell Line (Lane 2). The ~60Kda LMP1 protein was detected in the DG75tTALMP1 extract at 24hours post LMP1 induction (Lane3), but not in the BL41P3HR1-ER/E2 (9A) either before (Lane4) or after (lanes 5 and 6) induction of EBNA2. Thus EBNA2 up-regulates bfl-1 mRNA in the BL41P3HR1-ER/E2 (9A) cell line in an LMP1 independent manner.

It is clear from the ensemble of these experiments that EBNA2 up-regulates *bfl-1* mRNA and protein in (i) Bl41-EREBNA2-K3 in which it is the sole EBV protein and (ii) in BL41P3HR1-EREBNA2-9A in the absence of LMP1 expression. These results corroborate the findings in the DG75-tTA-EBNA2 cell line and together the results indicate that EBNA2 alone induces *bfl-1* mRNA and protein in two well-studied BL cell lines, BL41 and DG75. These findings were also confirmed by RPA analysis (See Figure 3.11 and 3.12).

3.1.8. Identifying Other Apoptosis-Related Transcriptional Targets of ER-EBNA2 in Bl41-EREBNA2-K3 and BL41P3HR1-EREBNA2-9A.

Having established that *bfl-1* is indeed a target gene of EBNA2 in these cell lines it was plausible to then investigate, in this system, if any of the other apoptosis-related/bcl-2 family genes were also EBNA2-responsive In order to determine this, RPA was carried out as before this time using the hAPO2C multiprobe (Pharmingen), a modified version of the hAPO2 probe set It can be seen from the RPAs (Figures 3 11 and 3 12) for each of the above cell lines, that elevated levels of bfl-1mRNA were once again induced in response to EBNA2 activation This is evident over the 24-hour time-course examined The GAPDH –corrected densitometry data indicated a 5 2 and 5 5 fold induction of *bfl-1* mRNA transcripts in the K3 and 9A cell lines respectively, in response to EBNA2 activation (Figure3 11 and 3 12) Interestingly (as in the DG75-tTA-EBNA2 cell line Figure 3 3) another apoptosis-related gene, *bik*, appeared to be down regulated by EBNA2 activation No significant changes in the mRNA levels from any of the other apoptosis-related genes was observed over the time-course investigated Using densitometry data, mRNA levels of the pro-apoptotic bik gene, were reduced by over 50% in both BL41-ER/E2-K3 and BL41P3HR1-ER/E2-9A, in response to EBNA2 activation over the 24-hour time-course (Figure 3 11, 3 12) Western blotting to detect the expression of Bik was also carried out on protein extracts harvested at the same time as the RPA samples, however no protein could be detected in control or sample cell lines using the only available antibody (Santa Cruz NBK (bik) Cat No SC1710) The combination of the up-regulation of transcription form the anti-apoptotic *bfl-1* and the down-regulation of transcription from the pro-apoptotic *bik* gene would suggest that EBNA2 expression may have a protective effect on BL cells under conditions where apoptosis is induced e g using ionomycin and serum starvation (See Figure 3 64) Down-regulation of *bik* mRNA was much less, and not observed until 72h in the DG75Tta-EBNA2 cell line when EBNA2 was induced (See Figure 3 3B)

Figure 3.11. Activation of EBNA2 Up-Regulates *bfl-1* mRNA Levels in an EBV-Negative Burkitt's Lymphoma Cell Line BL41-ER/EBNA2 -K3.

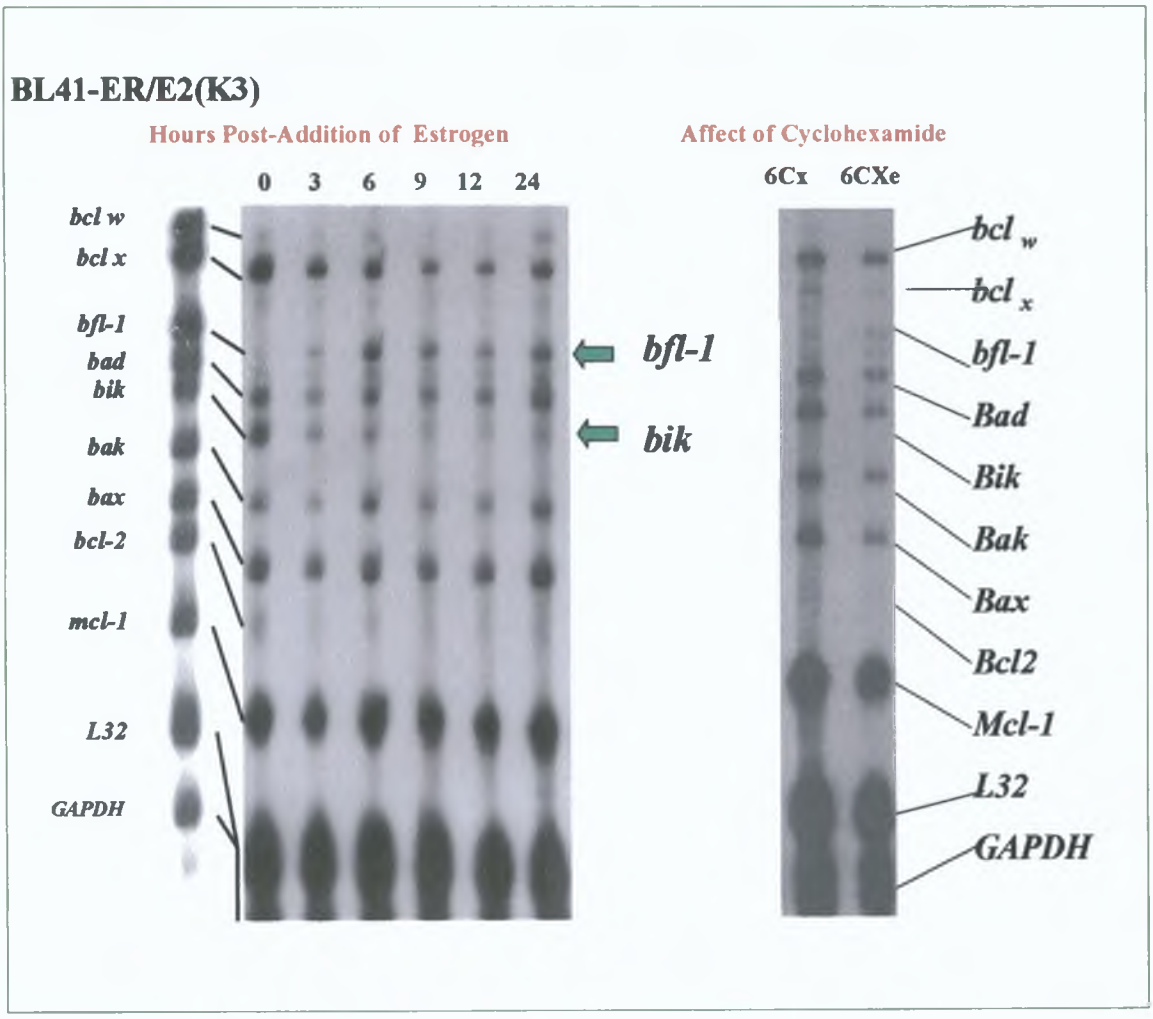


Figure 3.11. EBNA2 Up-Regulates *bfl-1* mRNA Levels and Down Regulates *bik* mRNA Levels in an EBV Negative BL Cell Line. EBNA2 was activated by addition of estrogen to the culture media as before. Total RNA was harvested at the indicated time points in the usual manner and the effect of EBNA2 activation on the bcl2 family of apoptosis related genes was analysed using multiprobe RPA analysis. Over the 24-hour time course, bfl-1 mRNA levels were up-regulated (5.2 fold) in response to EBNA2 activation, while bik mRNA levels were down regulated (~2 fold) in response to EBNA2 activation. In order to assess if induction of bfl-1mRNA was a direct effect of EBNA2 activation, cyclohexamide (Cx), an inhibitor of protein synthesis was added and cells harvested after six hours of exposure to cyclohexamide before (6Cx) and after (6Cxe) addition of estrogen. Total RNA was extracted in the same way as for other samples and RPA performed as described in the materials and methods.

Figure 3.12. EBNA2 Up-Regulates *bfl-1* mRNA Levels in an EBV-Positive Burkitt's Lymphoma Cell Line in an LMP1-Independent Manner.

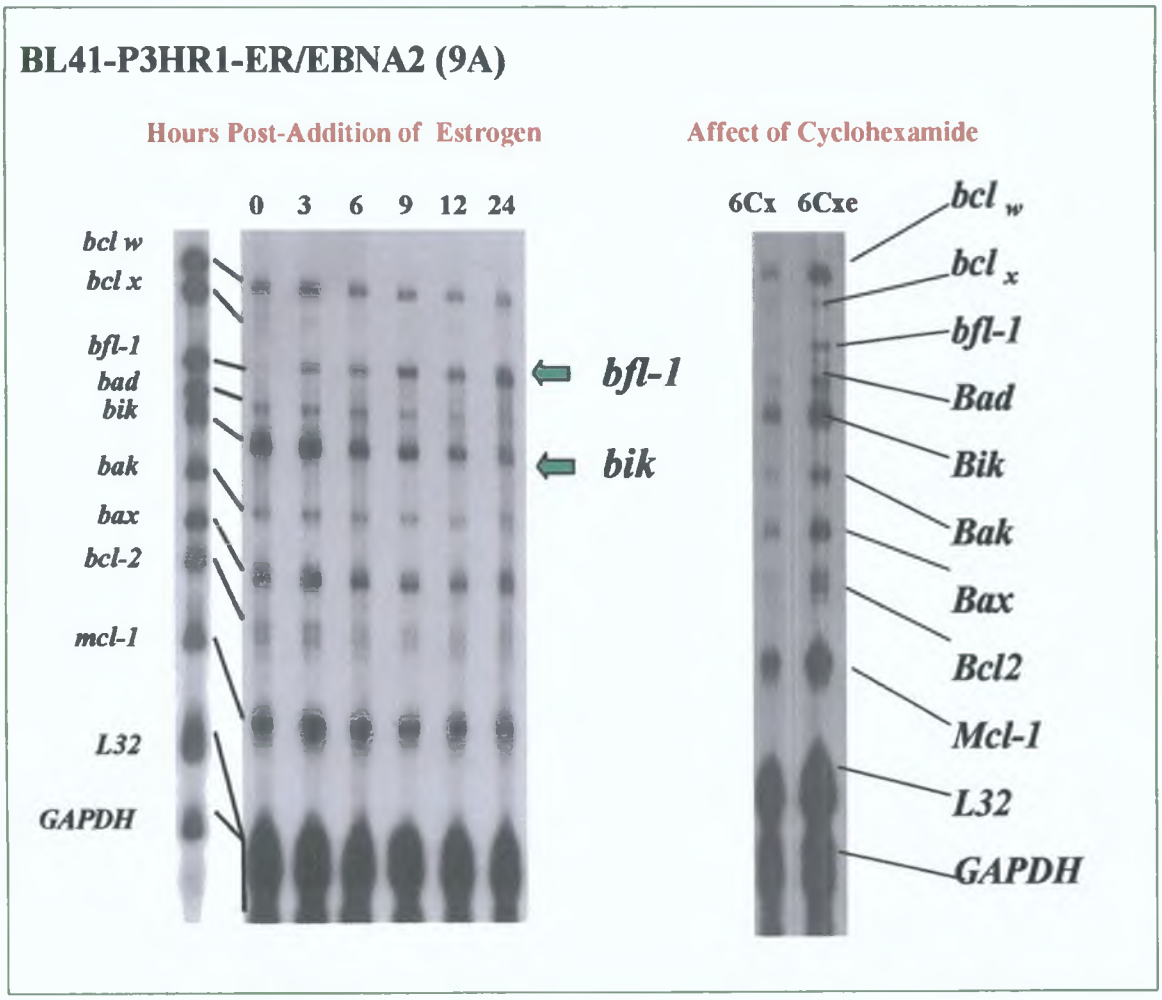


Figure 3.12. EBNA2 up-regulates bfl-1 mRNA levels and down regulates bik mRNA levels in an EBV positive BL cell line in an LMP1 independent manner. EBNA2 was activated by addition of estrogen to the culture media as before. Total RNA was harvested at the indicated time points in the usual manner and the effect of EBNA2 activation on the bcl2 family of apoptosis related genes was analysed using multiprobe RPA analysis. Over the 24-hour time course, bfl-1 mRNA levels were up-regulated (5.8 fold) in response to EBNA2 activation, while bik mRNA levels were down regulated (~2 fold) in response to EBNA2 activation. Western blotting had been used to show no LMP1 expression occurred in response to EBNA2 activation, thus the effects on the transcription from the various genes could be attributed to the activation of EBNA2 alone. In order to assess if induction of bfl-1mRNA was a direct effect of EBNA2 activation, cyclohexamide (Cx), an inhibitor of protein synthesis was added and cells harvested after six hours of exposure to cyclohexamide before (6Cx) and after (6Cxe) addition of estrogen. Total RNA was

extracted in the same way as for other samples and RPA performed as described in the materials and methods

Overall these results demonstrate that the anti-apoptotic gene bfl-1 is a target of EBNA2 in a BL-derived cell background EBNA2 up-regulates *bfl-1* mRNA levels in an EBV-positive cell line in the absence of LMP1 (Figure 3 12) In addition, the pro-apoptotic gene *bik* also appears to be a transcriptional target of EBNA2 in these BL cell lines, as the RPA experiments show a reduction of 50% in the steady-state level of its mRNA after 24 hours in both BL41-EREBNA2-K3 and BL41P3HR1-EREBNA2-9A in response to activation of ER-EBNA2 Transcription from the bik gene is also down regulated in the DG75EBNA2tTA system after 72 hours of EBNA2 expression

3.1.9. Cyclohexamide: Effect of protein synthesis on EBNA2 fusion protein activity.

Cyclohexamide is an antibiotic that inhibits protein synthesis BL41-ER/E2 (K3) and BL41P3HR1-ER/E2 (9A) cell lines were treated with 50ug/ml cyclohexamide for six hours with and without EBNA2 activation and the samples were analysed by both Northern blotting and RPA Cyclohexamide was used to determine if the effect of EBNA2 on *bfl-1* mRNA was specific to EBNA2 thus when all new protein synthesis is stopped, and EBNA2 alone is activated, EBNA2 alone is responsible for the effect on *bfl-1* mRNA In figures 3 11 and 3 12 it can be seen that in the presence of cyclohexamide without EBNA2 activation, bfl-1 mRNA is detected only at very low levels however upon activation of EBNA2 in the presence of cyclohexamide an increase in *bfl-1* mRNA is seen These results imply that *bfl-1* is a direct target gene of EBNA2

3.2.0. *bfl-1* Promoter Studies. Effect of EBNA2 on *bfl-1* Promoter Activity.

Having established that EBNA2 alone can up-regulate *bfl-1* mRNA and protein levels in B cell lines, it was then necessary to determine if this up-regulation was due to an enhanced rate of transcription from the promoter of the *bfl-1* gene In order to investigate if increased *bfl-1* promoter activity was involved, a suitable *bfl-1* promoter-reporter construct had first to be generated

Promoter reporter assays are one of the most common and convenient ways of assessing transcriptional activity The activity of the promoter of interest is assessed by transfection of a construct containing the promoter sequence fused to a reporter gene into an appropriate cell line Promoter activity in response to a chemical stimulus or a protein expressed from another plasmid that is cotransfected with the promoter-reporter construct is then measured by assaying for reporter activity Two of the commonly used reporter genes in promoter-reporter constructs encode the enzymes chloramphenicol acetyl transferase (CAT) and luciferase (luc) However, 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 the *bfl-1* promoter activity by EBNA2 using a luciferase based system

3.2.1. *bfl-1* Luciferase Promoter Reporter Construct –1374/+81 *bfl-1* Luc.

The 1 4kb DNA sequence from the 5' transcriptional regulatory region (-1374/+81) of the human *bfl-1* gene was obtained as a fragment cloned into the promoterless pCAT basic plasmid (Zong *et al*, 1999) In order to change the reporter context of the p-1374/+81Bfl-1-CAT construct to a luciferase based system, a cloning strategy based on replacing the CAT gene with the luciferase gene from pGL2-basic vector (Promega) in the pCAT basic background was employed The circle maps of pCAT –basic and pGL2 basic are shown in Figure 3 20 The sites in the pCAT basic polylinker into which the –1374/+81 region of the bfl-1 gene was cloned are indicated The –1374/+81 Bfl-1 Luc reporter plasmid contains the –1374/+81 region of *bfl-1* cloned just upstream of the luc gene and was

constructed in the laboratory by B D'Souza Using the same strategy as B D'Souza further promoter deletion constructs were generated at a later date (Figure 3 20)
Figure 3 13 shows the results of the restriction enzyme analysis used to confirm the organisation of the DNA fragment encoding -1374/+81 bfl-1 in the vector -1374/+81 Bfl-1 Luc

Figure 3.13. Characterizing -1374/+81 Bfl-1 Luc.

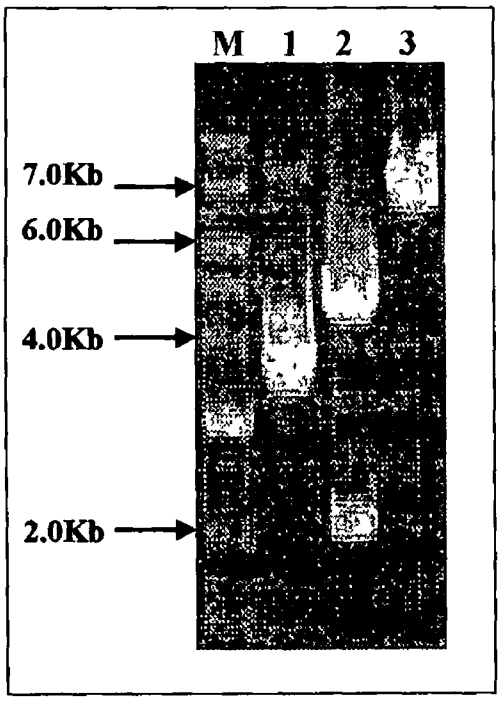


Figure 3.13 Characterizing -1374/+81 bfl-1 Luc. The EcoR1 restriction digestion pattern of p-1374/+81 Bfl-1 Luc plasmid confirms the expected organisation of the plasmid
Lane M is the Invitrogen 1Kb DNA Ladder Lanes 1-3 p-1374/+81 Bfl-1 Luc Lane 1 is undigested plasmid DNA Lane 2 shows the EcoR1 digestion pattern, resulting in a 4 7Kb fragment and a 2 0KB fragment Lane 3 shows the BamH1 digestion pattern Restriction with BamH1 linearizes the -1374/+81 bfl-1 Luc plasmid resulting in a 6 7Kb fragment

3.2.2. Characterising the EBNA2 Expression Plasmids.

Two EBNA2 expression plasmids (pPDL151) and (pPDL152), were obtained (from Diane Hayward, Table 2 2) pSG5EBNA2 (also known as pPDL151(Ling *et al*, 1993) consists of the wild type B95-8 EBNA2 gene cloned into the SG5 vector (Stratagene) pSG5EBNA2WW323SR (also known as pPDL152) had been constructed by replacing two tryptophan residues at positions 323 and 324 in the EBNA2 CBF1 binding domain with a serine and arginine residue respectively and in so doing abrogating its ability to bind CBF1 The mutant gene was then cloned into the pSG5 vector (Ling et al, 1993) Implicit in this mutation was the introduction of an extra XbaI restriction site in the pSG5EBNA2WW323SR construct, this extra site was used to distinguish the plasmids by

restriction analysis prior to use, as shown below Co-transfection experiments with these plasmids and the *bfl-1* promoter reporter constructs should elucidate not only the involvement of EBNA2 but also any possible involvement of CBF1 in trans-activation of the *bfl-1* promoter by EBNA2 All other gifted plasmids were similarly checked prior to use (Not Shown)

Figure 3.14. Characterising the pSG5EBNA2/pSG5EBNA2WW323SR Expression Plasmids.

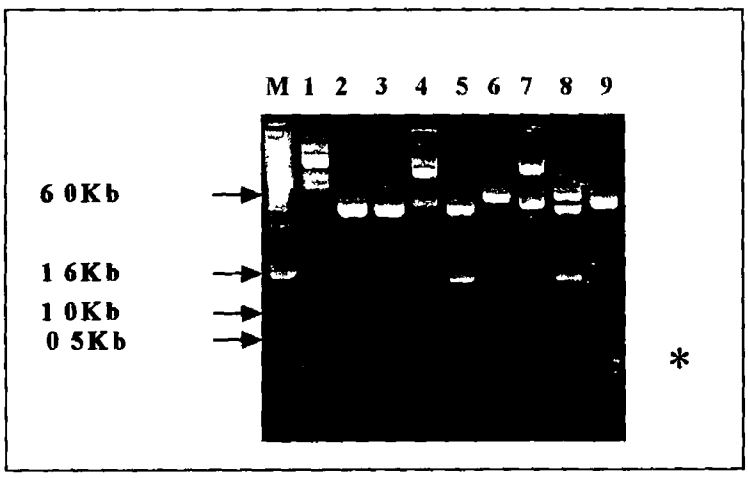


Figure 3 14 Characterising the pSG5EBNA2/pSG5EBNA2WW323SR Expression Plasmids Lane 1 1Kb DNA Ladder (Invitrogen) Lanes 1, 4 and 7 contain undigested plasmid DNA from the pSG5, pSG5EBNA2 and pSG5EBNA2WW323SR expression plasmids Lanes 2, 5 and 8 show bgl II digested DNA from the pSG5, pSG5EBNA2 and pSG5EBNA2WW323SR expression plasmids respectively Lanes 3, 6 and 9 show the restriction pattern for XbaI digestion for the pSG5, pSG5EBAN2 and pSG5EBNA2WW323SR expression plasmids respectively Restriction endonuclease digestion with bglII linearised the pSG5 plasmid to give an expected size of around 4Kb (Lane 2) Both pSG5EBNA2 and pSGEBNA2WW323SR had another BglII site introduced in the course of cloning the 1 6Kb EBNA2/EBNA2WW323SR fragments and thus digestion with BglII excises the 1 6Kb EBNA2 insert thus a band of this size should be visible here, another band representing the 4 1Kb pSG5 into which EBNA2/EBNA2WW323SR had been cloned should also be apparent (Lanes 5 and 8) Finally only one Xba I site is present in pSG5 thus restriction digestion of pSG5 with XbaI linearizes the pSG5 vector to create a DNA band at 4 1Kb (Lane 3) In the case of pSG5EBNA2 only the pSG5 XbaI site is present thus restriction digestion with XbaI linearizes the pSG5EBNA2 vector generating a DNA band of 5 7Kb * For the EBNA2WW323SR mutant, an extra XbaI site has been introduced in the course of the mutagenesis and thus another fragment of around 450bp is generated (Lane 9)

Figure 3.15. All DNA used in Transfection Studies was in the same Topological Condition and at a Similar Concentration.

Figure 3.15. DNA Used in Transfection Analyses was in the Same Topological Condition and at Similar Concentration. Although not digested, the condition and quality of the DNA can be assessed by resolving the DNA through an agarose gel. This is necessary to allow comparison of transfection data for each particular experiment. In this case 500ng of DNA from the pSG5, pSG5EBNA2 and pSG5EBNA2WW323SR expression plasmids are shown after electrophoresis (for 1 hour) through a 1% agarose gel made up in 1X TAE.

3.2.3. EBNA2 Trans-Activates the *bfl-1* Promoter in BL Cell Lines by a Mechanism Requiring CBF1.

pSG5EBNA2 and pSG5EBNA2WW323SR were titrated against the *bfl-1* promoter reporter construct in co-transfection studies. *bfl-1* promoter activity was then analysed using the luciferase assay. One, 3,5,7 and 10ug of the two EBNA2 expression plasmids were transiently co-transfected with 1ug of the bfl-1 promoter reporter construct, –1374/+81 bfl-1 Luc, in the EBV negative cell line DG75.

Figure 3.16 Titration of pSG5EBNA2 and pSG5EBNA2WW323SR with -1374/+81 *bfl-1* Luc

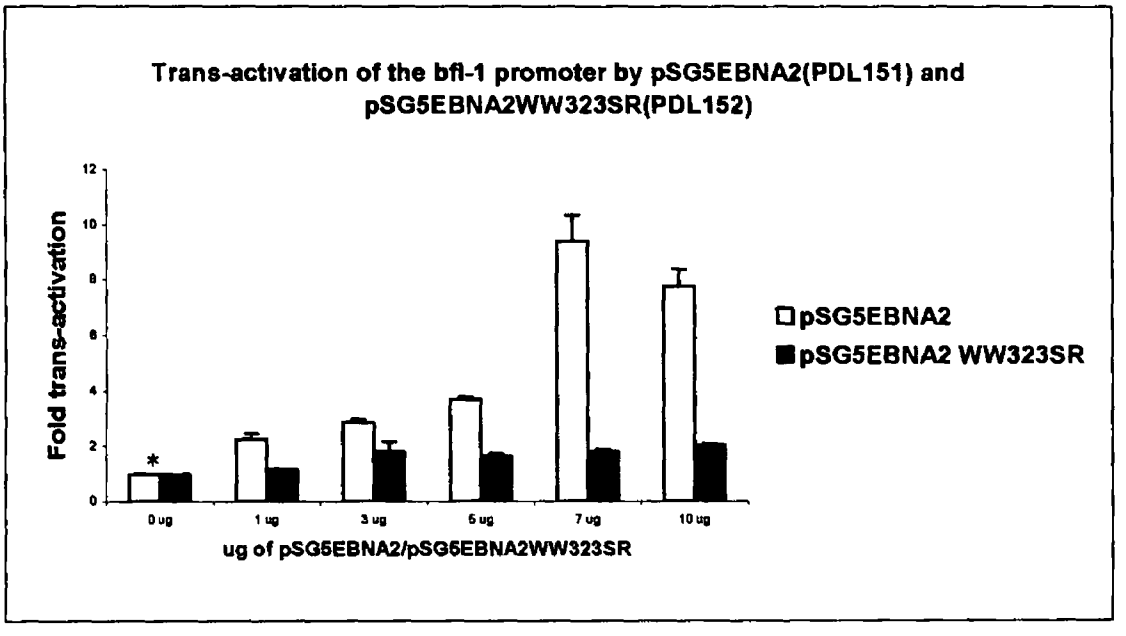


Figure 3 16. Activity of the *bfl-1*-luciferase promoter construct (-1374/+81 Bfl-1 Luc) Upon Titration of pSG5EBNA2 and pSG5EBNA2WW323SR Expression Plasmids 1ug of the bfl-1promoter reporter was cotransfected into DG75 cells with varying quantities of pSG5EBNA2 and pSG5EBNA2WW323SR. Cells were harvested 48 hr after transfection and analysed for luciferase activity. A dose-dependent increase in bfl-1 promoter activity was observed in response to co-transfection with increasing quantities of pSG5EBNA2 expression plasmid. PSG5EBNA2WW323SR only weakly transactivated the bfl-1 promoter over the range of quantities assayed. Thus binding of the CBF1 transcription factor may be important in mediating EBNA2 transactivation of the -1374/+81 bfl-1 Luc promoter. * Fold activation with the "empty" pSG5 vector (basal transactivation levels) is arbitrarily set to 1 and activation by the pSG5-related expression vectors are expressed relative to this. This is the case for all subsequent transfection experiments.

Transfections in the DG75 cell line were carried out using the DEAE dextran method (See Materials and Methods) unless otherwise stated. Briefly the principle of this method of transfection relies on the positively charged diethylaminoethyl (DEAE)-dextran interacting with the negatively charged phosphate backbone of DNA. The resulting complex is then absorbed into cells by endocytosis. Because of the toxicity of the DEAE

dextran this method of transfection is most commonly used in transient transfections The total amount of expression plasmid was adjusted in all cases to 10ug with the empty vector pSG5 Trans-activation rates were determined by standardizing the luciferase values obtained with luciferase values recorded from transfections with pSG5 alone A plasmid carrying the Lac Z (p7CMV Lac Z) gene was also included in each transfection and transfection efficiency was accounted for by measuring beta-galactosidase activity (Materials and Methods)

A clear dose dependent increase in *bfl-1* promoter activity occurs in response to addition of increasing quantities of the EBNA2 expression plasmid pSG5EBNA2 (Figure 3 16) Promoter activity increased up to an average of 9 fold with the addition of 7ug of pSG5EBNA2 Any further increase in the amount of EBNA2 expression plasmid added did not increase trans-activation of the promoter in this system Thus a 1 7 ratio of *bfl-1* promoter-reporter plasmid EBNA2 expression plasmid was deemed optimal in this system Optimal-fold trans-activation was recorded at an average value of 9 3 fold over the course of three independent experiments The CBF1- mutant pSG5EBNA2WW323SR EBNA2 expression plasmid trans-activated the promoter weakly (between 1 2 and 2 02 fold) over the range of concentrations used This result indicates that EBNA2 trans-activates the *bfl-1* promoter via a mechanism requiring the cellular DNA binding protein CBF1/RBP-jk

Total protein was extracted from the transfected cells and Western blotting performed using the PE2 antibody to detect the presence of EBNA2, however this antibody did not detect EBNA2 in the transiently transfected cells Transfections were scaled up to increase protein yields however Western Blotting still proved unsuccessful This problem has been experienced elsewhere (Zimber-Strobl et al , 1994) EBNA2 protein expression from these constructs (pSG5 EBNA2 and pSG5EBNA2WW323SR) were detectable however after stable transfection in the DG75 cell line (Figure 3 61)

3.2.4. EBNA2 Trans-activates the *bfl-1* Promoter via CBF1 in 2 Other BL Cell Lines.

It was important to establish if the trans-activational effect of EBNA2 on *bfl-1* was a feature in other BL cell lines (Figure 3 17) For this reason, similar experiments were carried out in two other well-studied BL cell lines, namely BL41 and BJAB (Described in materials and methods Table 1 0) Co-transfections for the BJAB cell line were performed using the DEAE-dextran method as before BL41 cells were transfected by electroporation (see materials and methods) In all cases, cells were harvested 24-48 hours post-transfection and lysates produced in an identical manner to DG75 cells before Activity of the *bfl-1* promoter was then assessed using the luciferase assay The optimized Promoter expression-plasmid ratio determined in the previous experiment was used with 1ug of -1374/+81 *bfl-1* Luc, co-transfected with 7ug of pSG5EBNA2/pSG5EBNA2WW323SR expression plasmids As before, fold trans-activation values were obtained using Beta-gal corrected luciferase values In both cell lines, there is significant trans-activation of the *bfl-1* promoter by pSG5EBNA2 In BL41 the promoter was trans-activated an average of 5 4 fold while in Bjab the fold-trans-activation was slightly higher at 5 7 fold pSG5EBNA2WW323SR only weakly trans-activates -1374/+81 *bfl-1*-Luc in both cell lines This data demonstrates the importance of the EBNA2 CBF1 binding site for the trans-activational effect of EBNA2 on the *bfl-1* promoter in both Bl41 and Bjab cell lines

Figure 3.17 EBNA2 Trans-activation of the *bfl-1* Promoter (-1374/+81 bfl-1 Luc) in both BL41 and Bjab Cell Lines Requires the EBNA2 CBF1 Binding Domain.

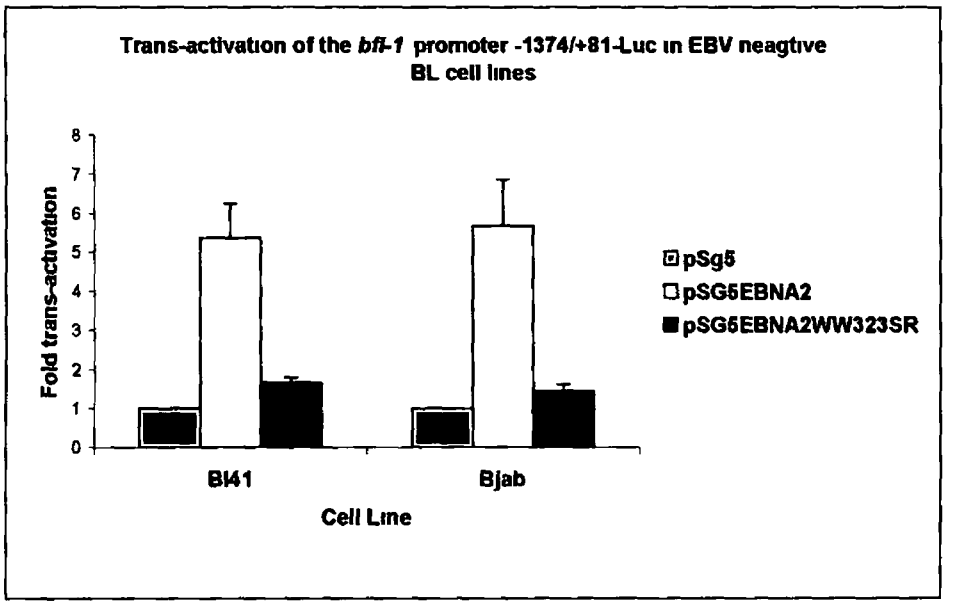


Figure 3.17 EBNA2 Trans-activates the *bfl-1* Promoter in a Range of BL Cell Lines by a Mechanism Dependent Upon its Ability to Bind to CBF1 BL41 and Bjab cells were transfected by electroporation and the DEAE-dextran methods respectively One microgram of the bfl-1 promoter reporter construct (-1374/+81 bfl-1 Luc), was co-transfected with 7ug of pSG5EBNA2 or pSG5EBNA2WW323SR. After 48 hours cells were lysed and promoter activity assessed by the luciferase assay The luciferase assay was performed using the Shaeff luminoskan 2000 All values are relative to the activity reported by the pSG5 vector alone All values are also corrected using B-Gal data It can be seen from the results that EBNA2 trans-activates the *bfl-1*luciferase reporter construct in these two BL cell lines It can be concluded from figures 2 2 and 2 3 that EBNA2 trans-activates the *bfl-1* promoter in a range of BL cell lines by a mechanism dependent upon its ability to bind to CBF1

3.2.5. Trans-activation of the *bfl-1* Promoter by EBNA2 May be a B Cell Specific Effect.

It was also necessary to investigate if the EBNA2 trans-activational effect on *bfl-1* was a B cell specific effect For this reason, similar co-transfection experiments were carried out with the EBNA2 expression plasmids and the *bfl-1* promoter reporter constructs in a T cell line Jurkat, an epithelial cell line C33A and a bovine vascular smooth muscle cell

line VSMC Each experiment included a transfection with the pGL2-Control vector alone, as a control for the efficiency of transfection (Results not shown) The pGL2-Control Vector (Promega) contains an SV40 promoter and enhancer sequences upstream of the luciferase gene, resulting in strong *luc* expression in many types of mammalian cells This plasmid is useful in monitoring transfection efficiency in general and is a convenient standard for promoter and enhancer activities expressed by pGL2 recombinants Transfections involving the Jurkat cell line were carried out by the DEAE-dextran method Transfections using the C33A cell line were carried out by electroporation and transfections involving the vascular cells used the Lipofectamine® method of transfection

Figure 3.18. EBNA2 Trans-Activation of the *bfl-1* Promoter May be a B Cell Specific Effect.

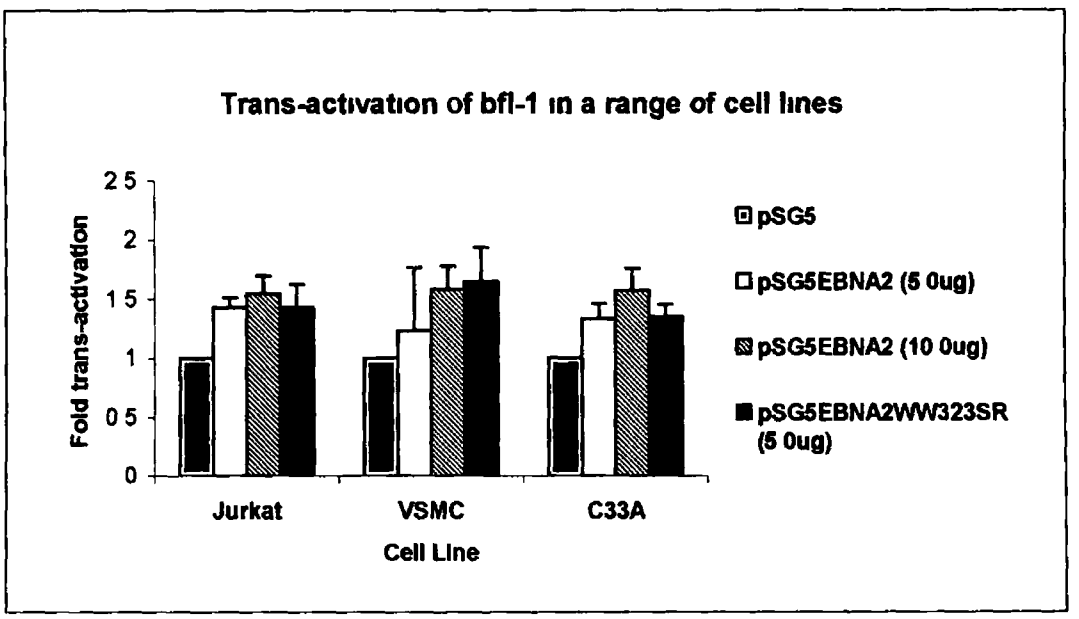


Figure 3.18 EBNA2 Weakly Transactivates the *bfl-1* promoter –1374/+81 bfl-1 Luc in T, Smooth Muscle Cells and Epithelial cells, thus EBNA2 transactivation of the *bfl-1* promoter may be a B cell specific effect. Co-transfections in three cell lines, Jurkat (T cell line), VSMC (a bovine vascular smooth muscle cellline) and C33A (a human epithelial cell line) 1ug of the –1374/+81 bfl-1 Luc promoter was co-transfected with 5ug/10ug of EBNA2 or 5ug of EBNA2WW323SR.Relative luciferase values were used to calulate fold-transactivation values All results are correceted using B-gal values

In the T cell line, 5ug of pSG5EBNA2 trans-activated the *bfl-1* promoter 1 4 fold, this figure increased to 1 6 fold when 10ug of pSG5EBNA2 was added, the non-CBF1 binding pSG5EBNA2WW323SR expression plasmid trans-activated the promoter 1 4 fold suggesting that CBF1 has no part in the small trans-activational effect of EBNA2 on the *bfl-1* promoter in this cell context In the vascular smooth muscle cell line, increasing the amount of pSG5EBNA2 expression plasmid increased the fold-trans-activation of the *bfl-1* promoter from 1 2 to 1 6, however in this cell line the mutant EBNA2 trans-activated the *bfl-1* promoter to a slightly higher extent than 10ug of EBNA2 itself The results obtained in this cell line however have larger error bars, as reproducibility obtained in the system was low, due to the small number of cells used in the Lipofectamine® assay With regard to the epithelial cell line C33A no significant difference in trans-activation of the *bfl-1* promoter occurred by increasing the amount of pSG5EBNA2 expression plasmid added In this cell line the promoter was trans-activated less than 1 5 fold The mutant EBNA2 pSG5EBNA2WW323SR trans-activated the promoter 1 4 fold Low trans-activation of the bfl-1 promoter was observed in all cases despite the efficient (Relative to transfection in B cell lines) trans-activation of the pGL2 control promoter reporter construct in each of the cell lines

Overall, despite comparable levels of trans-activation of the pGL2 control promoter reporter in each of the three cell lines, compared to transfections in the B cell lines, the *bfl-1* promoter was not trans-activated above 1 6 fold and the mutant did not trans-activate the promoter above 1 6 fold Although, increasing the amount of pSG5EBNA2 increased the fold-trans-activation of the promoter in each case, no significant increase in trans-activation was observed by increasing the amount of EBNA2 expression plasmid, in any of the cell lines therefore there may be no merit in continuing the titration of EBNA2 in these cell lines In total, these results show non-existant or very low levels of trans-activation of the *bfl-1* promoter in the non B cell lines used

In order to identify regions on the *bfl-1* promoter that mediate trans-activation by EBNA2, three luciferase-reporter plasmids (derived from –1374/+81 bfl-1 Luc) were generated which contained deletions from the 5' end of the promoter

3.2.6. Generation of Promoter Deletion Constructs, -1240/+81 *bfl-1* Luc, -367/+81 *bfl-1* Luc and -129/+81 *bfl-1* Luc.

In addition to the –1374/+81 bfl-1 CAT plasmid, three other bfl-1-CAT promoter reporter plasmids were obtained from Celine Gelinas (Center for Advanced Biotechnology and Medicine, University of Medicine and Dentistry of New Jersey–Robert Wood) These plasmids contained various truncated DNA sequences from the bfl-1 promoter cloned into the pCAT-basic (Promega) Nucleotides from positions –1240/+81, –367/+81 and –129/+81 respectively from the bfl-1 promoter had been inserted between the SalI and XbaI sites in the pCAT-basic vector, in the same manner as-1374/+81 *bfl-1* CAT (Zong *et al*, 1999) As before, relative to CAT assays, luciferase assays are easier to perform, non-radioactive and cheaper, and since a full length (-1374/+81 *bfl-1* Luc) bfl-1 luciferase reporter construct had already been generated (D'Souza *et al*, 2000) it was therefore decided to generate a series of bfl-1 promoter-luciferase reporter constructs for the purpose of investigating potential EBNA2-responsive elements within this promoter Three luciferase-promoter deletion constructs, -1240/+81 bfl-1 Luc (6 7Kb), -367/+81 bfl-1 Luc (5 9Kb) and –129/+81 bfl-1 Luc (5 7Kb) were generated according to the schematic in Figure 3 20 Briefly, the 3 truncated promoter regions were excised by double digestion with XbaI and BamHI of the –1240/+81 bfl-1 CAT, –367/+81 bfl-1 CAT and –129/+81 bfl-1 CAT constructs respectively, yielding promoter-containing fragments of 4 1Kb, 3 3Kb and 3 1Kb respectively These truncated bfl-1 promoter sequences were then ligated to the 2 6Kb BamH1-HindIII-ScaI digested fragment of the promoterless luciferase reporter plasmid pGL2 BASIC (Promega) which contained the luc gene In summary the ligation reaction involved blunt end ligation between the "filled in" XbaI and HindIII ends and a "sticky end ligation" between the BamH1 ends ScaI was also used to digest thepGL2-Basic vector to produce fragments of distinguishable sizes The same strategy was used to generate the –1374/+81 bfl-1 Luc reporter construct (See Flowsheet Figure 3 20) Recombinant vectors were verified by restriction analysis followed by agarose gel electrophoresis The presence of an EcorI site in the luciferase gene, as well as one inserted from the promoter containing pCAT basic vector make

identification of recombinants relatively simple (Figure 3.19B) These constructs were then used in transient co-transfection assays of BL-derived cell lines.

Figure 3.19. Generating *bfl-1-luc* Promoter Reporter Constructs. –1374/+81 *bfl-1* Luc, -1240/+81 bfl-1 Luc, -367/+81 bfl-1 Luc and –129/+81 bfl-1 Luc.

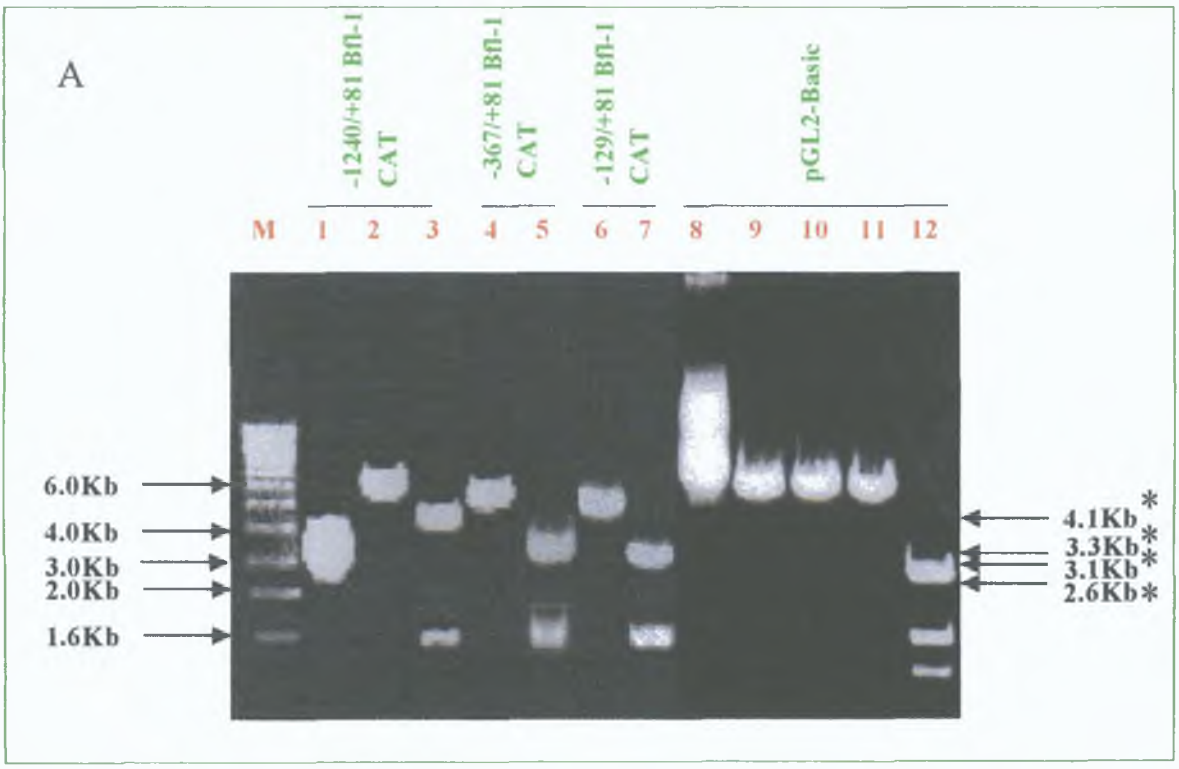
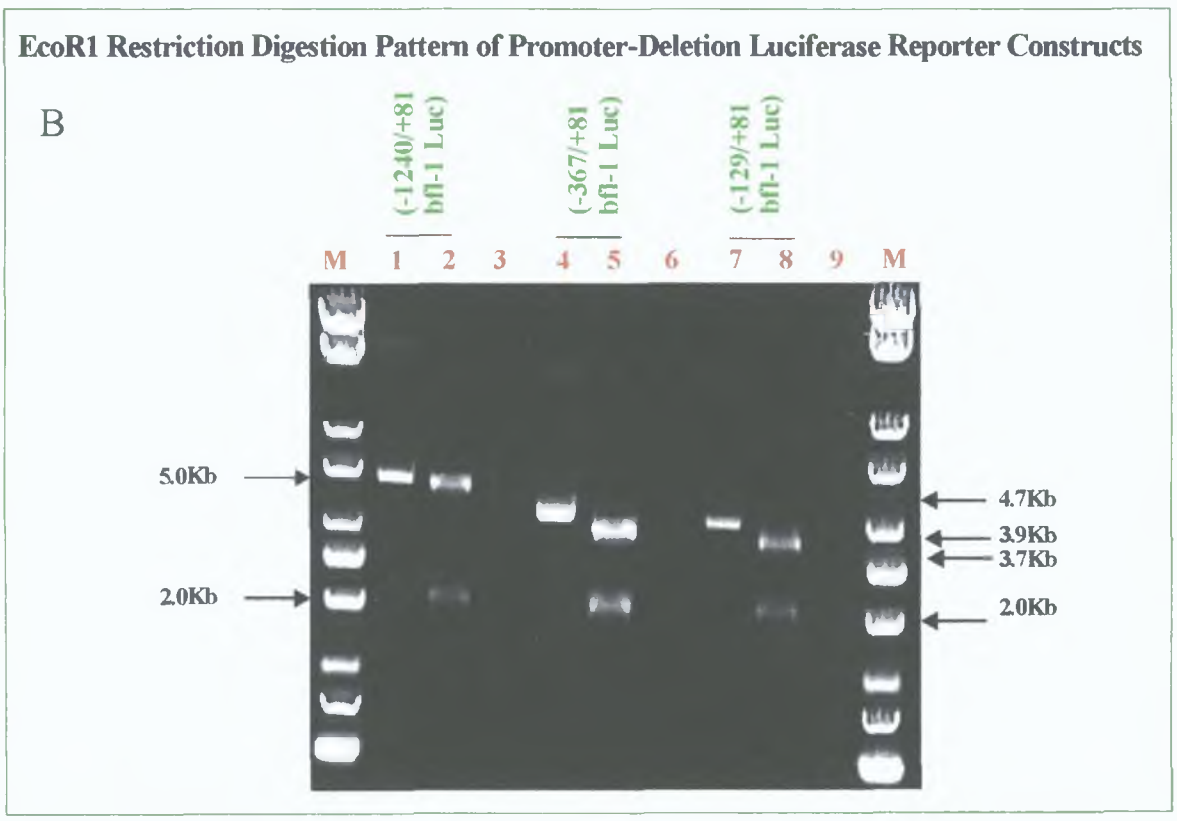


Figure 3.19. Generating *bfl-1-luc* Promoter Reporter Constructs. –1374/+81 *bfl-1* Luc, -1240/+81 bfl-1 Luc, -367/+81 bfl-1 Luc and –129/+81 bfl-1 Luc. (A). Lane M contains the Invitrogen 1Kb DNA Ladder. Lanes 1,2 and 3 contain –1240/+81 Bfl-1CAT plasmid DNA. Lanes 4 and 5 contain restriction digested –367/+81 Bfl-1 CAT plasmid DNA. Lanes 6 and 7 contain restriction digested –129/+81 Bfl-1 CAT plasmid DNA and lanes 8, 9, 10, 11 and 12 contain pGL2 Basic DNA (Separate gel). Lane 1 contains undigested plasmid DNA. Lanes 2, 4 and 6 contain BamHI digested plasmid DNA. Restriction digestion with BamH1 linearized the three CAT reporter constructs generating bands visible at ~5.7Kb for (-1240/+81 Bfl-1 Cat), lane 2) at ~4.8Kb for (-367/+81 Bfl-1 CAT, Lane 4) and at ~4.55Kb for (-129/+81 Bfl-1 CAT, lane 6). Lanes 3, 5 and 7 contain BamHI-XbaI double digested plasmid DNA. The 4.1Kb fragment in lane 3 contains the –1240/+81 region of the bfl-1 promoter. The 3.3Kb fragment in lane 5 contains the –367/+81 region of the bfl-1 promoter. The 3.1Kb fragment in lane 7 contains the –129/+81 region of the bfl-1 promoter. Lane 8 contains the undigested pGL2-Basic DNA. The 5.597Kb pGL2 Basic plasmid was digested with HindIII, BamH1 and ScaI to yield a 2.6Kb HindIII-BamHI fragment containing the luc gene. Lane 8 undigested plasmid DNA, lane 9: HindIII digested plasmid DNA, lane 10 BamHI

digested DNA, lane 11 ScaI digested plasmid DNA and lane 12, plasmid digested with HindIII, BamHI and ScaI. A 1% agarose 1X TAE gel was used and electrophoresis was carried out at 100v for 1Hr. The fragments marked by asterix were then ligated to generate the luciferase reporter constructs

Figure 3.19B. EcorI Restriction Digestion Pattern of bfl-1 Promoter-Deletion Luciferase Reporter Constructs.



(B). EcoR1 Restriction Digestion Pattern of bfl-1 Promoter-Deletion Luciferase Reporter Constructs. In generating the Luciferase resporter constructs. One EcoR1 restriction site is present in the luc gene (shown in the flowsheet overleaf) and one is present in the promoter-containing fragment from each of the CAT constructs. Restriction with EcoR1 should then exicse a 2.0Kb fragment from each of the luciferase reporter constructs and the remaining fragments should be 4.7Kb, 3.9Kb and 3.7Kb for the –1240/+81 bfl-1 Luc, -367/+81 bfl-1 Luc and –129/+81 bfl-1 Luc reporter constructs, respectively. Lane M is the 2-Log DNA Ladder.

Figure 3.20 Generating *bfl-1-luc* promoter reporter constructs. –1374/+81 *bfl-1* Luc, -1240/+81 bfl-1 Luc, -367/+81 bfl-1 Luc and –129/+81 bfl-1 Luc.

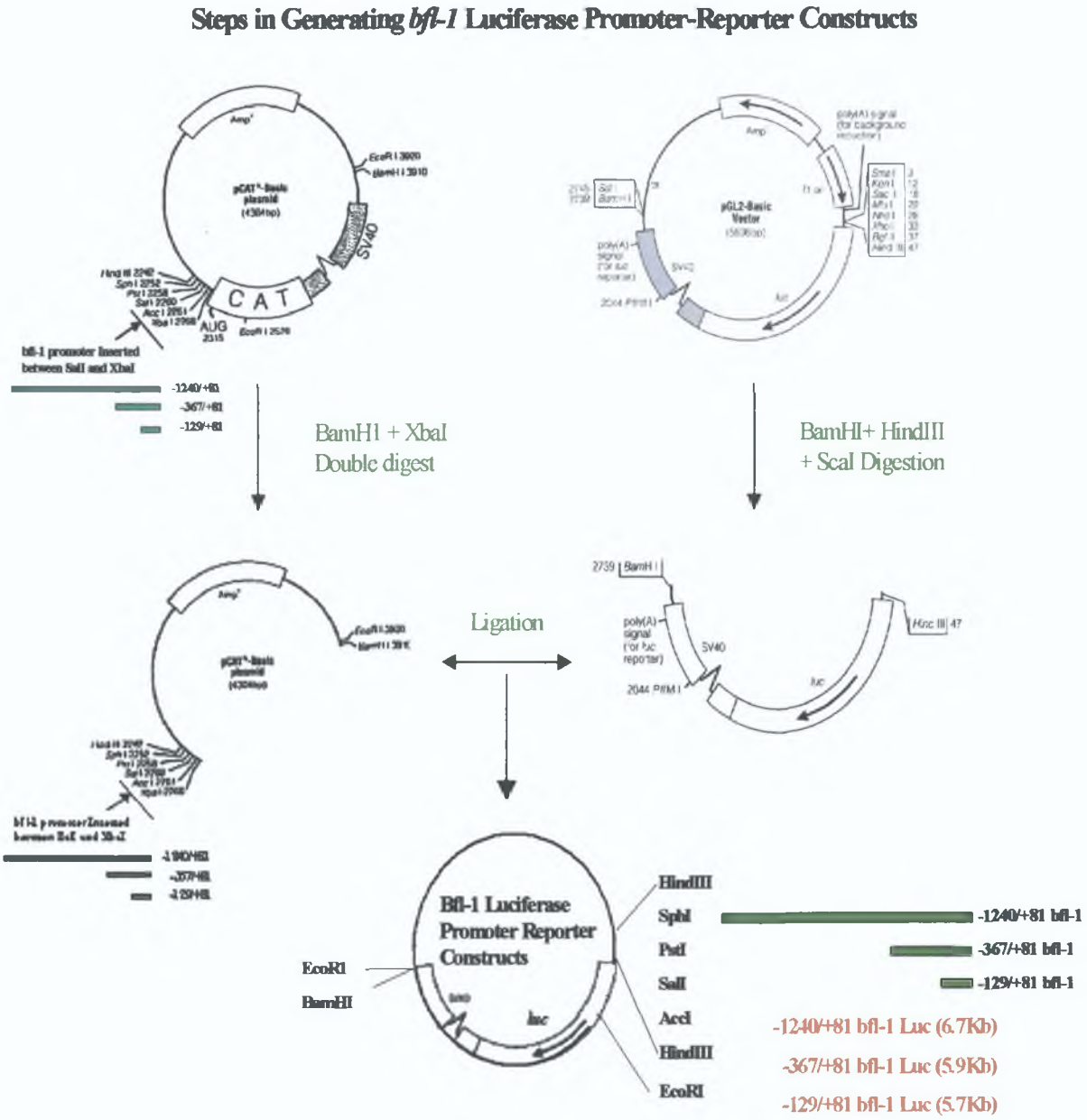


Figure 3.20 Generating *bfl-1-luc* promoter reporter constructs. -1240/+81 bfl-1 Luc, -367/+81 bfl-1 Luc and –129/+81 bfl-1 Luc. Flow diagram detailing the steps involved in making the Bfl-1 luciferase reporter constructs. The 4.1Kb 3.3Kb and 3.1Kb promoter containing fragments from the BamHI-XbaI digested CAT constructs were ligated to the 2.6Kb *luc*-containing fragment of the BamH1-HindIII-ScaI digested pGl2-basic vector. bfl-1 luciferase reporter constructs of 6.7Kb, 5.9Kb and 5.7Kb as induicated above were generated.

3.2.7. The *bfl-1* promoter sequence located between nucleotides -367 and-129 is essential for EBNA2 responsiveness in DG75 cells.

Transient co-transfections were carried out in DG75 with pSG5EBNA2 and the series of bfl-1 Luc promoter constructs containing the progressive deletions from the 5' end of the promoter described above (Figure 3 20) Transfections were carried out at a promoter to expression plasmid ratio of 1ug of bfl-1 promoter reporter construct to 7ug of EBNA2 expression plasmid This ratio had been established previously (see Figure 3 16) Cells were harvested at 48 hours post-transfection Promoter activity was assessed from luciferase assay results obtained using the luminoskan 1400 Results shown are representative of three independent experiments

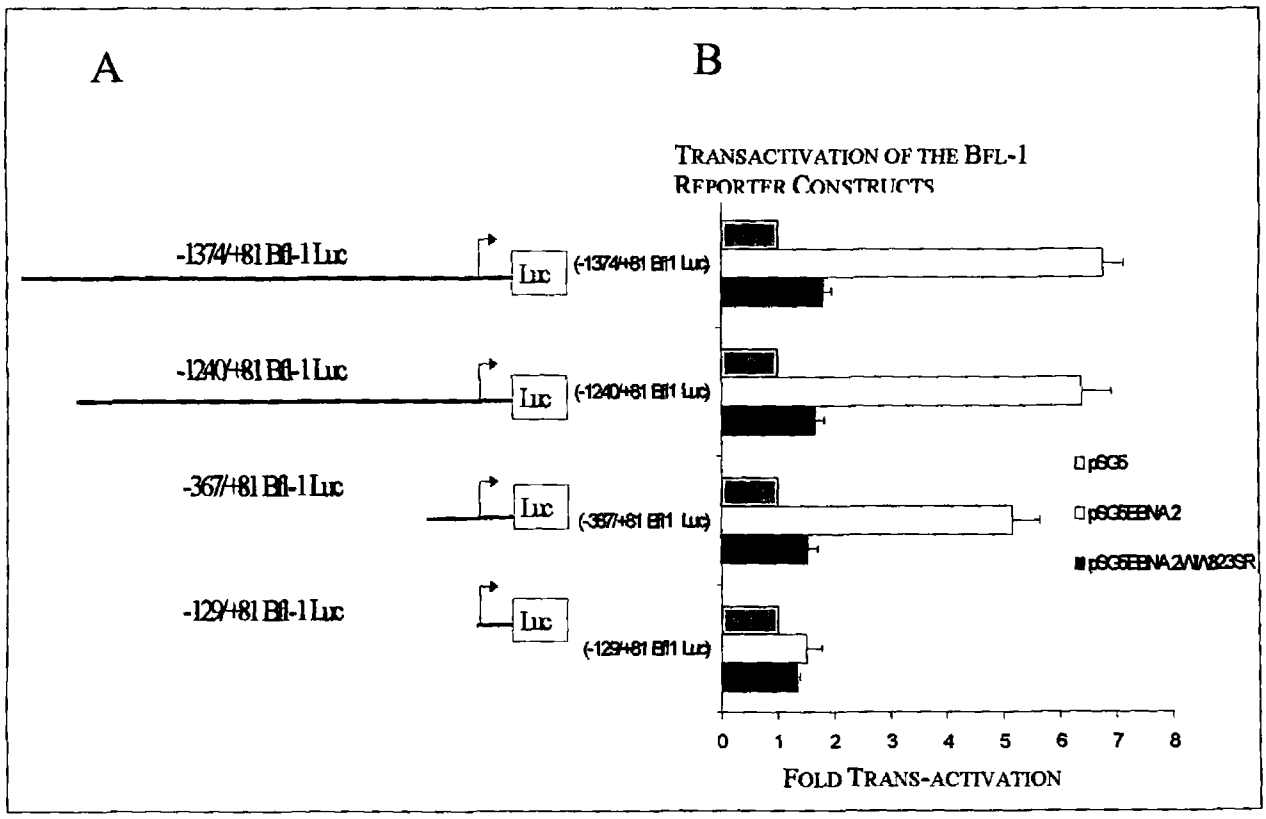


Figure 3.21 Co-transfections with EBNA2 and a series of bfl-1 promoter constructs containing progressive deletions from the 5' end show that sequences between –367 and –129 on the bfl-1 promoter are essential for EBNA2 responsiveness Dg75 cells were transfected with 1ug of each of the promoter constructs and 7ug of pSG5/pSG5EBNA2/pSG5EBNA2WW323SR. Both –1374/+81 bfl-1 Luc –and 1240/+81 bfl-1 Luc constructs were trans-activated an average of ~6 5 fold over the course of three

independent experiments in response to pSG5EBNA2 addition The -367/+81 bfl1 Luc promoter reporter construct was also trans-activated over 5 fold in the same experiments However the smallest promoter reporter construct -129/+81 bfl1 Luc was only very weakly transactivated by pSG5EBNA2 Thus sequences between -367 and -129 on the bfl-1 promoter are essential in mediating EBNA2 responsiveness to the bfl-1 promoter The pSG5EBNA2WW323SR did not significantly trans-activate any of the promoter reporter constructs

From Figure 3 21, it can be seen that both the -1374/+81bfl-1 Luc and -1240/+81 bfl-1 Luc promoter constructs are trans-activated over 6 5 fold by co-transfection with the EBNA2 expression plasmid pSG5EBNA2 The -367/+81 bfl1 Luc promoter reporter construct showed an average of approximately 5 fold trans-activation in the same experiments However the shortest promoter construct -129/+81 bfl1 Luc was trans-activated less than two fold when cotransfected with pSG5EBNA2 Thus sequences between position -367 and -129 on the bfl1 promoter are essential in mediating EBNA2 responsiveness to the bfl1 promoter In the case of all the promoter reporter constructs, substitution of EBNA2 with EBNA2WW323SR (in which CBF1 binding has been abolished See Figure 1 7 chapter 1) leads to a decline in trans-activation to less than two-fold The failure to trans-activate the shortest promoter construct -129/+81 bfl-1 Luc coupled with the requirement for a functional CBF1 binding site on EBNA2 itself, indicated that trans-activation most likely occurred via a CBF1-dependent mechanism, with the involvement of a possible CBF1 binding site located between -367 and -129 on the promoter sequence The difference between the activity of the longer promoter sequences (-1374/+81bfl1 Luc and -1240/+81 bfl1 Luc) and the activity of the -367/+81 portion of the promoter when co-transfected with EBNA2 suggests sequences between -1240/+81 bfl1 Luc and -367/+81 bfl1 Luc may enhance EBNA2 mediated trans-activation of the bfl1 promoter

3.2.7.1. Putative CBF1 and Ets-Family Transcription Factor Binding Sites Exist on the *bfl-1* Promoter Sequence.

Results from the transfections with the promoter deletion constructs indicated that the sequence of the bfl-1 promoter between nucleotides-367 and –129 contained elements essential for its EBNA2 responsiveness. Analysis of the –367 to –129 region of the promoter using Transcription Element Search Software website www.cbil.upenn.edu/tess, the MatInspector *V2.2* search tool from the Transfac database (http://transfac.gbf.de) and also the Alibaba transcription factor prediction database (http://www.alibaba2.com), revealed putative transcription factor binding sites for a number of relevant transcription factors. Analysis of the sequence between positions –367 and –129 indicated that the promoter contained the sequence motif GTGGGAA, in the reverse orientation at position –243 to –249 (see Figure 3.22A). This sequence has been found in all EBNA2-responsive promoters so far characterized. This motif is part of the consensus binding sequence for CBF1 (RBP-jk) (ie. CGTGGGAA; Tun et al 1994). Further analysis of the promoter sequence, revealed additional '5-GGAA-3' tracts, the core sequence motif known to be central in DNA binding of Ets family transcription factors [Karim, 1990]; [Gutman, 1991]. This was identified in four places between –367 and –129 on the promoter (see Figure 3.22A). Nucleotides adjacent to this core sequence partly determine the binding specificity for members of individual members of this family and so the Alibaba transcription factor search engine designated the four sites either 'Ets-1' or 'PU.1' binding sites: a putative Ets1 binding site at position –213 to -204, a 'double' Ets1 site between positions –176 and –163 and a near consensus PU.I binding site at position –143 to –134.

Figure 3.22. Putative CBF1 and Ets-Family Transcription Factor Binding Sites Identified on the *bfl-1* Promoter

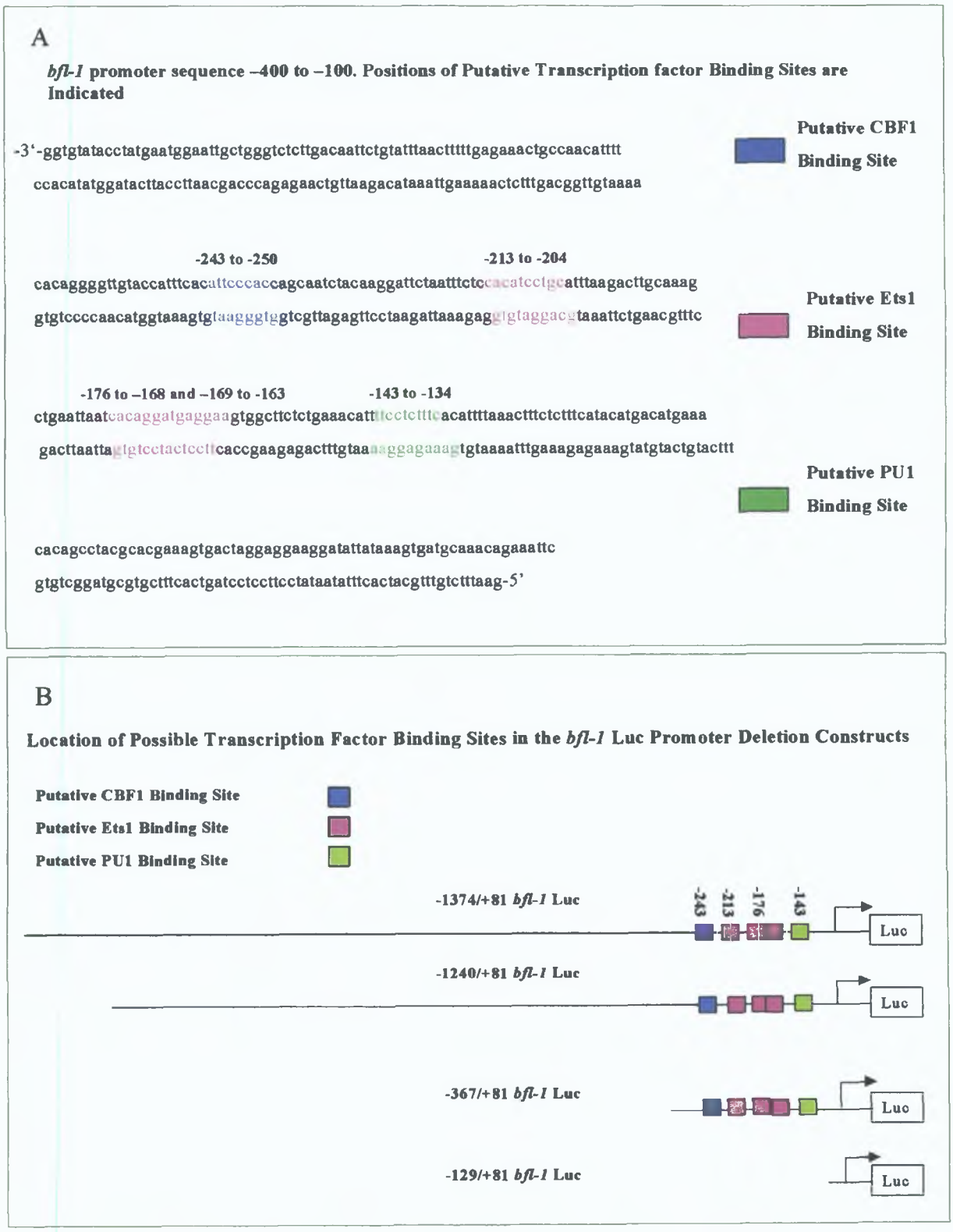


Figure 3.22. A number of Possible transcription factor binding sites were identified on the *bfl-1* promoter. (A). Analysis of the –367/+81 bfl-1 promoter sequence with Transfac and Alibaba databases identified putative transcription factor binding sites on the promoter.(B) shows the relative location of these putative transcription factor binding sites within the bfl-1 Luc promoter deletion constructs.

The presence of these potential transcription factor binding sites on the bfl-1 promoter is consistent with other EBNA2 responsive elements (E2REs) in EBNA2-responsive promoters such as LMP1, CD23, the EBV latent membrane protein (LMP) and EBNA Cp promoters. (Wang *et al.*, 1990; Zimber-Strobl *et al.*, 1991; Wang *et al.*, 1991; Tsang *et al.*, 1991; Fahraeus *et al.*, 1990; Sung *et al.*, 1991; Jin and Speck 1992) (Table 3.1 below adapted from Le Roux *et al.*, 1993; Ling *et al.*, 1993)

Table 3.1. Sequence Comparison of EBNA2 Responsive Elements of EBNA2 Target Genes.

Promoter	Homology I (CBF1 sites in EBNA2 responsive promoters)	Homology II (PU.1/Ets family binding sites in EBNA2 responsive promoters
Bfl-1*	-237 G G T GTGGGAA TGT G A -251	-143 AAAAGGAGAAAG -134
TP1	-255 C T C GTGGGAA AAT G G -241	
	-230 A C C GTGGGAA AAT A G –216	
Cp	-378 G C C GTGGGAA AAA A T –364	
CD23	-174 C C T GTGGGAA CTT G C -160	
LMP1*	-290 G T T GTGGGAA GCG G C -304	-169 AAAGGGGAAGTA -161
	-215 G C T GTGGGAA TGC G C –229	

Table 3.1. The boxes in the figure above show an alignment of two different sequences (denoted homologies I and II) from EBNA2 responsive elements in several promoters. The nucleotide coordinates are given relative to the known transcription initiation sites for each promoter. * It is to be noted that LMP and bfl-1 promoter sequences are given from the opposite DNA strand relative to the CAP site of these promoters.

The contribution of these sites in facilitating EBNA2 trans-activation of the bfl-1 promoter was next investigated. To this end site directed mutagenesis was employed to "knock out" the relevant possible transcription factor binding sites and thus assess their potential contribution to EBNA2 responsiveness on the bfl-1 promoter.

3.2.7.2. SITE-DIRECTED MUTAGENESIS OF THE PUTATIVE CBF1-BINDING SITE AT –243 to –249.

CBF1 binding has been shown to be important in mediating EBNA2 responsiveness in a number of promoters including the viral Cp, LMP1, and LMP2 promoters and the cellular CD23 promoter. [Zimber-Strobl, 1993]; [Ling, 1993]; [Laux, 1994]; [Ling, 1994]. At least one CBF1 site has been identified in all these E2REs, though often interaction with other transcription factors is also necessary to induce gene expression [Meitinger, 1994]. With regard to bfl-1, the fact that the pSG5EBNA2WW323SR which cannot bind CBF1 cannot trans-activate the bfl-1 promoter, combined with the presence of a possible CBF1 binding site in the bfl-1 promoter provide compelling circumstantial evidence that CBF1 binding likely plays a role in facilitating EBNA2 transactivation of the bfl-1 promoter. Thus the CBF1 binding site was initially chosen for mutagenesis and assessment.
The contribution of the putative CBF1-binding site located at –243 to –249 on the bfl-1 promoter was assessed by generating a bfl-1 promoter construct in which this site has been eliminated by site-directed mutagenesis (Promega Altered Sites® Mutagenesis Kit).(-1374/+81 mCBF1 bfl1 Luc).

A 40bp single stranded oligonucleotide was designed as being complimentary to the regions flanking the CBF1 site on the bfl-1 promoter but which included a six base pair region of mismatch at the centre corresponding to the CBF1 site (see Figure 3.23 below). This region of mismatch was designed to contain the restriction site for the enzyme Xba I, so that the mutated DNA sequence could be identified and easily distinguished from the wild type promoter, by restriction analysis with this enzyme. The oligo (below) was designed to minimize secondary structure formation within itself while also ensuring that the altered sequence did not contain any new transcriptional activator sites. The six base

pairs constituting the core CBF1 recognition sequence in the bfl-1 promoter were therefore replaced with the XbaI restriction site TCTAGA.

Figure 3.23. Mutagenesis of the Putative CBF1 binding site of the bfl-1 Promoter

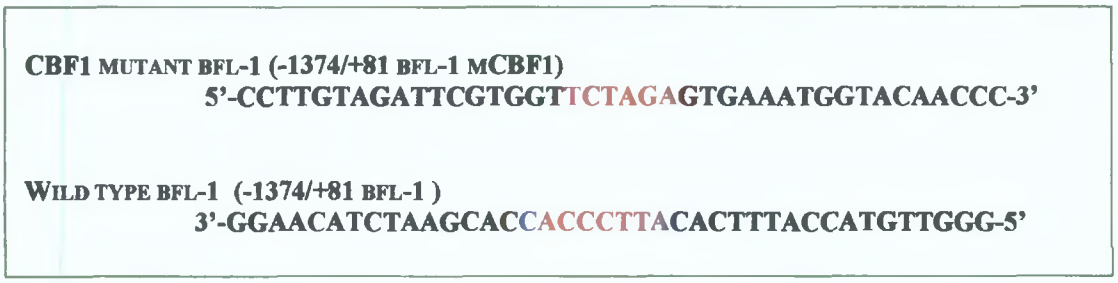


Figure 3.23. Site directed mutagenesis was employed to replace the putative CBF1 binding site in the bfl-1 promoter with an XbaI restriction site.

A diagram of the complete cloning strategy can be seen in Figure 3.24

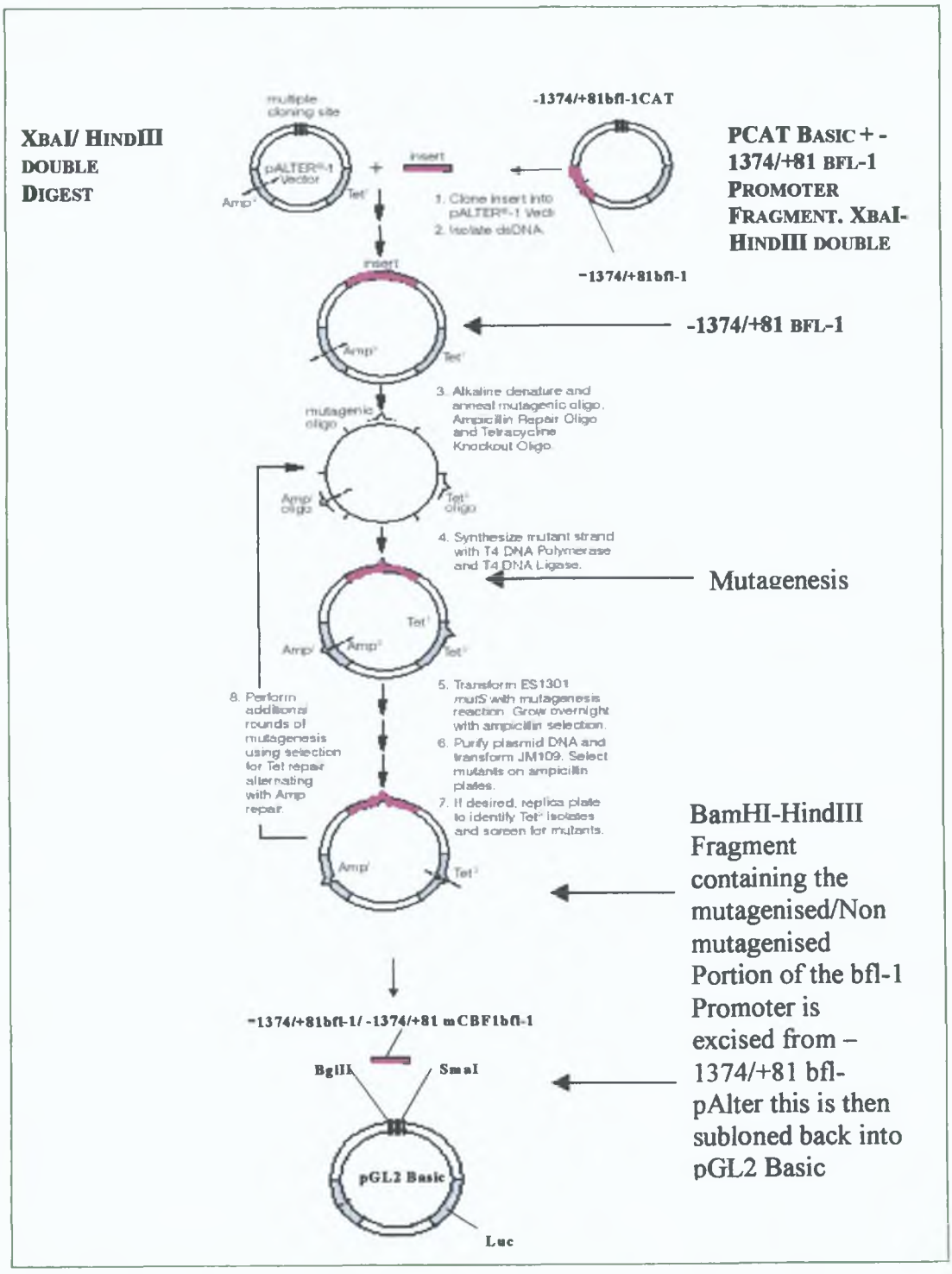
1. The full length promoter was excised from the pCAT-basic-1374/+81 after double digestion with XbaI and HindIII .
2. The 1.4kb fragment was inserted into the double digested pAlter-1 mutagenesis vector between XbaI and HindIII. This vector (Figure 3.24) contains a multiple cloning site flanked by the opposing SP6 and T7 RNA polymerase promoters inserted into the DNA encoding the lacZ alpha-peptide. This allowed blue-white screening of the clones produced. When plated on X-Gal indicator plates, colonies containing recombinant plasmids were white against a background of blue colonies. The pAlter-1 vector also carried sequences for both ampicillin and tetracycline resistance. However the plasmid is ampicillin sensitive because a frameshift mutation has been introduced into the amp^r gene by removing the Pst I site. Thus propagation of this plasmid and non-mutated bfl-1-pAlter recombinants was performed under tetracycline selection.
3. Site directed mutagenesis was then performed on the –1374/+81 *bfl-1* pAlter recombinants in order to knockout the CBF1 consensus binding sequence in the bfl-1 promoter. The mutagenesis was carried out by hybridizing single stranded DNA to the synthetic mCBF1oligonucleotide (Materials and Methods) that was

complimentary to the single stranded template except for the region of mismatch near the centre containing the XbaI site. Following hybridization the oligonucleotide was extended with DNA polymerase to create a double stranded structure. DNA ligase was then used to seal the nick, and the duplex structure was transformed into *E .coli* Es1301mutS, a mismatch repair minus strain. Use of this strain prevented repair of the newly synthesized unmethylated strand.

4. In the course of the mutagenesis reaction, an ampicillin repair oligonucleotide was added thus restoring ampicillin resistance to the mutant strand after mutation had occurred. This oligonucleotide was annealed to the single stranded DNA template at the same time as the CBF-1-mutagenic oligonucleotide. In this way, the CBF1 mutant clones could be isolated under positive selection for ampicillin resistance.
5. The *bfl-1*CBF1mutant sequence and also it unmutated counerpart –1374/+81bfl-1 (which had also been cloned into pAlter 1 in the same way but not mutated were excised from pAlter by double digestion with BamH1 and HindIII. The fragments were then cloned directionally between Sma I and Bgl II into the multiple cloning site of pGL2-basic.
6. In this way a paired set of luciferase promoter reporter constructs (–1374/+81 wtbfl-1 Luc) and (–1374/+81 mCBF1bfl-1 Luc) were created and the singular difference between the two is that one promoter sequence no longer contains the CBF1 binding site. The insertion of an extra Xba I site in the mutant promoter proved a useful tool in distinguishing the wild-type promoter from the CBF1 mutant promoter. The extra Xba I site resulted in the presence of a 300bp fragment in the restriction pattern for the mutant promoter, when digested with XbaI, this is not seen for the wild-type promoter.

As these two promoter reporter constructs are a paired set, transfections invoving the CBF1 mutated *bfl1* promoter reporter construct (-1374/+81 mCBF1 bfl1 Luc) are always done in conjunction with its non-mutated counterpart (-1374/+81 wtbfl1 Luc). In cases where the promoter deletion constructs are used the other (-1374/+81 bfl1 Luc) promoter construct is used as it contains the same background as the deletion constructs as described in the flowsheet Figure 3.20

Figure 3.24(i). Flowsheet of steps involved in generating –1374/+81wtbfl-1Luc and –. 1374/+81 mCBF1bfl-1 Luc.



3.25 Generating –1374/+81 wtbfl-1 Luc and –1374/+81 mCBF1bfl-1 Luc.

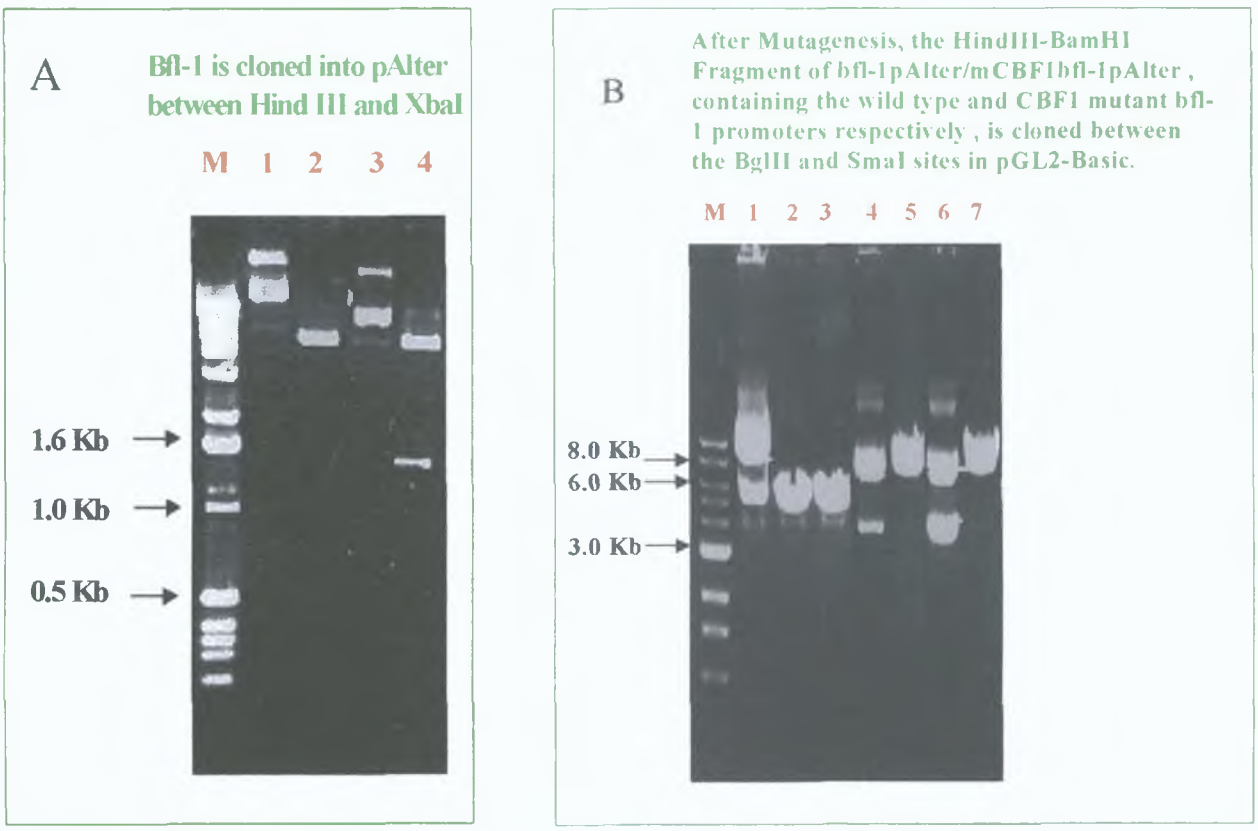
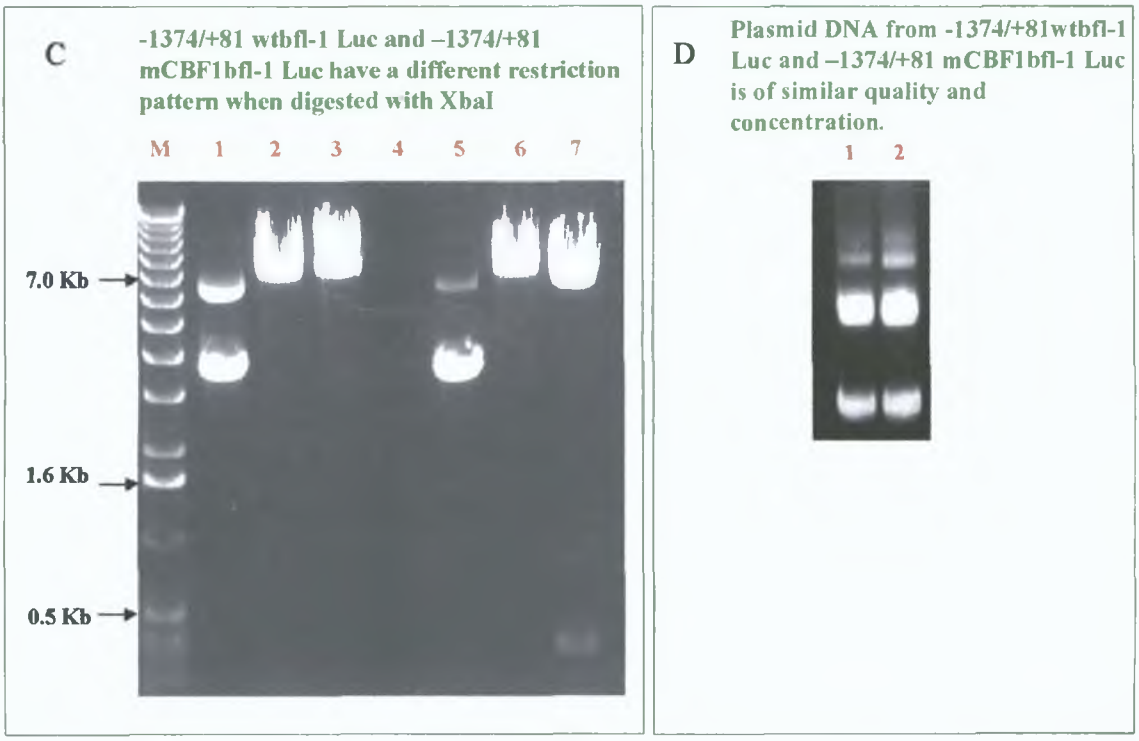


Figure 3.25. Generating –1374/+81 wtbfl-1 Luc and –1374/+81 mCBF1bfl-1 Luc. (A). The –1374/+81 HindIII-XbaI bfl-1 fragment from –1374/+81bfl-1CAT was inserted into the pAlter vector between HindIII and XbaI. M= Invitrogen 1Kb DNA Ladder. Lanes 1 and 2 purified plasmid DNA from pAlter. Lanes 3 and 4 DNA from –1374/+81 bfl-1 pAlter. Undigested DNA is shown in lanes 1 and 3. Lanes 2 and 4 show HindIII-EcoR1 restriction digestion pattern. The ~1.5Kb bfl-1 insert is clearly visible in lane 4. (B). Mutagenesis was undertaken in the pAlter vector and the BamH1-HindIII fragment of -1374/+81 bfl-1 pAlter and a non mutated counterpart were cloned between the BglII and SmaI sites of pGL2-Basic, generating –1374/+81 wtbfl-1 Luc and –1374/+81 mCBF1bfl-1 Luc. M= the NEB 2-Log DNA Ladder.Lanes 1-3 pGL2-basic. Lanes 4-&5 –1374/+81 wtbfl-1 Luc and Lanes 6&7 –1374/+81 mCBF1bfl1 Luc. Lanes 1, 4, and 6 = undigested purified plasmid DNA. Lanes 2, 5, and 7 HindIII Restriction pattern. Digestion with HindIII linearises the pGL2-Basic, –1374/+81 wtbfl-1 Luc and –1374/+81 mCBF1bfl-1 Luc DNA to produce fragments of ~5.5Kb, 7.0Kb and 7.0Kb respectively. Lane 3, is the BamHI restriction pattern for pGL2-Basic.

Figure 3.25 C and D. Site Directed mutagenesis of the Putative CBF1 site on the bfl-1 Promoter Introduces an extra XbaI site in –1374/+81 mCBF1bfl-1 Luc relative to –1374/+81 wtbfl-1 Luc



(C). Mutagenesis introduced an extra XbaI site in the –1374/+81 mCBF1bfl1 Luc relative to the -1374/+81 wtbfl1 Luc reporter construct. M= Invitrogen 1Kb DNA ladder. Lanes 1-3 –1374/+81 wtbfl-1 Luc. Lane 4= empty, Lanes 5-7 –1374/+81 mCBF1bfl1 Luc. Lanes 1 and 5 undigested plasmid DNA. Lanes 2 and 6, restriction digestion with HindIII linerises both promoter reporter construct to generate a fragment of the expected size ~7.0Kb. Lanes 3 and 7 digestion with XbaI. Only one XbaI site is present in wild type promoter reporter construct thus again the –1374/+81 wtbfl-1 Luc is linearised to generate a fragment of 7.0Kb upon digestion with eth restriction endinuclease XbaI. Mutagenesis of the putative CBF1 site simoultaneously introduced another XbaI site thus digestion with XbaI of the mutated promoter reporter construct should result in a fragment of around ~320bp and a fragment of around 6.68Kb. These two fragments can be clearly seen in lane 7. **(D).** Plasmid DNA from –1374/+81 wtbfl-1 Luc and –1374/+81mCBF1bfl-1 Luc is of similar qulaity and concentration and may be used in subsequent transfection experiments.

3.2.7.3. The Putative CBF1-Binding Site at –243 to –249 is Essential for EBNA2-Mediated Trans-activation of the *bfl-1* Promoter in a Range of BL Cell Lines.

Transient co-transfections, were carried out using the wild type bfl-1 luciferase reporter construct –1374/+81 wtbfl-1Luc) and the bfl-1 CBF1 mutated promoter reporter construct –1374/+81 mCBF1 bfl-1 Luc The transfections were performed in a range of BL cell lines to assess the contribution of the CBF1 binding site in the bfl-1 promoter with regard to promoter trans-activation by EBNA2 Initially transient co-transfections were carried out in DG75 cells Promoter activity was assessed after 48hr by luciferase assay and the results are shown in Figures 3 26 –3 28 It can be seen that mutation of the CBF1 binding site in the promoter significantly reduces trans-activation by EBNA2 from an average of 5 7 fold to an average of 1 9 fold, thus implying that this putative CBF1-binding site is essential for EBNA2-mediated trans-activation of the bfl-1 promoter

Figure 3.26A. Mutation of the Putative CBF1 site on the bfl-1 Promoter, Abolishes EBNA2-Mediated Trans-activation of the Promoter in the Dg75 Cell Line.

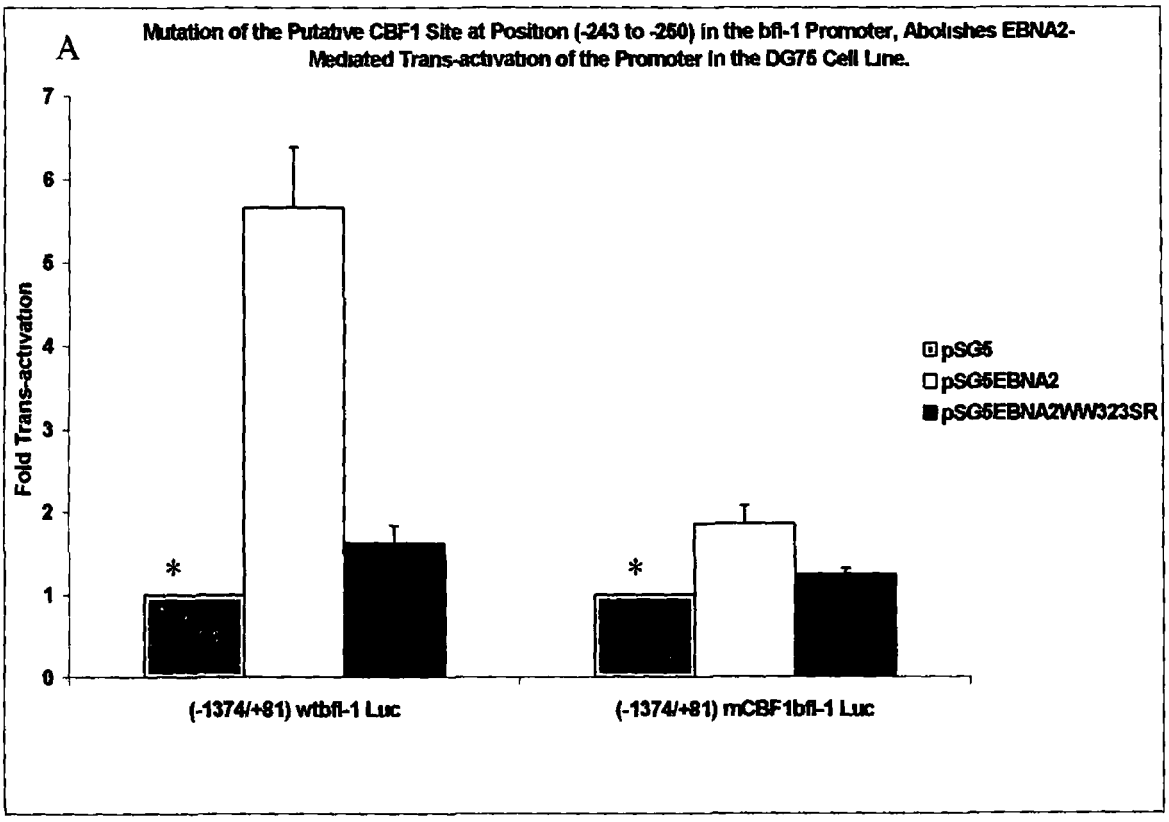


Figure 3 26. (A). Mutation of the CBF1 site abrogates EBNA2 ability to transactivate the *bfl-1* promoter in DG75 Cells. Co-Transfection of pSG5EBNA2 with –1374/+81 wtbfl-1 Luc induces a 5 7 fold up-regulation in promoter activity Co-transfection of the same EBNA2 expression plasmid with -1374/+81 mCBF1bfl-1 Luc shows only a 1 8 fold trans-activation of the promoter Thus Mutation of the bfl-1 CBF1 site causes EBNA2 trans-activation of the bfl-1 promoter to fall to less than 2 fold This correlates with the inability of the EBNA2 CBF1 binding mutant pSG5EBNA2WW323SR to trans-activate the bfl-1 promoter In the case of all transfections luciferase reporter activity obtained with the empty (pSG5) vector was arbitrarily assigned a value of 1 0 and beta-gal fold induction values were calculated relative to this

* Although basal levels of trans-activation were arbitrarily assigned a value of 1 actual basal levels of transactivation were consistenly slightly higher with the mutated promoter (-1374/+81 mCBF1bfl-1 Luc) relative to the wild type promoter (-1374/+81 wtbfl-1 Luc) See Figure 3 26B below

Figure 3.26B Basal Levels of Transcription are Higher for the CBF1 Mutant *bfl-1* Promoter (-1374/+81 mCBF1bfl1 Luc) Relative to the Wild Type Promoter (-1374/+81 wtbfl1 Luc).

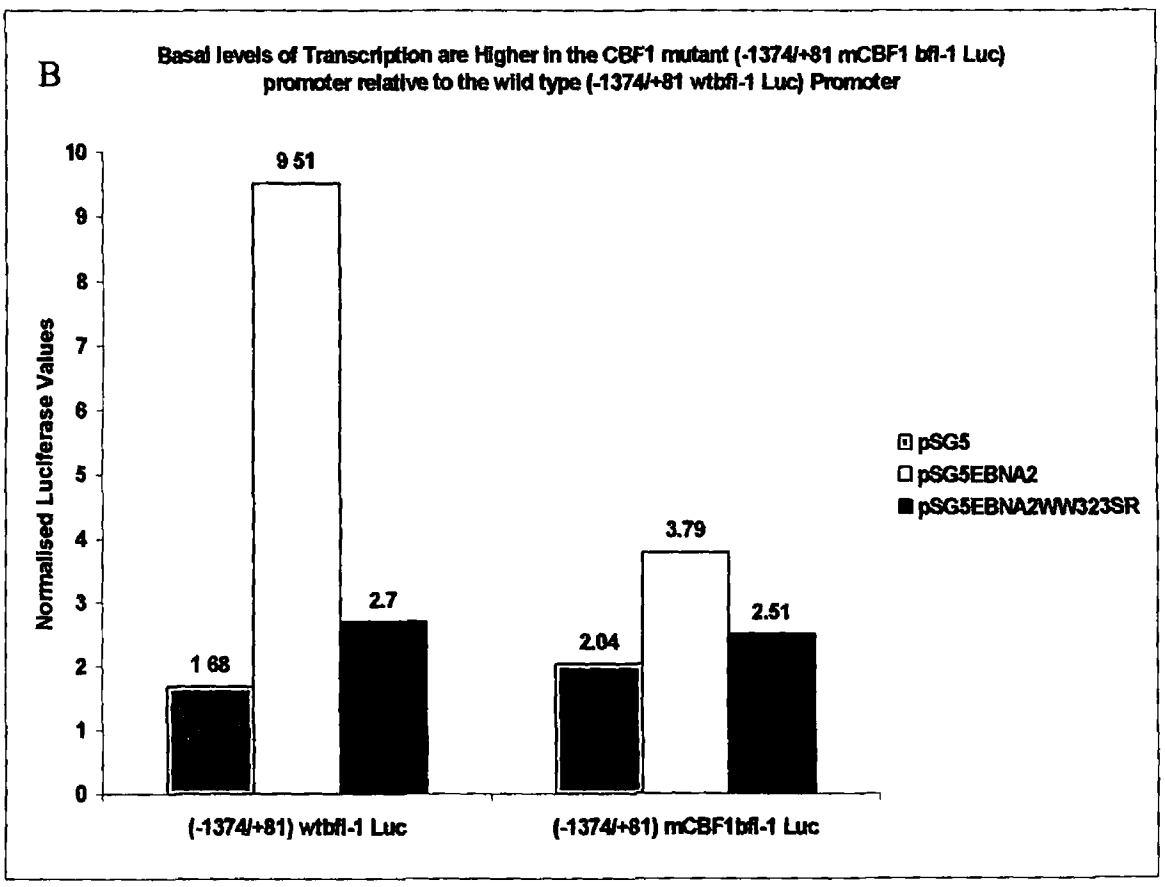


Figure 3.26 B. Basal transcription levels are higher in the –1374/+81 mCBF1bfl-1 luc than the –1374/+81 wtbfl-1 Luc promoter. Actual average Beta galactosidase normalised luciferase values over the course of the three independent experiments used for Figure 3 26A are shown. Although in the case of 3 26A basal transcription which occurs with co-transfection with the pSG5 empty vector is assigned a value of 1 0 it can be seen that the basal level of transcription from the mutated promoter (-1374/+81 mCBF1bfl-1 Luc) is consistently higher than in the wild type counterpart This may be due to dislocation of the CBF1 repressor complex from the promoter when its CBF1 binding site is destroyed

In the case of all transfections the basal levels of promoter activity (both mutated and wild-type) are normalised to 1 fold when co-transfected with the pSG5 vector However it should be noted that the basal levels of activity of the CBF1-mutated *bfl-1* promoter (-1374/+81 mCBF1bfl-1 Luc) are consistently marginally higher than the levels recorded for the wild type promoter (-1374/+81 wtbfl-1 Luc) This may be due to corruption of the putative CBF1 repressor complex on the promoter allowing higher levels of promoter activity even in the absence of EBNA2 To demonstrate this, the average beta-galactosidose normalised luciferase values for the above transfection are shown in Figure 3 26B

The significance of this mutation was further substantiated in an experiment in which the EBNA2 expression plasmid was titrated against the wild type *bfl-1* promoter (–1374/+81 wt*bfl-1* Luc) and the bfl-1CBF1 mutant promoter (–1374/+81 mCBF1*bfl-1* Luc) in the Dg75 cell line (See Figure 3 27 below) It can be seen that EBNA2 cannot trans-activate the bfl-1 CBF1 mutant promoter (–1374/+81 mCBF1*bfl-1* Luc) above 1 8 fold, despite its ability to trans-activate the wild-type *bfl-1* promoter (–1374/+81 wt*bfl-1* Luc) in a dose dependent manner in the same experiment Thus, although functional EBNA2 is expressed, as shown by the trans-activation of the wild-type *bfl-1* promoter, (–1374/+81 wt*bfl-1* Luc), EBNA2 does not trans-activate the *bfl-1* promoter when the CBF1 site at position –243 to 249 is altered by site-directed mutagenesis These results combined with the inability of the pSG5EBNA2WW323SR mutant (which cannot bind CBF1) to trans-activate the *bfl-1* promoter, confirm the likely requirement of CBF1 for EBNA2 mediated trans-activation of the *bfl-1* promoter

Figure 3.27. EBNA2 Trans-activates the *bfl-1* Promoter in a Dose Dependent Manner

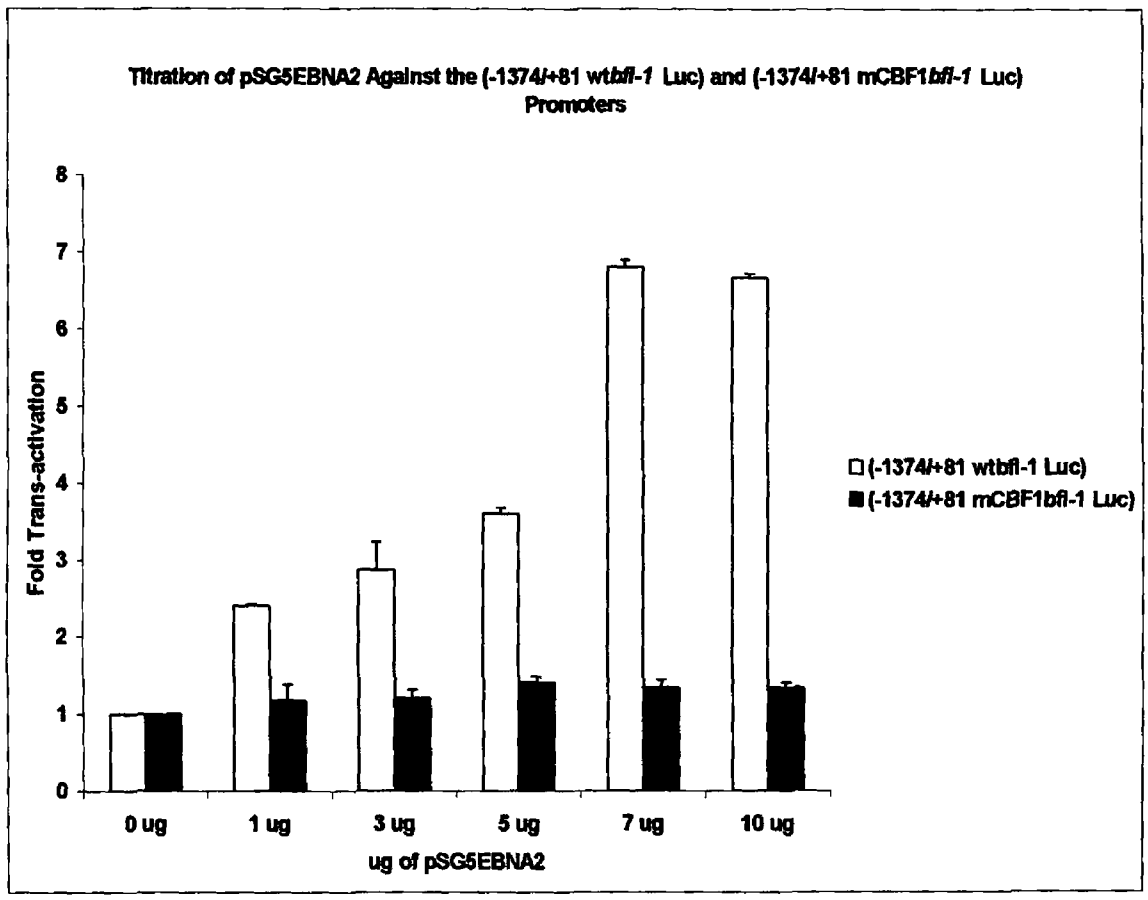


Figure 3 27 pSG5EBNA2 Trans-activates (-1374/+81 wt*bfl-1* Luc) in a dose dependent fashion. It does not Trans-activate the CBF1 mutant bfl-1 promoter (-1374/+81 mCBF1*bfl-1* Luc) at any of the quantities used. Titrating the pSG5EBNA2 EBNA2 expression plasmid with the paired bfl-1 promoter constructs shows the importance of the promoter CBF1binding site in mediating EBNA2 responsiveness

It was important to establish that the effects observed were reproducible in other well-established BL cell lines and to this end a similar series of transfections were carried out using the BL41 and BJAB cell lines In all cases 7ug of the expression plasmids pSG5/pSG5EBNA2/pSG5EBNA2WW323SR were transfected with 1ug of the promoter reporter constructs With regard to the BL41 cell line, pSG5EBNA2 trans-activated the wild type *bfl-1* promoter (-1374/+81 wtbfl-1 Luc) an average of 4 2 fold, this trans-activational effect of EBNA2 is reduced to 1 4 fold when the CBF1 binding site in the promoter is removed by mutation Figure 3 28 Similar observations were made using the

BJAB cell line in which the trans-activation of the promoter is reduced from 4 8 fold to 1 6 fold upon mutation of the putative CBF-1-binding site The EBNA2 mutant which cannot bind to CBF1 (pSG5EBNA2WW323SR) does not trans-activate the *bfl-1* or the mutated *bfl-1* promoter, by a factor of more than 1 6 fold in either cell line These results show that this putative CBF1 binding site is a critical DNA element that mediates EBNA2 trans-activation of this promoter in BL cells

Figure 3.28. EBNA2 Trans-activation the *bfl-1* Promoter in a Range of EBV Negative Bl Cell Lines Requires the Putative *bfl-1* CBF1 binding site

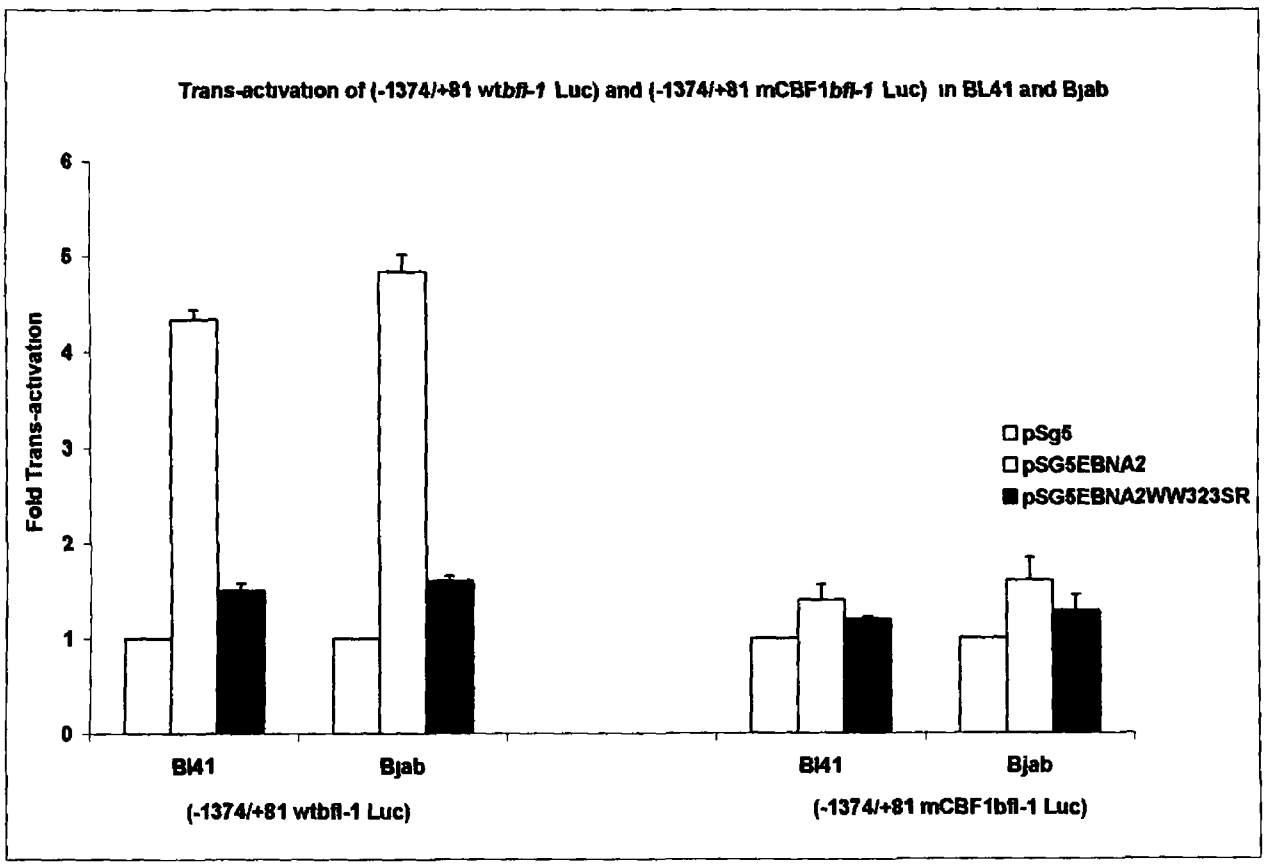


Figure 3.28. Mutation of the CBF1 site in the bfl-1 promoter prevents EBNA2 mediated trans-activation in a range of B cell lines In both the Bl41 and Bjab cell lines pSG5EBNA2 trans-activates the wild type bfl-1 promoter (-1374/+81 wtbfl-1 Luc) but fails to trans-activate its CBF1 mutated counterpart (-1374/+81 mCBF1bfl-1 Luc) Also the pSG5EBNA2WW323SR mutant, which also cannot bind CBF1 fails to trans-activate the wild type bfl-1 promoter These results provide further evidence of the importance of CBF1 binding in mediating EBNA2 responsiveness on the bfl-1 promoter

3.2.8.0 Trans-activation of the *bfl-1* Promoter in Response to EBNA2 Activation in BL41- ER/EBNA2 (K3) and BL41P3HR1-ER/EBNA2 (9A) Cell Lines.

The importance of the CBF1 site in the *bfl-1* promoter for conferring EBNA2 responsiveness was also examined in the BL41-ER/EBNA2 (K3) and BL41P3HR1-ER/EBNA2 (9A) cell lines, where EBNA2 function was controlled by the presence of estrogen addition These cell lines are stable transfectants of the BL41 cell line and its superinfected derivative BL41-P3HR1 in which EBNA2 is activated in response to the addition The two cell lines were transiently transfected with the –1374/+81 wtbfl-1 Luc and –1374/+81 mCBF1bfl-1 Luc promoters After transfection the cells were cultured in the presence/absence of estrogen (estradiol), harvested and cell extracts were assayed for luciferase activity As a control for assaying the functionality of EBNA2 in this system one transfection in each cell line was carried out using the pGa981-6 promoter construct (Minoguchi *et al*, 1997) This plasmid contains 12 CBF1 binding sites (essentially six copies of a portion of the TP1 promoter which contains 2 CBF1 binding sites), positioned upstream of a minimal (SV40) promoter and luciferase-coding gene pGa981-6 was constructed as described in the legend Figure 3 29 below A partner for the pGa981-6 plasmid, is pGa50-7 which consists of the same plasmid background as the pGa981-6 construct however without the CBF1 inserts (Minoguchi *et al*, 1997) Trans-activation of the pGA981-6 vector in the presence of active EBNA2, is relative to trans-activation in the absence of estrogen/active EBNA2 which has been arbitrarily designated a fold activation of 1 This is also true for the pGa50-7 promoter reporter construct

Figure 3.29. EBNA2 is Functionally Active and Trans-activates the wild type bfl-1 Promoter in the BL41-ER/E2 (K3) Cell Line.

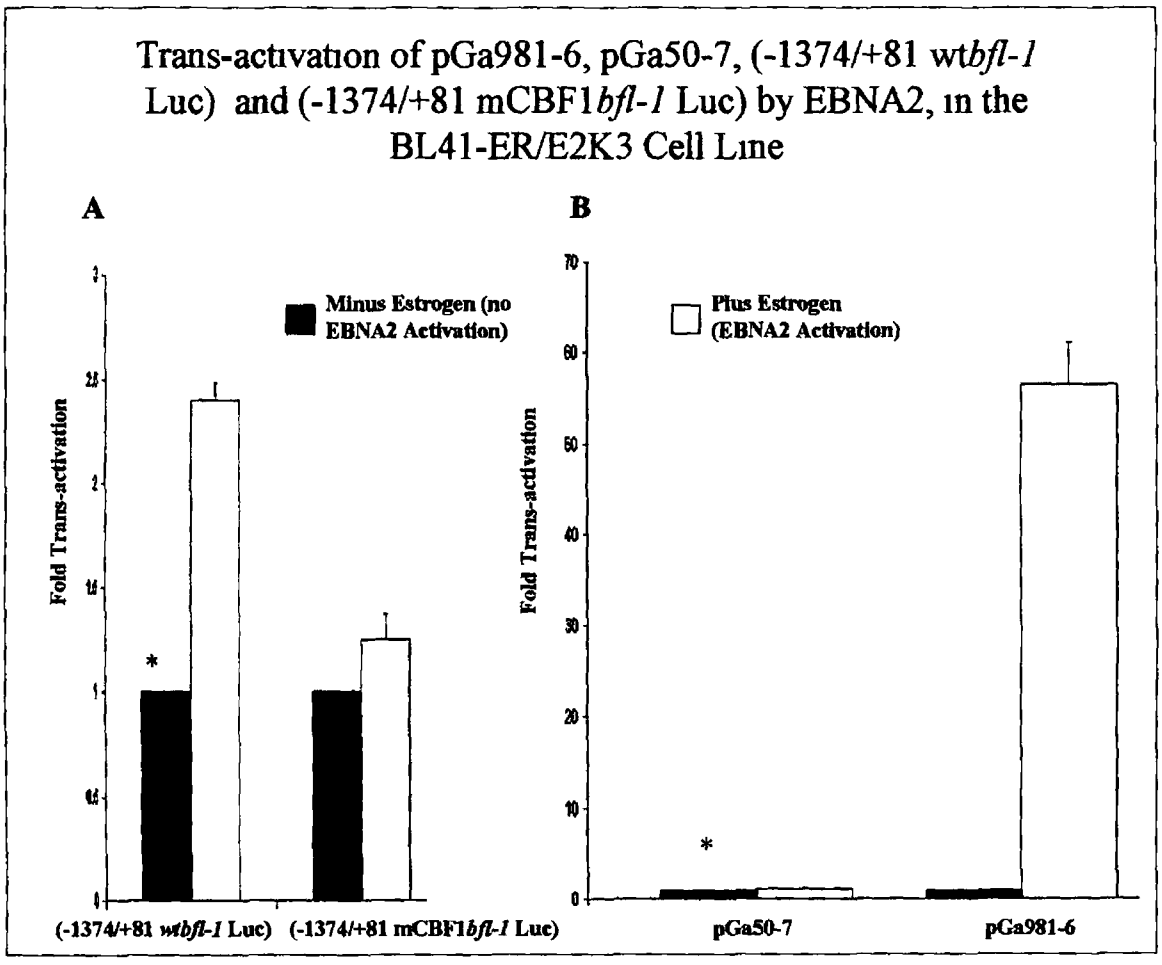


Figure 3.29 EBNA2 is Functionally Active and Trans-activates the wild type bfl-1 promoter (-1374/+81 wtbfl-1 Luc) but not the CBF1 mutant bfl-1 promoter in the BL41-ER/E2 (K3) cell line In this case transfection was carried out using electroporation. Panel (A) shows the trans-activation of (-1374/+81 wtbfl-1 Luc) and (-1374/+81 mCBF1bfl-1 Luc) before and after Estrogen (EBNA2 activation) in the BL41-ER/E2 (K3) cell line Panel B shows that EBNA2 is activated/functional in response to estrogen addition in the K3 cell line as the pGA981-6 promoter reporter construct is trans-activated after estrogen addition. * In the case of the bfl-1 promoter reporter constructs, activation of the promoters prior to estrogen addition was arbitrarily assigned a fold activation of 1 and activation due to estrogen addition/EBNA2 activation was relative to this Similarly the pGa50-7 vector and the pGa981-6 were arbitrarily assigned a fold activation of 1 before estrogen addition and fold trans-activation in response to EBNA2 activation was calculated relative to this This is the case for all subsequent transfections in the stably transfected BL41 cell Lines The pGa981-16 reporter construct was generated using a 50-bp oligonucleotide harboring both RBP-J binding sites of the EBV *TP1* promoter,

(GGATCCCGACTCGTGGGAAAATGGGCGG AAG GGCACCGTGGGAAAATAGTAGATCT), which was then ligated as a hexamer into plasmid pGa50-7 (Minoguchi *et al*, 1997)

It can be seen from figure 3 29 that EBNA2 trans-activated the pGa981-6 an average of 56 fold (relative to transactivation of the prior to estrogen addition set to 1) demonstrating the functionality of EBNA2 in the BL41 ER-EBNA2 K3 cell line upon addition of oestrogen In the same experiment EBNA2 trans-activated the wild-type *bfl-1* luciferase reporter construct (-1374/+81 wtbfl-1 Luc) an average of 2 4 fold The CBF1 mutated *bfl-1* promoter reporter construct was not trans-activated above 1 25 fold Although less promoter trans-activation is observed when compared to the transient transfections shown previously, the consistently higher trans-activational effect of EBNA2 on the wild-type compared to the mutated *bfl-1* promoter construct demonstrates an important role for this sequence in mediating EBNA2 responsiveness on the *bfl-1* promoter The results from BL41P3HR1 ER-EBNA2 9A cell line showed similar levels of EBNA2 associated trans-activation of the *bfl-1* promoter In this case the *bfl-1* promoter (-1374/+81 wtbfl-1 Luc) was trans-activated an average of 2 67 fold over the course of three independent experiments As before trans-activation of the *bfl-1* CBF1 mutant promoter (-1374/+81 mCBF1bfl-1 Luc) was less than 1 5 fold These results further confirm the requirement for the putative CBF1- binding site for EBNA2-mediated trans-activation of the *bfl-1* promoter It can also be seen that in the BL41P3HR1 ER-EBNA2 9A experiment that EBNA2 was functional when one compares fold trans-activation of the pGa981-6 promoter reporter construct before and after estrogen addition

Figure 3.30. EBNA2 is Functionally Active and Trans-activates the wild type *bfl-1* Promoter in the BL41P3HR1-ER/E2 (9A) Cell Line.

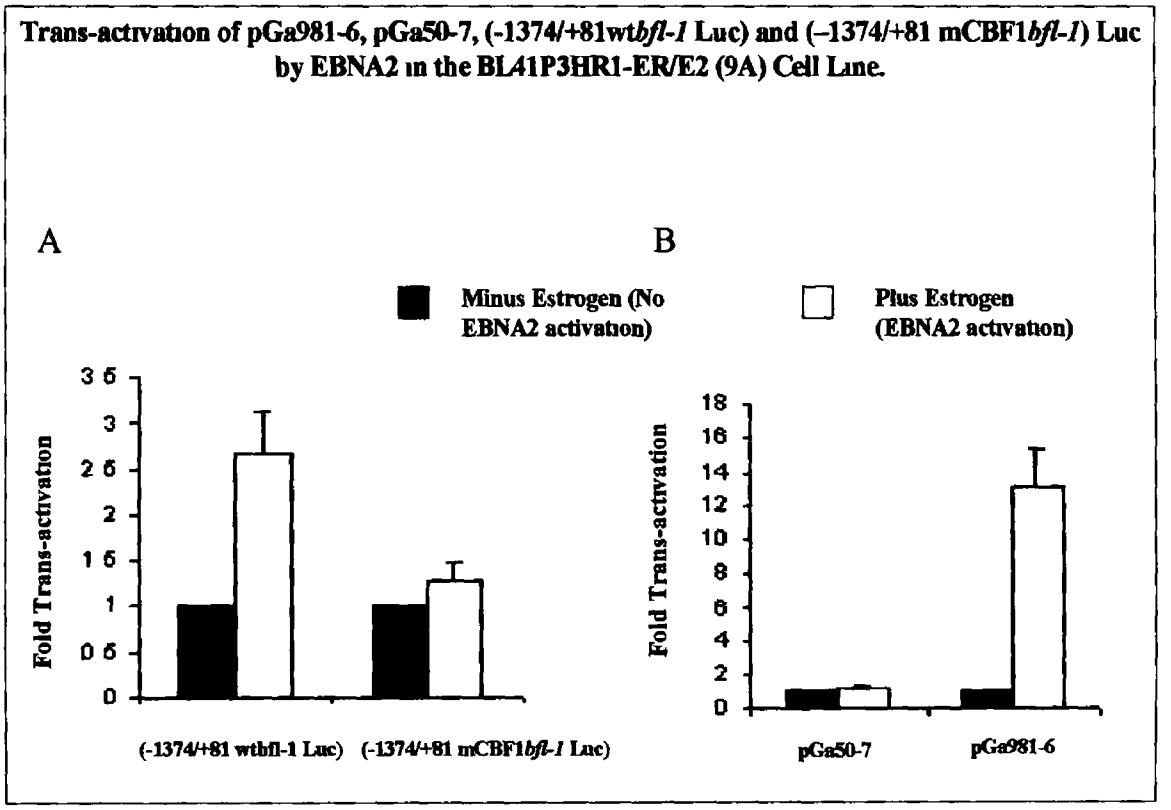


Figure 3 30. EBNA2 is Functionally Active and Trans-activates the wild type bfl-1 promoter (-1374/+81 wtbfl-1 Luc) but not the CBF1 mutant bfl-1 promoter in the BL41P3HR1-ER/E2 (9A) cell line Again electroporation was used to transfect this cell line Panel (A) shows the trans-activation of (-1374/+81 wtbfl-1 Luc) and (-1374/+81 mCBF1bfl-1 Luc) before and after Estrogen (EBNA2 activation) Panel B shows that EBNA2 is activated/functional in response to estrogen addition in the 9A cell line as the pGA981-6 promoter reporter construct is trans-activated after estrogen addition. As before Fold activation of the (-1374/+81 wtbfl-1 Luc and -1374/+81 mCBF1bfl1 Luc promoters are set to 1 prior to estrogen addition. Fold trans-activation of theses promoters as a result of EBNA2 activation are relative to these values The same is true with regard to transactivation of the pGa981-6 and pGa50-7 reporters Activation of these promoters after estrogen addition is relative to their pre-estrogen addition counterparts in which fold activation has been set to 1

The relative values recorded for fold trans-activation of the promoter in the control pGa981-16 are lower in the BL41P3HR1 ER-EBNA2 9A cell line than in the BL41 ER-EBNA2 K3 cell line and levels of trans-activation of the *bfl-1* promoter are also much lower in this stably transfected cell line background than in the DG75 cell system as well

as all other transfections in the non stably transfected cell lines such as Bjab and BL41 This may be due to a number of reasons,

(i) The ER system cannot be mampulated in the same way as pre-expressed endogenous EBNA2 in this case EBNA2 is activated by the presence of estrogen thus, there is potentially a limited quantity of EBNA2 relative to the co-transfection system in the DG75 (and non stably transfected cell lines) background With regard to the non stably transfected cell lines an optimal expression plasmid to promoter reporter ratio has already been obtained by titrating the EBNA2 expression plasmid against the promoter reporter construct and so optimal trans-activation conditions are easier to manipulate (ii) It is also possible that the recombinant ER-EBNA2 is a weaker trans-activator than wild type EBNA2 for example in Dg75 the pGA981-6 reporter is trans-activated an average of 200 fold (Not shown) however in these cell lines the average trans-activation of the same reporter construct is less than 60 fold in the BL41-ER/E2 (K3) cell line and less than 20 fold in the BL41P3HR1-ER/E2 (9A) cell line Also (iii) with reference to the BL41P3HR1-ER/E2 cell line, this does not express a full length EBNA-LP (an EBV protein known to coactivate certain promoters with EBNA2 See below) which has been shown elsewhere to co-operate with EBNA2 as a co-trans-activator of certain promoters, nonetheless the lack of EBNA-LP in all other transfections carried out in EBV negative cell lines has not hindered EBNA2 trans-activation of the *bfl-1* promoter

In summary, the co-transfections involving the EBNA2 CBF1 binding mutant (pSG5EBNA2WW323SR), and the bfl-1 CBF1 binding mutant –1374/+81 wtbfl-1 Luc in a range of BL cell lines indicate a key role for the putative CBF1- binding site for mediating EBNA2 trans-activation of the *bfl-1* promoter in BL cells

3.2.9.0. EBNA3A, EBNA3B and EBNA3C Repress EBNA2-Mediated Trans-activation of the *bfl-1* Promoter.

Previous studies have demonstrated that all three EBNA3 proteins, EBNA3A, 3B and 3C can also bind CBF1, negatively regulating the expression of both EBNA2 and Notch target genes including CD21 and the viral LMP2A, LMP1 and Cp promoters (Johannsen *et al*, 1996, Radkov *et al*, 1997, Robertson and Kieff 1995, Waltzer *et al*, 1996, Zhao *et al*, 1996) Furthermore EBNA3C can form complexes with a member of the CBF1 repression complex HDAC1 (Radkov *et al*, 1999) and binding of EBNA3A to CBF1 has been shown to down regulate c-myc expression and EBV transformed lymphoblast growth (Cooper *et al*, 2003) Since CBF1 binding seems to be important in facilitating EBNA2 trans-activation of the *bfl-1* promoter, it was important to assess the effect of the EBNA3 proteins on EBNA2 mediated trans-activation of the bfl-1 promoter To this end, co-transfections with EBNA2 and EBNA3 expression plasmids (p7CMV EBNA3A 3B and 3C) were undertaken The three EBNA3 expression plasmids consisted of EBNA3A/3B or 3C cloned into the multiple cloning site of the p7CMV vector Transient transfection analysis showed that all three EBNA3s can individually inhibit EBNA-2 mediated trans-activation of the *bfl-1* promoter (Figures 3 31-3 33) These findings, again imply a key role for CBF1 in mediating EBNA2 responsiveness to the *bfl-1* promoter under the conditions examined, as the ability of the EBNA3 proteins to repress EBNA2 mediated trans-activation is often due to their ability to bind CBF1 and members of the CBF1 repression complex

Figure 3.31 EBNA3A Represses EBNA2 Mediated-Trans-Activation of the *bfl-1* Promoter.

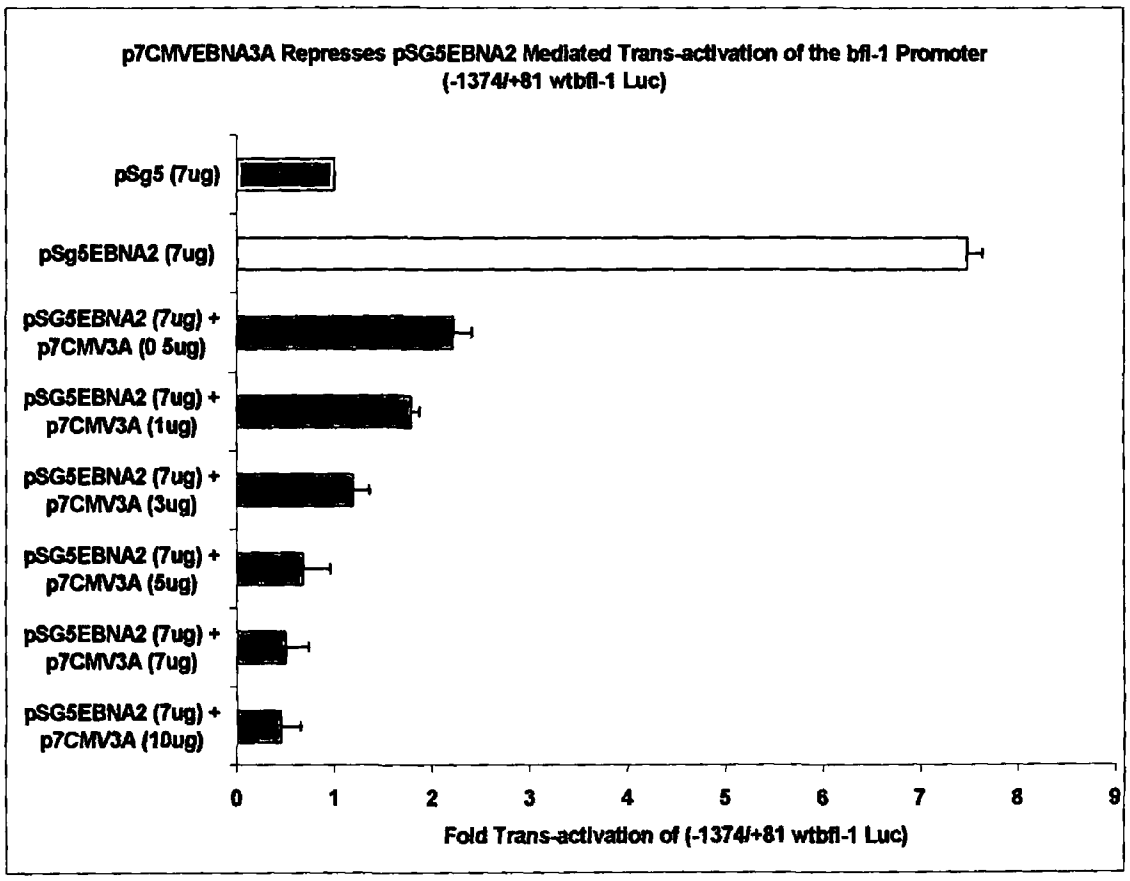


Figure 3.31 EBNA3A Represses EBNA2-Mediated Trans-activation of the *bfl-1* promoter Transfections were carried out in the DG75 cell line Increasing quantities of EBNA3A expression plasmid (p7CMV EBNA3A) resulted in a corresponding decrease in trans-activation of the *bfl-1* promoter by EBNA2 These results add further weight to the theory that EBNA2 trans-activates *bfl-1* promoter through CBF1 as increasing quantities of EBNA3A compete with EBNA2 for binding to the CBF1 binding site.

Different quantities of p7CMVEBNA3A, p7CMVEBNA3B and p7CMVEBNA3C were all titrated with pSG5EBNA2 against the *bfl-1* promoter (-1374/+81 wtbfl-1 Luc) in co-transfection studies and all transfections were transfected with the same total quantity of DNA equalized by addition of the empty p7CMV vector It can be seen from the results, EBNA2 trans-activation is decreased from 6 fold down to ~0 5 fold with addition of increasing quantities of either EBNA3A EBNA3B or EBNA3C expression plasmids (Figures 3 30-3 32) EBNA2 trans-activated the *bfl-1* promoter an average of 7 5 fold in

the transient transfections above (Figure 3 30) This level of trans-activation was reduced to 2 3 fold upon addition of 0 5ug of EBNA3A expression plasmid Increasing the amount of EBNA3A further reduced trans-activation down to a level of 0 3 fold Since EBNA3A binds to the CBF1 repressor complex and the addition of EBNA3A affects the trans-activation of *bfl-1* by EBNA2, one possible conclusion is that *bfl-1* promoter trans-activation by EBNA2 involves CBF1

Figure 3.32 EBNA3B Represses EBNA2-Mediated Trans-Activation of the *bfl-1* Promoter.

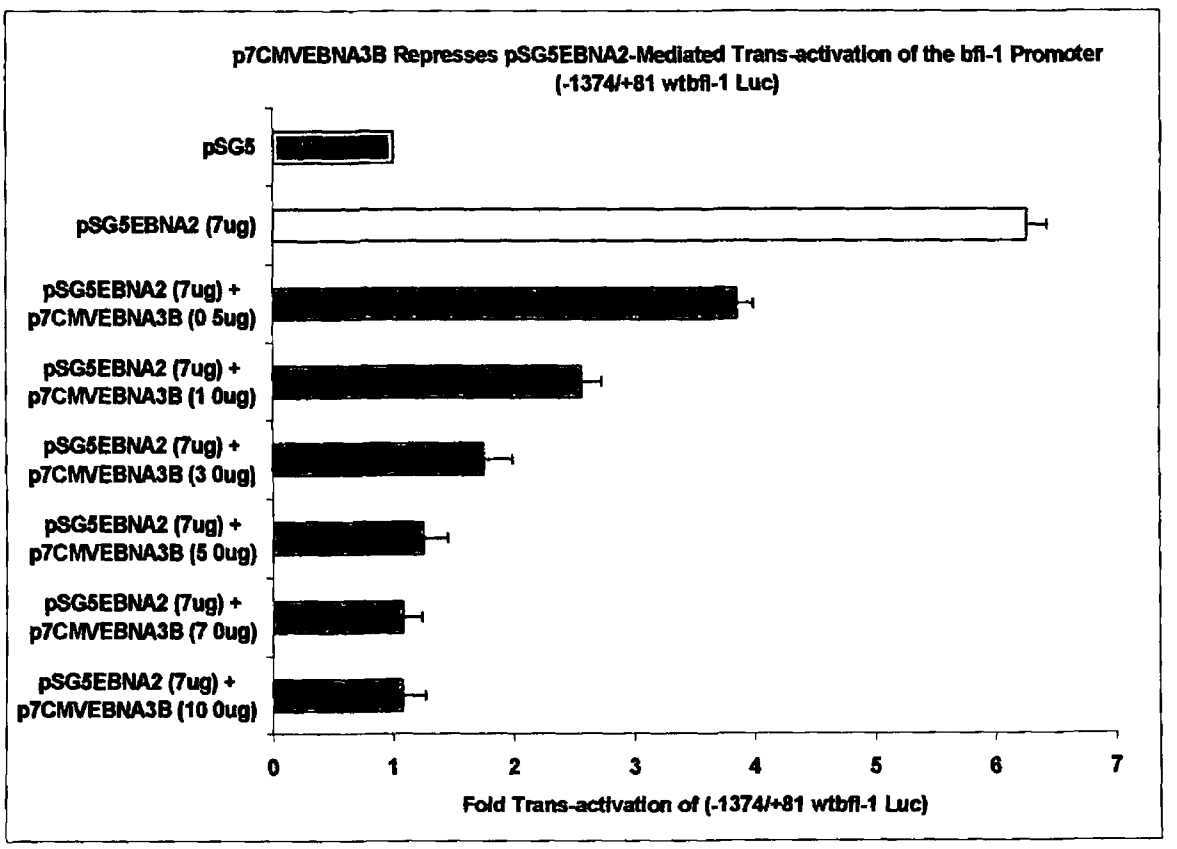


Figure 3 32 EBNA3B represses EBNA2 mediated trans-activation of the *bfl-1* promoter As with EBNA3A, co-transfection with increasing amounts of the EBNA3B expression plasmid resulted in corresponding decrease in EBNA2 mediated trans-activation of the bfl-1promoter (-1374/+81 wtbfl-1 Luc)

Figure 3.33 EBNA3C Repressess EBNA2-Mediated Trans-activation of the *bfl-1* Promoter

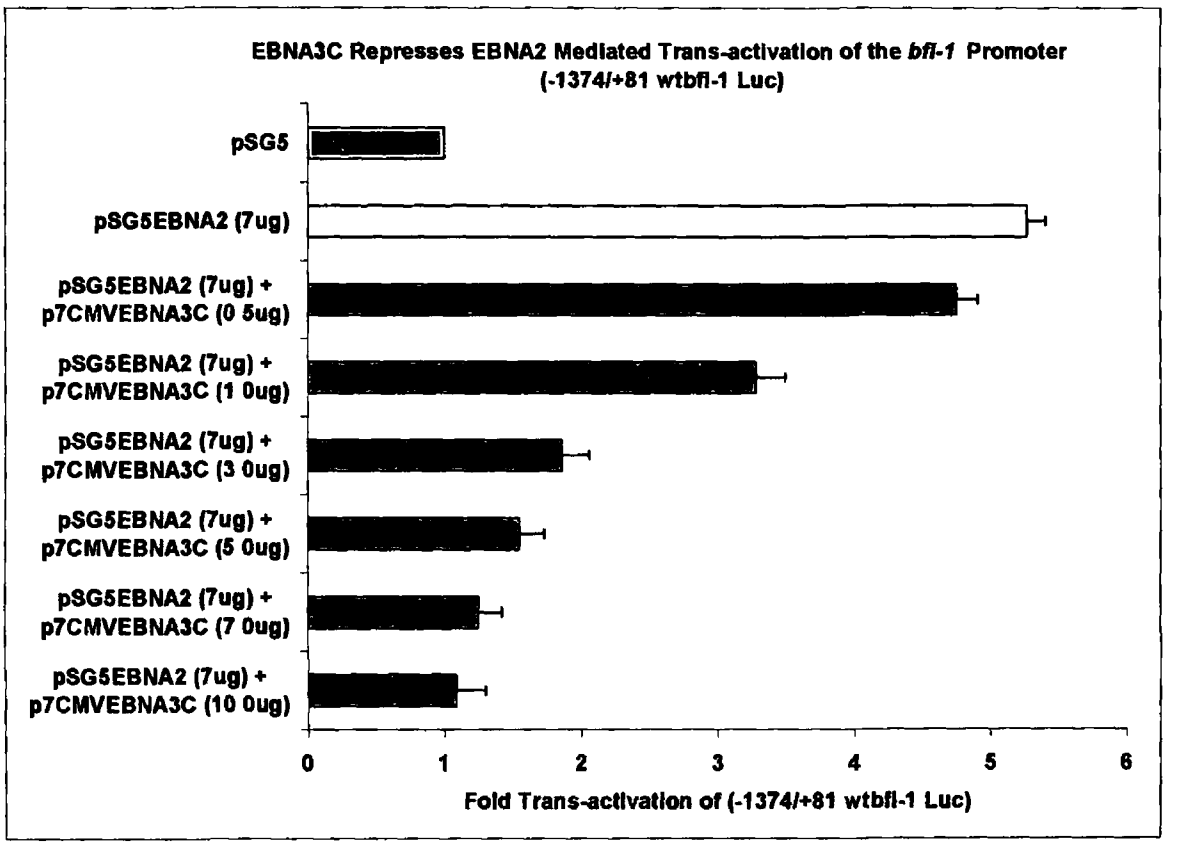


Figure 3.33. EBNA3C Represses EBNA2 mediated Trans-activation of the bfl-1 promoter. p7CMVEBNA3C was also titrated with pSG5EBNA2 against the bfl-1 promoter in the same manner as for EBNA3A and 3B In this instance EBNA3C, down-regulates EBNA2 trans-activation of the bfl-1 promoter (-1374/+81 wtbfl-1 Luc)

EBNA3C however may also be regarded as a trans-activator of viral and cellular genes, which up-regulates transcription of the LMP1, CD21 and vimentin proteins via CBF1 and CBF1 independent means (Wang *et al*, 1990, Allday *et al*, 1993, Ring 1994, Zhao *et al*, 2000) Thus transfections were carried out with the three EBNA3 expression plasmids and the *bfl-1* promoter reporter construct (-1374/+81 wtbfl-1 Luc) and its CBF1 mutant counterpart (-1374/+81 mCBF1bfl-1 Luc) It can be seen that on their own EBNA3A 3B and EBNA3C could not trans-activate the *bfl-1* promoter (-1374/+81 wtbfl-1 Luc) or the mutant (-1374/+81 mCBF1bfl-1 Luc) reporter construct (Figure 3 34)

Figure 3.34 EBNA3A, 3B and 3C Do Not Trans-activate the Wild Type or CBF1 Mutated *bfl-1* Promoter.

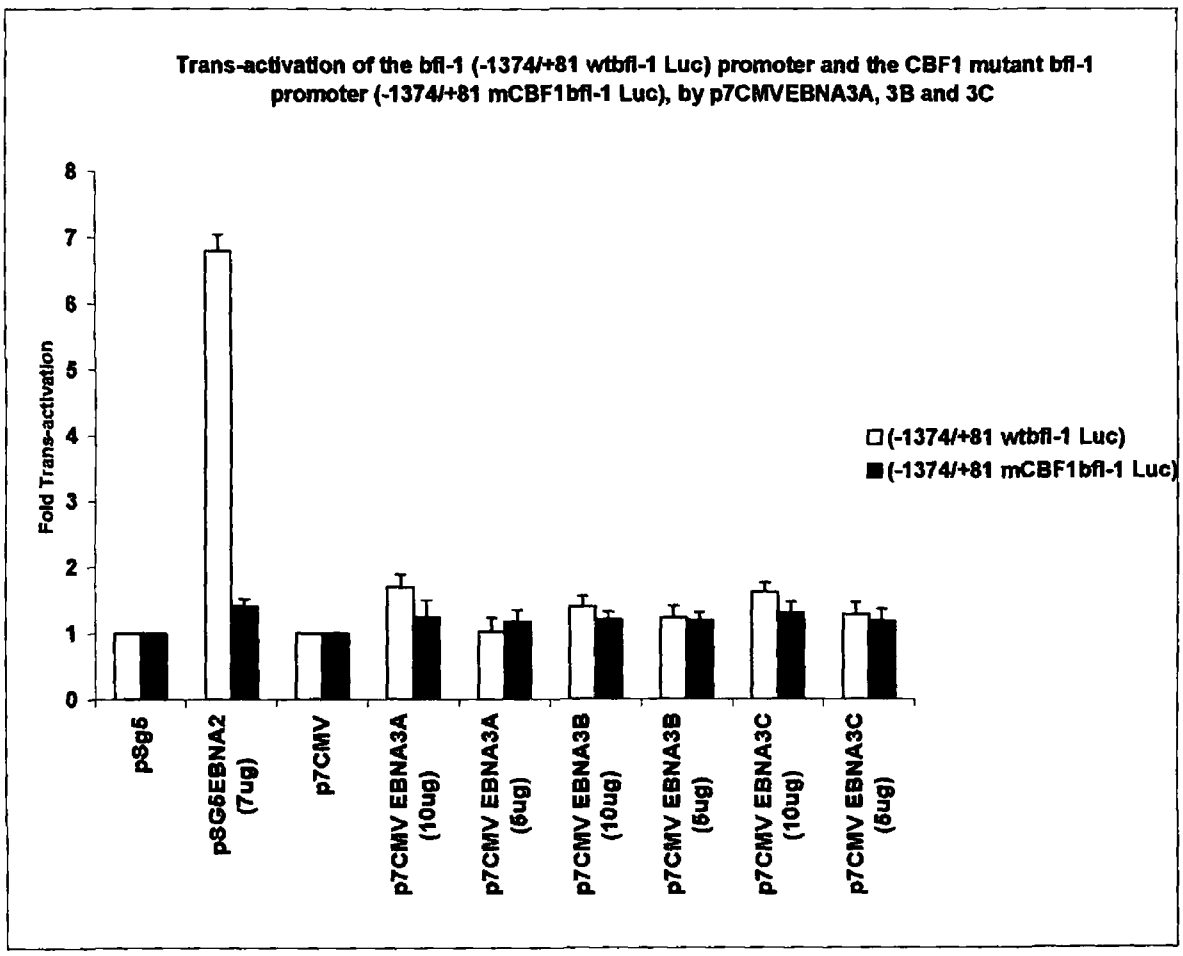


Figure 3.34. EBNA3A, 3B and 3C do not trans-activate the wild type *bfl-1* promoter (-1374/+81 wtbfl-1 Luc) or the CBF1 mutated *bfl-1* promoter (-1374/+81 mCBF1bfl-1 Luc) in the Dg75 cell line. The three p7CMVEBNA3 expression plasmids were co-transfected with the *bfl-1* and the CBF1 mutant *bfl-1* promoters (-1374/+81 wtbfl-1 Luc and –1374/+81 mCBF1bfl-1 Luc respectively) None of the EBNA3 expression plasmids trans-activated either promoters at either concentration used thus although EBNA3C has been shown to be a trans-activator of certain viral and cellular genes, *bfl-1* does not appear to be one of them under these conditions

3.2.10.0. The Product of the CST RPMS1, also known as RPMS1 Represses EBNA2 Trans-activation of the *bfl-1* Promoter in DG75 Cells

In addition to the well-established EBNA, LMP, and EBER genes, Epstein Barr virus (EBV) has been found to express various spliced RNAs transcribed rightward from the region spanning 150,000 to 161,000 on the B95-8 EBV genetic map This family of viral RNAs have been referred to as complementary strand transcripts (CSTs), BamH1 A rightward transcripts (BARTs), or the BARF0 RNAs (See Introduction Figure 1 14) The product of one of these CSTs RPMS1, a nuclear protein, has recently been characterized and shown to interact with the DNA-binding protein CBF1 (Fries *et al*, 1997, Smith, *et al*, 2000, Zhang *et al*, 2001) Studies by Smith *et al*, 2000 showed that RMPS1 can inhibit transcriptional activation induced via CBF1 by both EBNA2 and NotchIC

Our study to date has shown that the *bfl-1* promoter is trans-activated by EBNA2 via a mechanism that is likely to involve CBF1 To investigate any possible inhibitory effect of RPMS1 on EBNA2 trans-activation of the *bfl-1* promoter, transient co-transfections with EBNA2 and RPMS1 expression plasmids and the *bfl-1* promoter were undertaken One microgram of the *bfl-1* promoter reporter construct (-1374/+81 wtbfl-1 Luc) and 7ug of the EBNA2 expression plasmid pSG5EBNA2 were co-transfected into DG75 cells along with increasing quantities of an RPMS1 expression plasmid pcDNA3RPMS1 (a gift from Prof P Farrell (described by Smith *et al*, 2000) In all cases the total amount of DNA in each transfection was equalized by addition of either background vectors pSG5 or pcDNA3 where appropriate It can be seen from the reporter assay results (figure3 35) that RPMS1 can act as an antagonist of EBNA2 transcriptional activation of the *bfl-1* promoter under these conditions Addition of increasing amounts of RPSM1-expression vector resulted in a dose dependent reduction in EBNA2-mediated trans-activation of the bfl-1 promoter (-1374/+81 wtbfl-1 Luc) In these experiment, EBNA2 trans-activated the promoter by 6 9 fold, and this dropped to 2 03 fold upon addition of vector expressing RMPS1 Since RPMS1 has been shown to interact with CBF1 via other proteins in the repressor complex, the effects of RPMS1 on EBNA2 trans-activation of the *bfl-1* promoter provides further evidence that CBF1 or at least removal of the CBF1 repression complex, has a key role in the trans-activation of the *bfl-1* promoter by EBNA2

Figure 3.35. RPMS1 Represses EBNA2-Mediated Trans-Activation of the *bfl-1* Promoter (-1374/+81 wtbfl-1 Luc).

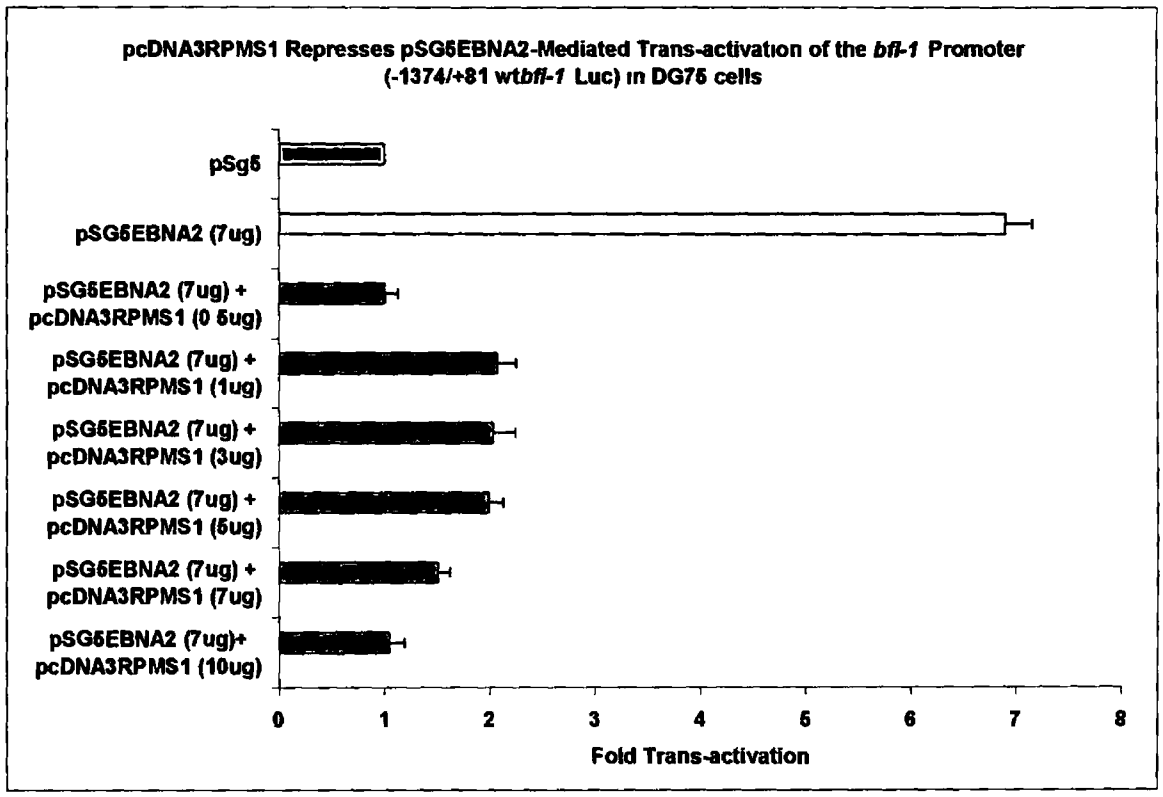


Figure 3.35. RPMS1 Represses EBNA2 Mediated Trans-activation of the *bfl-1* promoter The pcDNA3RPMS1 expression plasmid was co-transfected in increasing quantities with 1ug of the *bfl-1* promoter reporter construct and 7ug of pSG5EBNA2, the EBNA2 expression plasmid Increasing amounts of pcDNA3RPMS1 resulted in a dose dependent reduction in the trans-activation of the *bfl-1*promoter (-1374/+81 wtbfl-1Luc) by EBNA2 Since RPMS1 has been shown to interact with CBF1 via other proteins in the repressor complex, the effects of RPMS1 on EBNA2 trans-activation of *bfl-1* implies a key role for CBF1 in the trans-activation of *bfl-1* by EBNA2

Overall the repressive effects of EBNA3A, B, C and RPMS1 on EBNA2 mediated trans-activation of the *bfl-1* promoter imply a crucial role for CBF1 binding in the regulation of bfl-1 by EBNA2, as each of these proteins have been shown to inhibit EBNA2 associated transactivation by either binding directly to CBF1 or members of the CBF1 repression complex This can be seen clearly in Figure 3 36 where co-transfection with each of the p7CMV EBNA3 expression plasmids or the pcDNA3 RPMS1 expression plasmid down-

regulates EBNA2 associated trans-activation of the *bfl-1* promoter (-1374/+81 wtbfl-1 Luc) These results combined with the inability of the pSG5EBNA2WW323SR mutant (which cannot bind CBF1) to trans-activate the *bfl-1* promoter and the inability of EBNA2 to trans-activate the *bfl-1* CBF1 mutant promoter (-1374/+81 mCBF1bfl-1 Luc) suggest EBNA2 trans-activates *bfl-1* via a mechanism involving CBF1 binding Also co-transfection of the EBNA3 and RPMS1 expression plasmids does not affect the lack of EBNA2 trans-activation observed with the CBF1 mutant *bfl-1* promoter

Figure 3.36. The Combined Effects of EBNA3A, EBNA3B, EBNA3C and RPMS1 Imply a Key Role for CBF1 in the EBNA2 Mediated Trans-activation of the *bfl-1* Promoter.

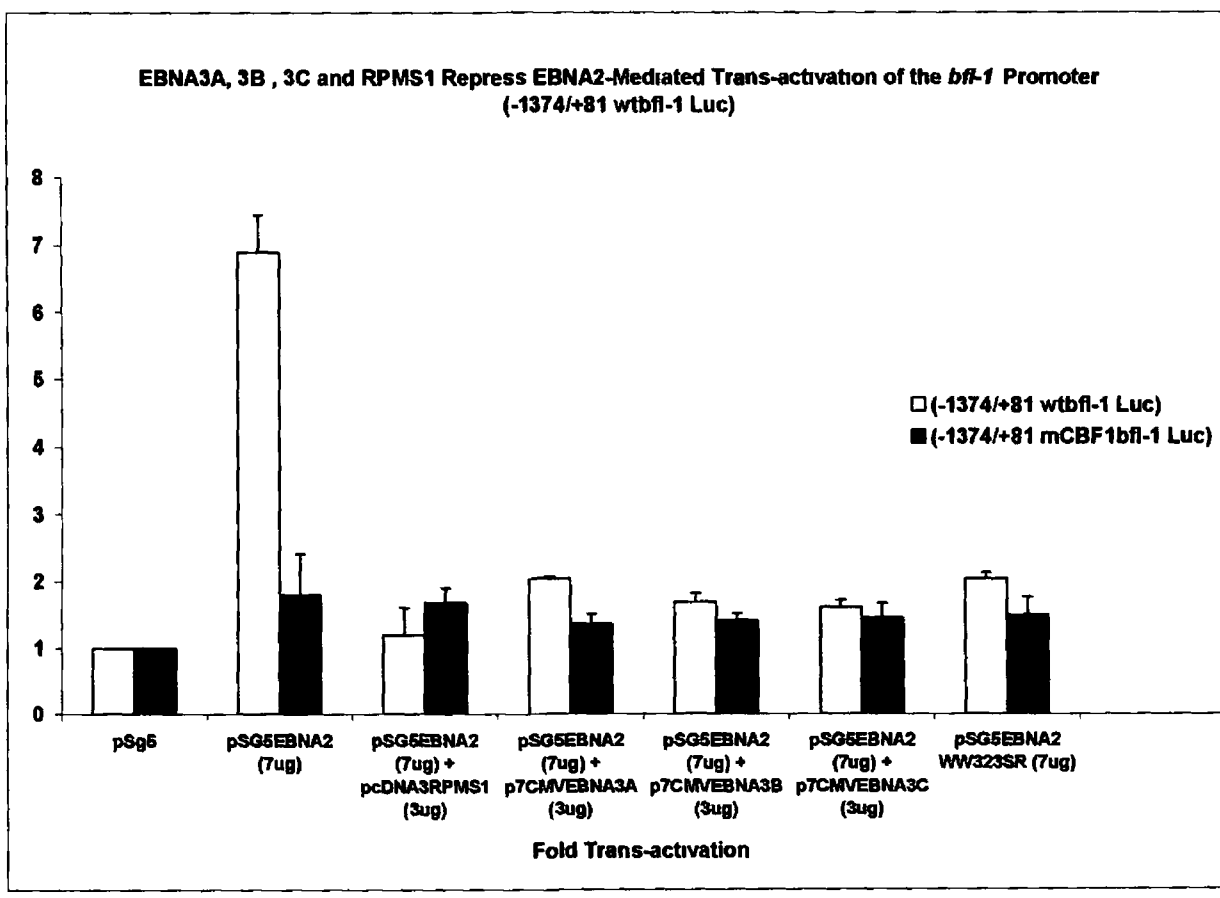


Figure 3 36 **The Combined Effects of EBNA3A, EBNA3B, EBNA3C and RPMS1 Imply a Key Role for CBF1 in the EBNA2 Mediated Trans-activation of the *bfl-1* Promoter.** In a single experiment each of the EBNA3 expression plasmids (p7CMV EBNA3a, 3B and 3C) and the RPMS1 expression plsmid

(pcdna3RPMS1) repressed EBNA2 mediated transactivation of the bfl-1 promoter The EBNA2 mutant psg5EBNA2WW323SR which cannot bind CBF1 also failed to trans-activate the bfl-1 promoter (-1374/+81 wtbfl-1 Luc) EBNA2 trans-activation of the mutant bfl-1 promoter (-1374/+81 mCBF1bfl-1 Luc) is not affected by addition of the EBNA3 or RPMS1 expression plasmids.

3.2.11.0. LMP1 Trans-activates the *bfl-1* Promoter via a Mechanism Which Does Not Require the Putative CBF1-Binding Site at –243.

In separate experiments both EBNA2 and LMP1 have been shown to trans-activate the *bfl-1* promoter when expressed as the sole EBV protein (Figure 3 37B) The potential for co-operativity between EBNA2 and LMP1 was therefore investigated in this regard Initially pSG5LMp1 was titrated against both the *bfl-1* promoter (1374/+81 wtbfl1Luc) and the CBF1 mutant *bfl-1* promoter -1374/+81mCBF1bfl1 Luc (Figure 3 37A) LMP1 trans-activated both promoters, thus the CBF1 site is not required to confer LMP1 responsiveness to the *bfl-1* promoter Increasing the quantity of pSG5LMP1 DNA transfected in the 1 to 10ug range examined did not increase the trans-activational effect of LMP1 on the *bfl-1* promoter LMP1 trans-activated both the mutated and wild type promoter constructs to an average of 4 5 fold over the range of concentrations tested The fact that LMP1 can trans-activate the mutated promoter, also demonstrates that the *bfl-1* promoter is still functional in the absence of the CBF1 site at position –264 to-269 and that this site is not critical for promoter function in the absence of EBNA2 A second similar experiment was also carried out (Figure 3 37B) This experiment is similar in that it shows the trans-activational effect of LMP1 on both the wild type (–1374/+81 wtbfl-1 Luc) and CBF1 mutated promoters (-1374/+81 mCBF1bfl-1 Luc) however also included in this experiment is a transfection with both promoters singly and the EBNA2 expression plasmid pSG5EBNA2 The importance of the CBF1 site for EBNA2 mediated trans-activation of the *bfl-1* promoter is especially evident when EBNA2 and LMP1 are transfected singly m the same experiment (figure 3 37B) Although both pSG5LMP1 and pSG5EBNA2 trans-activate the wild-type promoter (–1374/+81 wtbfl-1 Luc), only pSG5LMP1 can trans-activate the CBF1 mutaed *bfl-1* promoter (-1374/+81 mCBF1bfl-1 Luc) EBNA2 trans-activates the wild-type promoter 6 8 fold while LMP1 trans-activates the wild type promoter 5 2 fold Again, when the promoter CBF1 binding site is absent,

EBNA2 trans-activation is reduced to 1 5 fold This is not the case for LMP1, which trans-activates the mutated promoter 4 5 fold in the absence of the CBF1 binding site in the promoter

Figure 3.37A LMP1 Trans-activates Both Wild-type *bfl-1* Promoter (-1374/+81 wtbfl1 Luc) and the CBF1 Mutated *bfl-1* Promoter (-1374/+81 mCBF1bfl1 Luc).

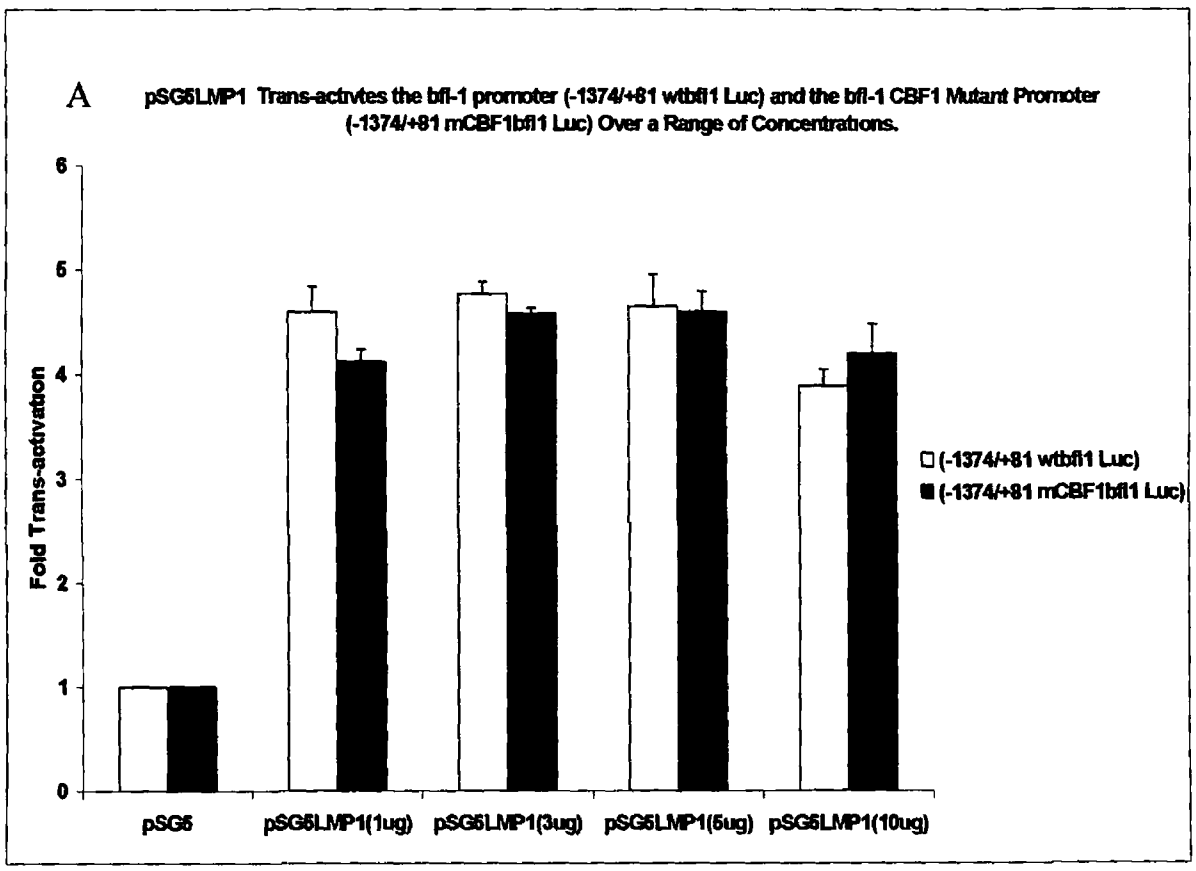


Figure 3 37(A). LMP1 trans-activates both –1374/+81wtbfl-1 Luc and –1374/+81mCBF1bfl-1 Luc promoters, over the range of concentrations of LMP1 expression plasmid used. pSG5LMP1 was titrated against the bfl-1 promoter at various quantities between 1ug and 10ug Transfections were carried out in DG75 cells using the DEAE dextran method Quantities of expression plasmid used are as outlined in the legend above One of promoter reporter constructs was used in each case All transfections were made up to equal quantities of DNA by addition of pSG5 vector

Figure 3.37B. LMP1 Trans-activates the *bfl-1* Promoter in a CBF1 Independent Manner.

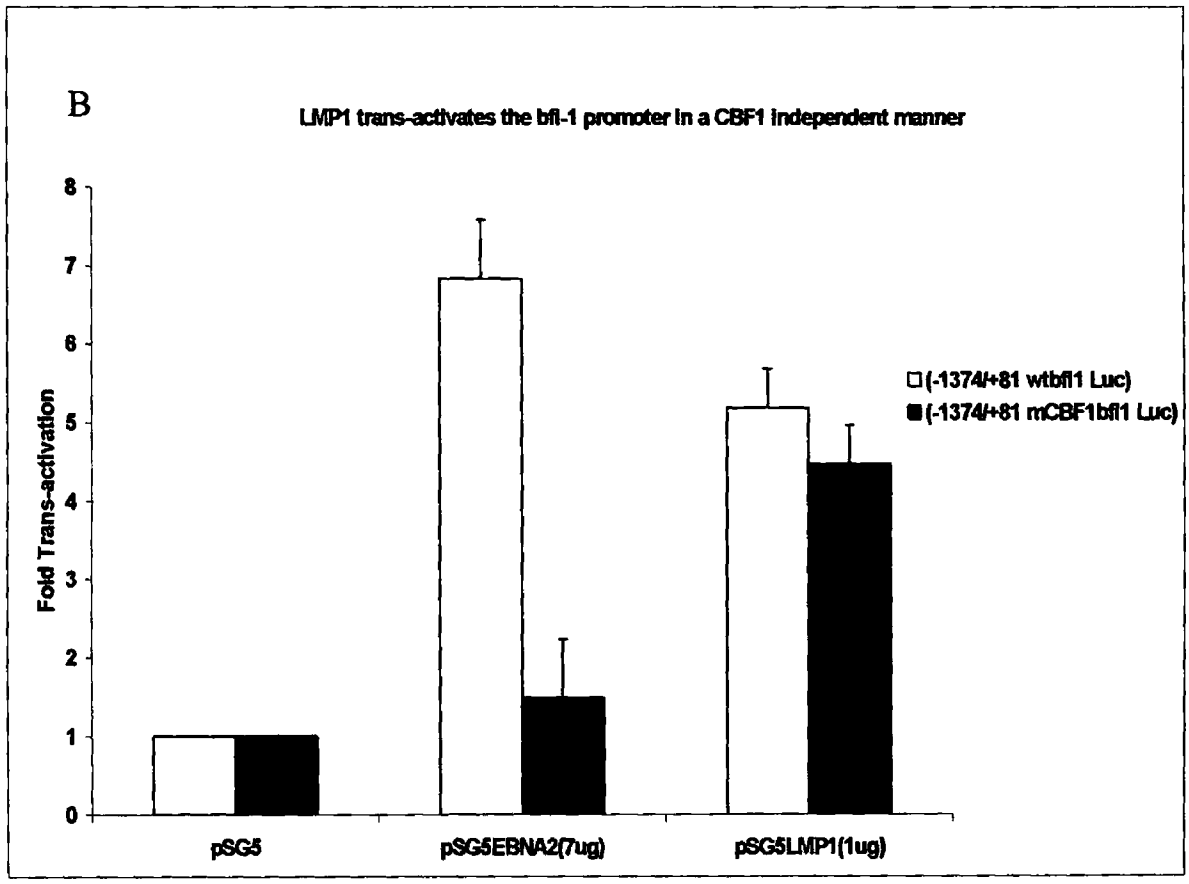


Figure 3.37B. LMP1 Trans-activates the bfl-1 Promoter in a CBF1 Independent Manner. DG75 cells were transfected using the expression plasmid indicated above (quantities of vector used given in brackets) All transfections were carried out using 1ug of promoter reporter construct. Total quantities of DNA in each transfection were normalised using pSG5 vector Promoter activity was determined using the luciferase assay as before Luciferase values were normalised using the B-Gal assay The results are representative of three independent experiments –error bars show variation

3.2.11.1. EBNA2 and LMP1 Do Not Co-operate to Trans-activate the *bfl-1* Promoter

Results from further transfections below showed that co-transfection of pSG5EBNA2 and pSG5LMP1 did not increase promoter trans-activation In fact co-transfection of increasing quantities of LMP1 expression plasmid with a standard 7ug input of EBNA2 expression plasmid resulted in a dose dependent decrease in EBNA2 mediated trans-activation of the *bfl-1* promoter

Figure 3.38 EBNA2 Does Not Co-operate with LMP1 to Trans-activate the *bfl-1* Promoter. Co-Transfection with LMP1 reduces EBNA2 Associated Transactivation of the *bfl-1* Promoter

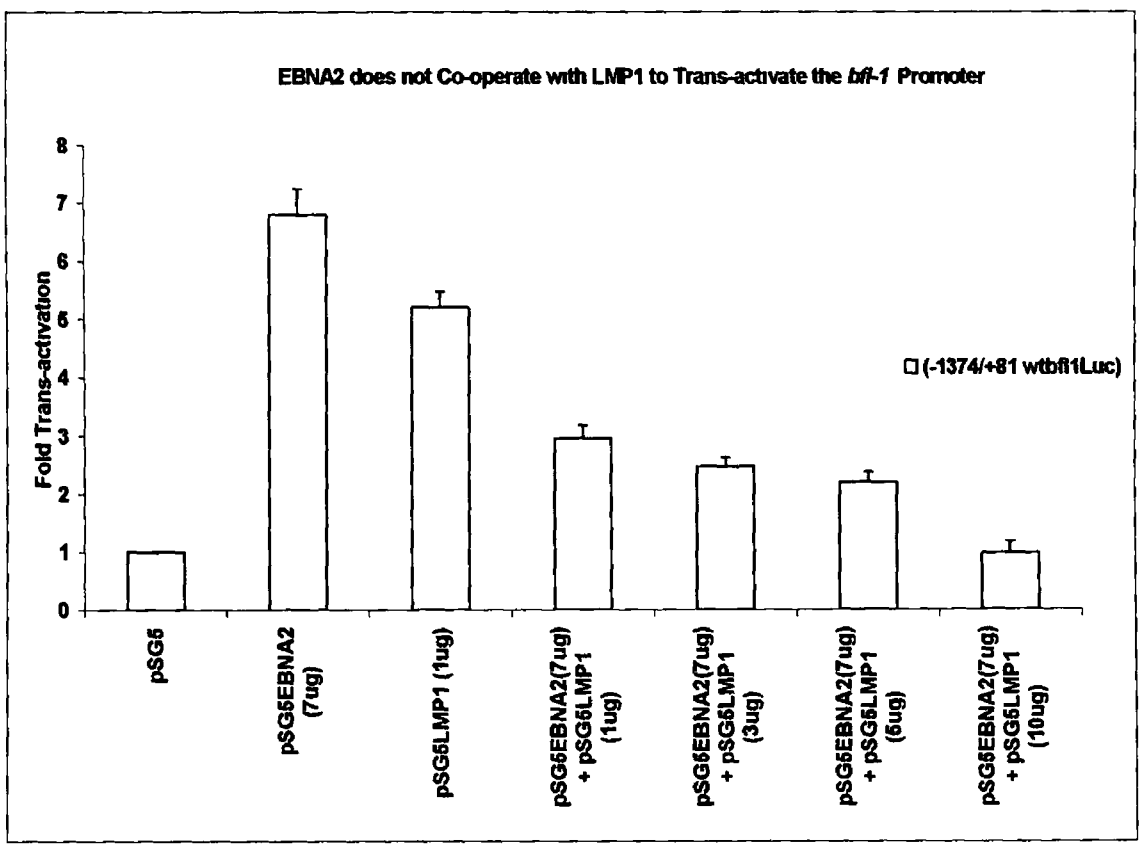


Figure 3.38. EBNA2 does not Cooperate with LMP1 to Trans-activate the *bfl-1* Promoter Co-Transfection with LMP1 reduces EBNA2 Transactivation of the *bfl-1* Promoter One microgram of the bfl-1 luciferase reporter plasmid (-1374/+81 wtbfl1 Luc) was transfected in each case Both EBNA2 and LMP1 trans-activated the promter when transfected individually however in the same experiment, co-transfection of the LMP1 and EBNA2 expression plasmids resulted in a decrease in promoter activity

Transfections were carried out in DG75 cells using the DEAE dextran method Expression plasmids were transefected according to the quantities indicated in the legend above DNA quantities used in all transfections were normalised using the empty SG5 vector Luciferase activity in the co-transfections was measured at 48 hours post transfection and the results are expressed as fold activation over the luciferase activity seen with pSG5 alone

EBNA2 alone trans-activated the promoter an average of 6 8 fold In the same experiments, LMP1 alone trans-activated the bfl-1 promoter an average of 5 2 fold When LMP1 was co-transfected with EBNA2, trans-activation of the promoter is reduced Addition of even 1ug of the pSG5LMP1 expression plasmid resulted in promoter transactivation falling to less than 3 fold Increasing the quantity of LMP1 transfected further reduces trans-activation of the promoter until the promoter is no longer activated above the background levels obtained with the empty pSG5 vector These results suggest that although both EBNA2 and LMP1 can trans-activate the promoter independently, they may share some common pathway or interact with each other or impede each other from interacting with the promoter

The fact that LMP1 and EBNA2 fail to co-operate and that overexpression of LMP1 may hinder EBNA2-mediated transactivation could imply that both EBV proteins recruit an as yet unidentified common transcription factor/promoter element and thus serve to inhibit each other by preventing the formation of stable trans-activation complexes

3.2.12.0. EBNA-LP Potentiates the EBNA2 Activation of the *bfl-1* Promoter in the Dg75 Cell Line.

EBNA-LP, is the first viral gene product to be expressed together with EBNA-2 during EBV-induced B-cell immortalization (Alfieri *et al*, 1991) EBNA-LP consists of a multirepeat domain (W1W2) and a unique carboxyl-terminal domain (Y1Y2) (See Chapter 1 Figure 1 10 and Figure 3 38 below) and is known primarily as a transcriptional co-activator of EBNA-2 It has also been shown that EBNA-LP co-operates with EBNA-2 in up-regulating the expression of the essential viral transforming gene product LMP1 in B cells (Harada and Kieff 1997, Nitsche *et al*, 1997) and that EBNA-LP and EBNA-2

co-operatively stimulate expression of cyclin D2 in resting **B** cells and the progression of these cells from G0 to G1 in the cell cycle (Sinclair *et al*, 1994)

Since EBNA-LP is a known co-activator of EBNA2, the aim of this part of the study was to determine if there were any cooperative effects between EBNA2 and EBNA-LP in the trans-activation of the *bfl-1* promoter To this end a number of EBNA-LP expression plasmids were obtained (2 paired sets of EBNA-LP expression plasmids and corresponding non functional mutants of each EBNA-LP were obtained as gifts from Elliot Kieff Channing Laboratory, Departments of Medicine and Microbiology and Molecular Genetics, Brigham and Women'sHospital and Harvard Medical School, Boston, Massachusetts and Paul Ling Division of Molecular Biology Baylor College of Medicine Houston Texas) The construction of these plasmids has been published elsewhere (Harada and Kieff 1997, Peng et al, 2000) however Figure 3 38 below shows a brief schematic representation of the main features of the EBNA-LP expression plasmids used in this study

Figure 3.38 Map of EBNA-LP showing the exons derived from the EBV long internal repeat (W) and from the 3' unique DNA (Y1 and Y2) and the deletion mutants used in this study.

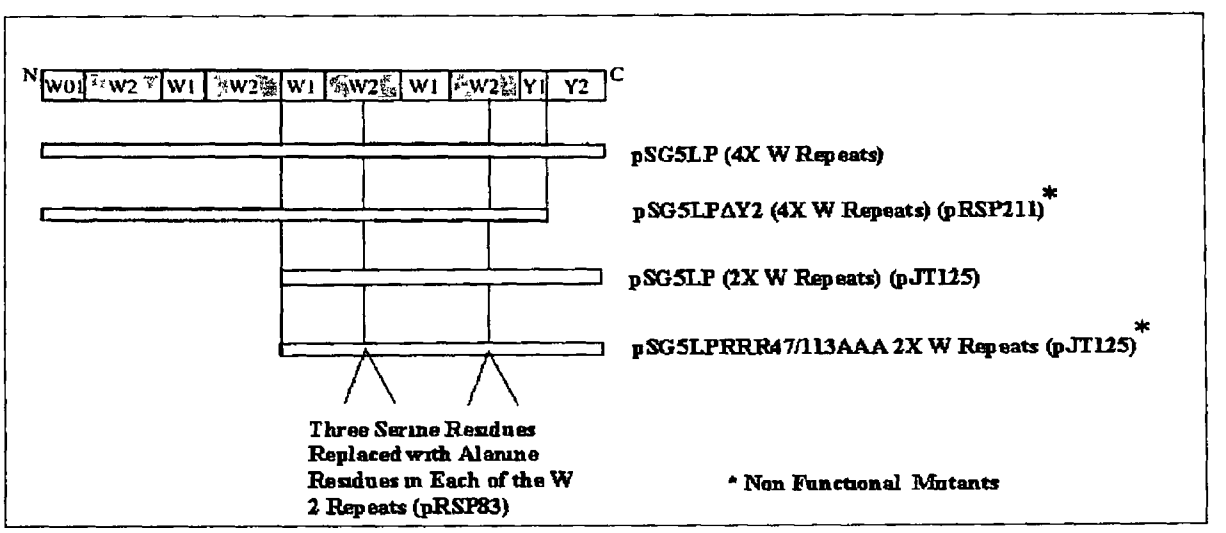


Figure 3.38 Map of EBNA-LP showing the exons derived from the EBV long internal repeat (W) and from the 3' unique DNA (Y1 and Y2) and the deletion mutants used in this study The W1 and

W2 exons are 22 and 44 codons, respectively, and together encode 66-amino-acid repeats All the expression plasmids had been generated using the pSG5 vector (Stratagene) The descriptive name for each of the plasmids is listed first followed by the actual lab names which will be used henceforth A wild type EBNA Lp with four W repeats, (SG5LP) [Harada, 1997] and a non functional partner for pSG5LP, pRSP211 were obtained [Peng, 2000] The mutant partner for SG5LP is known as pRSP211/ SG5LP ΔY2 This plasmid contains the four W repeats however theY2 domain has been removed. Another EBNA Lp expression plasmid was also used, in this case the wild type Lp had two W repeats (pJT125) [Peng, 2000] The mutated partner to pJT125 is pRSP83 in this case again the mutant is identical to its wild-type partner in that it also contains two W repeats however three of the serine residues in the W2 region have been replaced with alanine residues SG5LP RRR47/113AAA (Peng *et al*, 2000) The two non functional mutants had been generated by PCR mutagenesis of EBNA Lp cDNA as described (Peng *et al*, 2000) (Adapted from`Harada and Kieff 1997 and Peng et al, 2000)

3.2.12.1. EBNA-LP Co-Operates with EBNA2 to Trans-activate the *bfl-1* Promoter in the Dg75 Cell Line

In non-EBV infected DG75 cells, transfection of the SV40 promoter driven EBNA2 expression plasmid (pSG5EBNA2) with the bfl-1 promoter (-1374/+81 wtbfl1 Luc) resulted in eight fold higher luciferase reporter activity than that with the pSg5 vector-transfected controls as before EBNA2 did not trans-activate the CBF1 mutated *bfl-1* promoter (-1374/+81 mCBF1bfl1 Luc) Initially experiments were carried out with the pSG5-LP expression plasmid containing the 4xW repeats It can be seen from Figure 3 39A that pSG5LP alone, consistently trans-activated the *bfl-1* promoter from 2 fold to 5 3 fold over the range of concentrations of pSG5LP used during transfection This trans-activational effect appears to be dependent on the CBF1 site in the *bfl-1* promoter since trans-activation of the CBF1 mutated *bfl-1* promoter (-1374/+81 mCBF1bfl1 Luc) is reduced by half compared to the trans-activational effect of pSG5LP on the wild-type promoter over the range of concentrations of pSG5LP transfected Co transfection experiments with pSG5EBNA2 and pSG5LP showed a dose dependent increase in EBNA2 mediated trans-activation of the *bfl-1* promoter (-1374/+81 wtbfl1 Luc) in response to the addition of increased quantities of pSG5LP Cotransfection of EBNA-LP

increases EBNA2 trans-activation of the *bfl-1* promoter from around 8 fold (in the absence of EBNA-LP) to about forty fold when co-transfected with 10ug of pSG5LP

Figure 3.39A. pSG5LP (EBNALp 4X W Repeats) Co-operates with EBNA2 in Trans-activating the *bfl-1* Promoter.

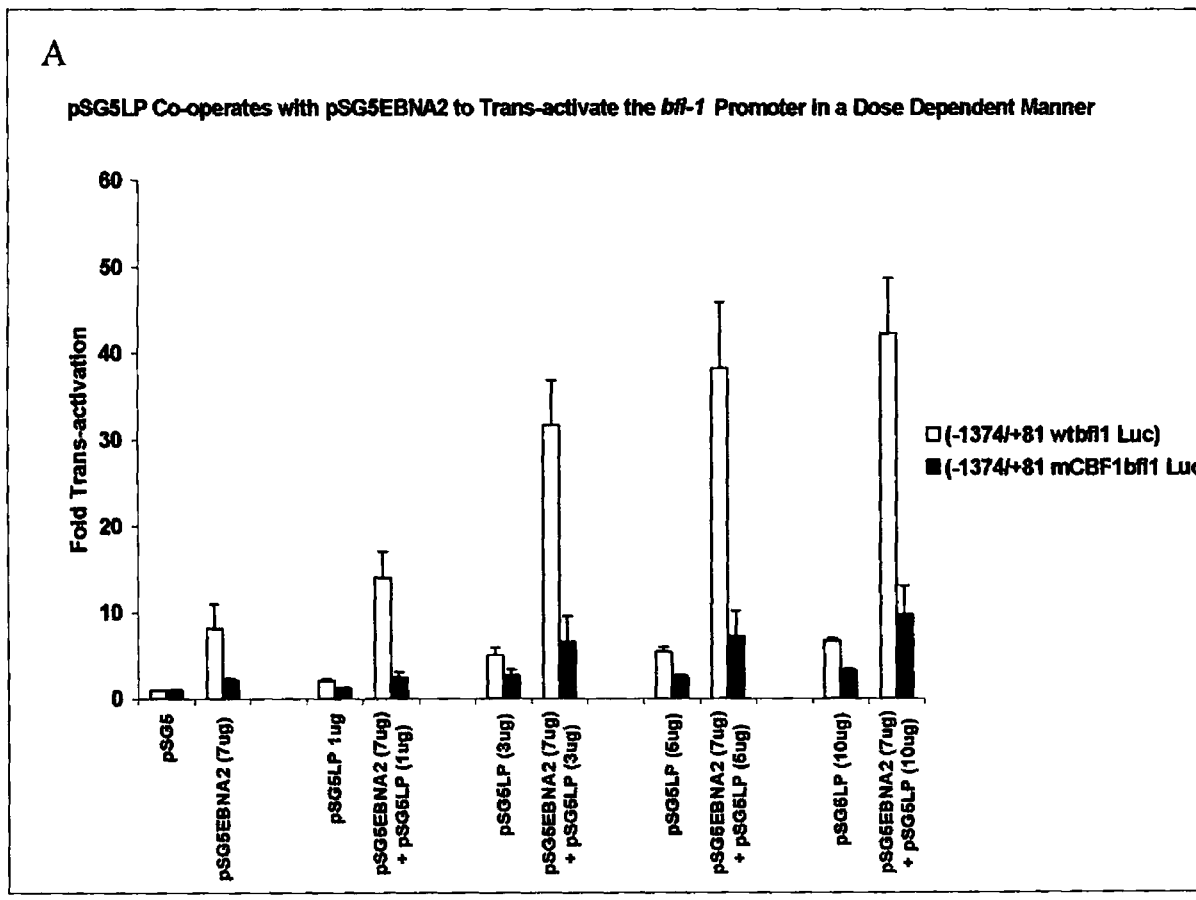


Figure 3 39A. EBNA-LP Stimulates EBNA2-Mediated Trans-activation of the *bfl-1* Promoter in a Dose Dependent Manner Bfl-1 luciferase reporter plasmids weretransfected into Dg75 cells along with the indicated amounts of pSG5EBNA2 and/or pSG5LP Transfections were performed using the DEAE dextran method and the total amount of transfected DNA in each sample was equalized with pSG5 vector DNA Fold activation relative to that with pSG5 is indicated

The same titrational and co-operation experiments were performed in the Dg75 cell line with the shorter pJT125 LP expression plasmid containing just two W repeats and the results can be seen in figure 3 39B below The average results of experiments where EBNA-LP input was titrated across a 1 to 10ug range in the presence or absence of a

standard input (7ug) of pSG5EBNA2 can be seen In this experiment as before, transfection with pSG5EBNA2 alone compared to the pSG5 vector, results in an average increase in promoter activity of about 7 fold Again the mutated promoter reporter construct (-1374/+81 mCBF1bfl1 Luc) was not trans-activated by EBNA2 pJT125 also trans-activated the *bfl-1* promoter (-1374/+81 wtbfl1 Luc) in a dose dependent manner when transfected in the absence of EBNA2 Luciferase reporter activity increased from 2 fold to 6 25 fold over the range (1 to 10ug) of (pJT125) EBNA-LP expression plasmid transfected EBNA-LP trans-activation of 1374/+81 bfl-1luc mut (in which the CBF1 binding site has been removed) was approximately half that seen for the wild type promoter Thus the CBF1 site in the promoter appears to play a role in the trans-activational effect of (pJT125)/EBNALP on the *bfl-1* promoter Co-transfection of EBNA2 with EBNA-LP resulted in an increase in promoter activity from 7 fold with EBNA2 alone to 85 fold with cotransfection of 5ug of the EBNA-LP expression plasmid Thus pJT125/EBNALP increases EBNA2 transactivation of *bfl-1* by an additional factor of about 12 The co-operative effect of EBNA-LP on EBNA2 mediated trans-activation of the *bfl-1* promoter (-1374/+81 wt1bfl1 Luc) is dose dependent since increasing the amount of EBNA-LP expression plasmid increases EBNA2 trans-activation of the promoter over the range of EBNA-LP levels tested It should be noted also that transfection with the EBNA-LP isoform with just 2 W repeats (pJT125) consistently produced higher levels of cooperative trans-activation of the *bfl-1* promoter (-1374/+81 wt1bfl1 Luc) than its 4X W repeat counterpart pSG5LP Co-transfection of EBNA2 and EBNA-LP expression plasmids also showed trans-activation of the *bfl-1* CBF1 mutated promoter (-1374/+81 mCBF1bfl1 Luc) however trans-activation recorded is probably due to the additive effect of background trans-activation by the two expression plasmids

Figure 3.39(B). pJT125(EBNALP 2X W Repeats) Co-operates with EBNA2 in Trans-activating the *bfl-1* Promoter.

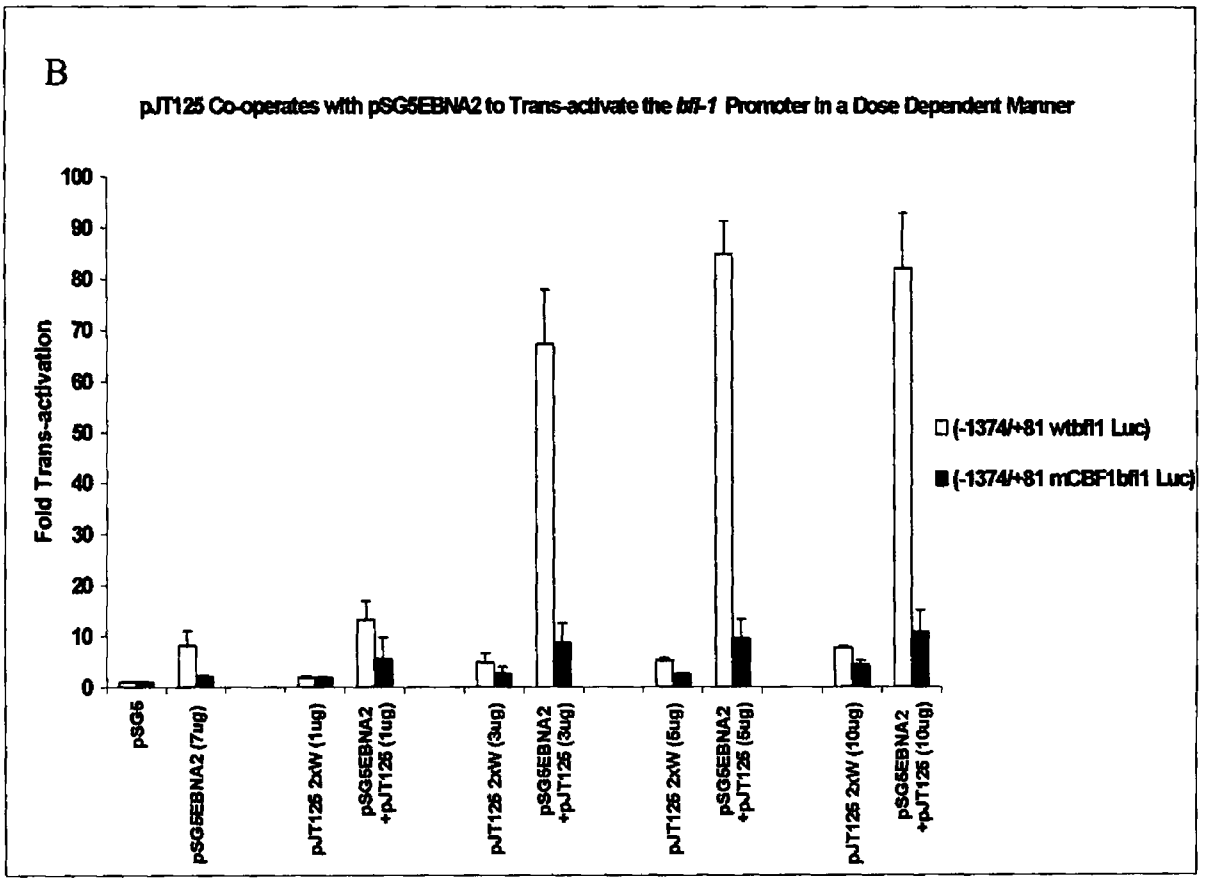


Fig 3 39B. EBNA-LP (pJT125) stimulates EBNA2-mediated trans-activation of the bfl-1 promoter in a dose dependent manner Bfl-1 luciferase reporter plasmids were transfected into Dg75 cells along with the indicated amounts of pSG5EBNA2 and/or pJT125 (psg5EBNALP 2xW Repeats) Transfections were performed using the DEAE dextran method and the total amount of transfected DNA in each sample was equalized with pSG5 vector DNA Fold activation relative to that with pSG5 is indicated.

Further transfections were carried out in the Dg75 cell line with the non-functioning mutant pairs for each of the EBNA-LP expression plasmids (See Asterix marked panels in Figure 3 38) A summary of the results can be seen below in Figure 3 39C The SG5LP (pRSP211) mutant pair contains the four W repeats but has no Y2 region, transfection with this plasmid alone or in co-transfections with EBNA2, does not trans-activate the wild type *bfl-1* promoter (-1374/+81 wtbfl1 Luc) When co-transfected with EBNA2 it does not affect EBNA2 mediated trans-activation of the *bfl-1* promoter which remains at

an average of 7 fold Similar results were obtained with the pRSP83 vector This expression plasmid is the mutant partner for pJT125 and consists of the EBNA-LP isoform which conatins 2 W repeat regions, however it has had a number of serine residues replaced by alanine residues rendering it non functional This EBNA-LP expression plasmid does not trans-activate the promoter either alone or when co-transfected with EBNA2 Again co-transfection with pRSP83 does not affect EBNA2 trans-activation of the wild type *bfl-1* promoter 9-1374/+81 wtbfl1 Luc)

Figure 3.39 C. Summary of EBNA-LP Effects on EBNA2 Mediated Trans-activation of the *bfl-1* Promoter.

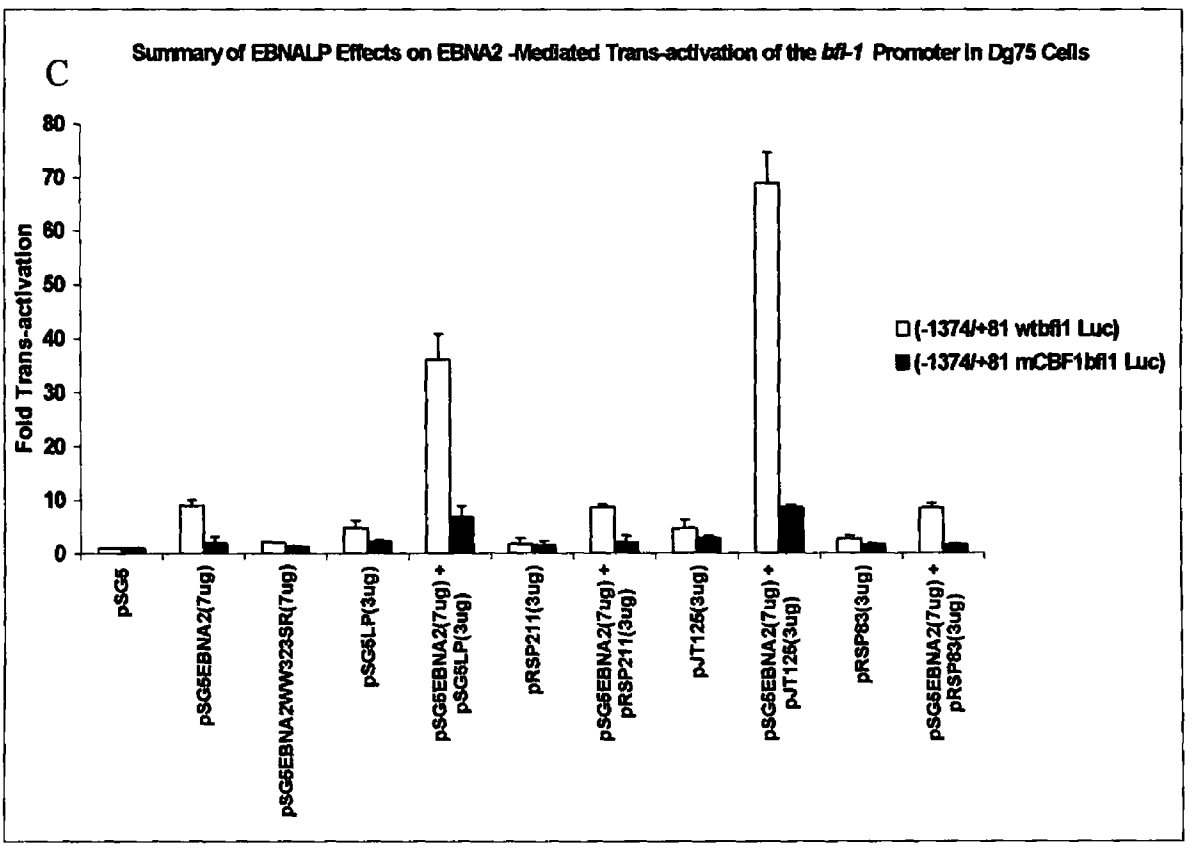


Figure3.39(C). Summary of the effects of pSG5EBNA-Lp and pJT125 on pSG5EBNA-2-mediated trans-activation of the wild type *bfl-1* (-1374/+81 wtbfl-1 Luc) and *bfl-1* CBF1 mutant promoters (-1374/+81 mCBF1bfl1 Luc) in DG75 cells Luciferase reporter plasmids were transfected into Dg75 cells along with the indicated amounts of pSG-EBNA-2/pJT125 and/or pSG-EBNA-LP The total amount of transfected DNA in each sample wasequalized with pSG5 vector DNA. Fold activation relative to that with pSG5 is indicated. These are representative results of 3 independent experiments. Also shown are the

effects of the non functional mutants of the two EBNALP isoforms used in these experiments, pRSP211 and pRSP83

3.2.12.2. EBNA-LP Enhances EBNA2 Mediated Trans-activation of the *bfl-1* Promoter in the Bjab Cell Line.

Transfections were also carried out in another EBV negative B-lymphoma cell line, BJAB The results (Figure 3 40) showed much lower levels of trans-activation for the promoter in all cases (only the pJT125 EBNA-LP expression plasmid was used as it had showed consistently higher levels of *bfl-1* promoter activation in the previous co-transfection experiments in DG75 cells) EBNA2 trans-activated the *bfl-1* promoter just over four fold compared to the pSG5 control vector Although less trans-activation was observed in this experiment relative to that presented earlier (4 fold as compared to around 8 fold in the DG75 cell line), co-transfection with pJT125/EBNALP increased psg5EBNA2 mediated trans-activation of the *bfl-1* promoter (-1374/+81 wt*bfl1* Luc) from 4 fold to 12 46 fold (Figure 3 40) Thus, in this cell line also, EBNA-LP co-operates with EBNA2 to trans-activate the *bfl-1* promoter Transfection with the pJT125/EBNALP expression vector alone showed a 2 5 fold trans-activation of the *bfl-1* promoter The CBF1 mutant bfl-1 (1374/+81 mCBF1*bfl1* Luc) was not trans-activated by EBNA2 the EBNA2 mutant (pSG5EBNA2WW323SR) or the EBNALP expression plasmid (pJT125) Co-transfection of pSG5EBNA2 and pJT125 with the CBF Mutant promoter (-1374/+81 mCBF1bfl1 Luc) showed a 3-fold trans-activation of the promoter and again this may be due to addition of residual trans-activation by both EBNA2 (pSG5EBNA2) and EBNA-LP (pJT125)

Figure 3.40. EBNA-LP (pJT125), Co-operates with pSG5EBNA2 to Trans-activate the *bfl-1* Promoter (-1374/+81 wtbfl1 Luc) in the Bjab Cell Line.

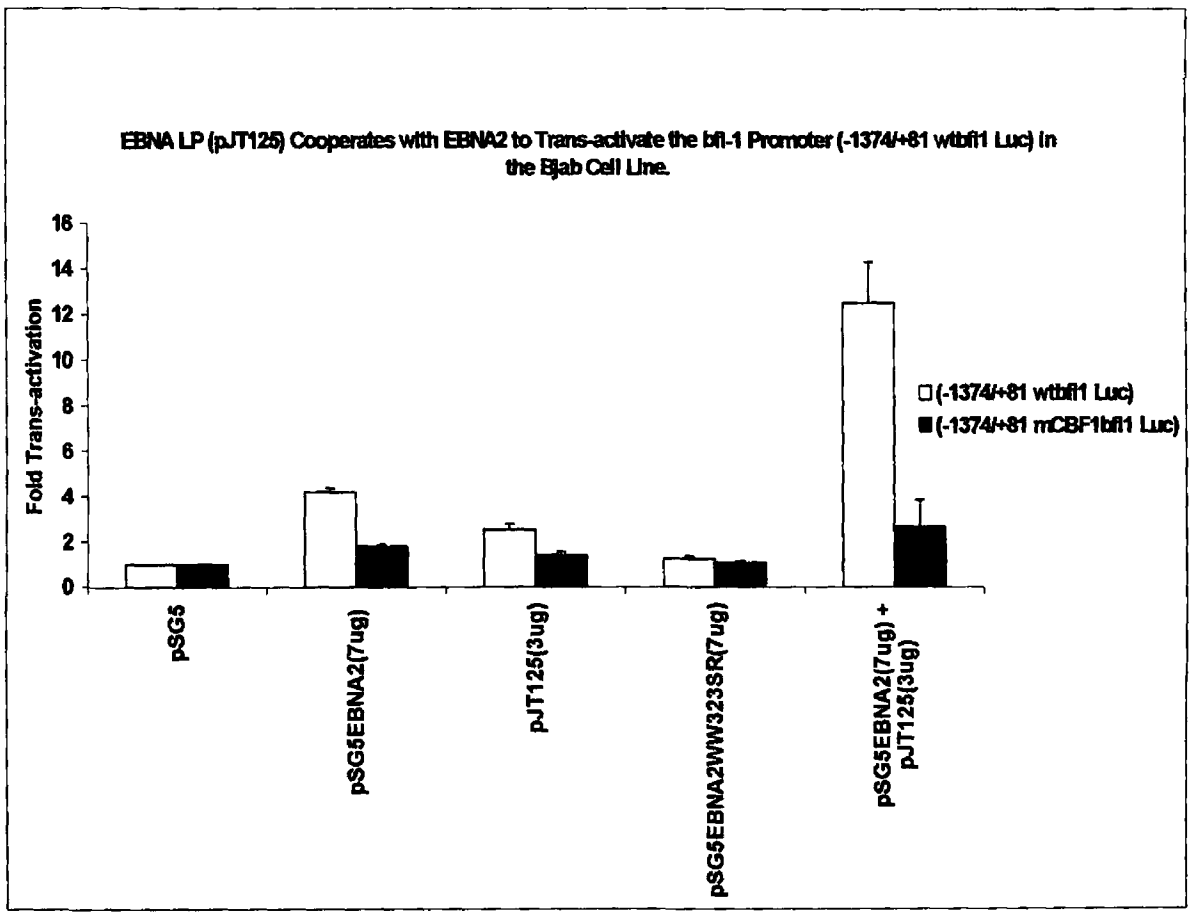


Figure 3 40 pJT125 (EBNALP) stimulates pSG5EBNA2 mediated trans-activation of the bfl-1 promoter in the Bjab cell line The activating plasmid pSG5EBNA2 and the reporter plasmids (-1374/+81 wtbfl1 Luc) and (-1374/+81 mCBF1bfl1 Luc) were transfected into Bjab cells with or without pJT125/EBNALp Average fold activity among 2 independent experiments is shown Fold activation is relative to that with the pSG5 control vector Error bars indicate standard deviations

In summary, (i) EBNA2 and EBNA-LP co-operate to enhance *bfl-1* promoter activity in 2 EBV negative B cell Lines DG75 and Bjab (ii) Co-transfection with the EBNA-LP isoform containing only two W1W2 repeats (pJT125) consistently showed a higher level of promoter trans-activation compared to the LP isoform which contained the 4W repeats (pSG5LP) (iii)Both EBNA-LP isoforms trans-activate the wild type *bfl-1* promoter (-1374/+81 wtbfl1 Luc) (iv) however, the EBNA-LP mutants lose their background when transfected with the *bfl-1* CBF1 binding mutant promoter reporter(-1374/+81 mCBF1bfl1

Luc), thus the CBF1 site appears to play a role in the trans-activational effect of EBNA-LP on the *bfl-1* promoter (v) Although there appears to be a cooperative trans-activation of the bfl-1 mutant promoter (-1374/+81 mCBF1bfl1 Luc) by pSG5EBNA2 and the EBNALp expression plasmids, (albeit relatively low), this can be attributed to the additive effect of basal trans-activation of the mutant promoter (-1374/+81 mCBF1bfl1 Luc) by the pSG5EBNA2 and pJT125/pSG5LPEBNALp expression plasmids (vi)The EBNA-LP mutants pRSP211 and pRSP83 don't co-operate with pSG5EBNA2 in trans-activating the *bfl-1* promoter (-1374/+81 wtbfl1 Luc), neither do they inhibit EBNA2 trans-activation of this promoter

3.2.13.0 Investigating the Importance of the Putative Ets and Pu1 Binding Sites in the *bfl-1* Promoter and their Effect on EBNA2 Associated Trans-activation of the *bfl-1* Promoter.

Ets proteins have been implicated in the regulation of genes involved in diverse cellular processes, such as proliferation, differentiation, development, transformation, and apoptosis (Sementchenko and Watson 2000, Yordy and Muise-Helmericks 2000, Li *et al*, 2000) Ets factors typically consist of a conserved winged helix-turn-helix DNA-binding domain that recognize a core motif 5'GGA(A/T)3' whose flanking nucleotides determine specificity (Sementchenko and Watson 2000) Members of this family include Ets-1, Ets-2, PU 1, Fli-1, GABPα, Elf-1, Sap-1, PEA-3, Elk-1, Elk-2, erg and ergB A key characteristic that Ets transcription factors display is their ability to regulate transcription through interactions with other nuclear factors For example, Ets proteins functionally interact with NFκB (Bassuk *et al*, 1997), AP-1 (Bassuk and Leiden 1995), Pax (Fitzsimmons et al, 1996), Tax (Dittmer *et al*, 1997, Gitlin *et al*, 1993), and Sp1 (Dittmer *et al*, 1997, Block *et al*, 1996)

PU1(Spi-1) is a transcriptional regulator whose DNA binding domain presents 40% identity with the DNA binding domain of the c-Ets-1 So far Pu 1 binding sites have been characterised in B cell specific transcription regulatory elements such as the intronic enhancer of the immunoglobulin heavy chain The 3' enhancer of the Ig k (Pongubala *et*

al, 1992) and λ light chains (Eisenbeis *et al*, 1993) and the promoter of the ig J chain (Shin and Koshland 1993) Others have been identified in the promoter regions of myeloid specific genes the integrin CD11b (Pahl *et al*, 1993), the receptor of the macrophage growth factor (Zhang *et al*, 1994) and the Fcγ receptor (Perez *et al*, 1994) PU 1 functional specificity seems to depend on both the flanking nucleotides adjacent to the minimal core RGAA conserved between all PU 1 binding sites presently identified

The '5-GGAA-3'core sequence motif known to be the core DNA sequence involved in DNA binding of Ets family transcription factors, (Karim *et al*, 1990, Gutman *et al*, 1991) was identified at four points in the –376/+81 portion of the bfl-1 promoter (See Figure 3 22A and B) Transcription factor databases identified three possible ets-1 binding sites at positions –213, -176, -163 and a possible PU 1 binding site at position –143 on the basis of flanking sequences

Since Ets transcription factors interact with other nuclear factors and since PU 1 and Ets1 transcription factor binding sites have been identified and found to be essential in the E2REs of other EBNA2 responsive promoters such as LMP1 [Johannsen, 1996], the importance of the putative Ets-1 and PU 1 sites identified on the *bfl-1* promoter had to be addressed To this end site directed mutagenesis was undertaken to destroy the 5'-AGGA-3' consensus sequences which make up the Ets family transcription factor binding sites on that portion of the *bfl-1* promoter being examined

3.2.13.1.Mutating the Putative ets-1 and PU 1 Binding Sites on the bfl-1 Promoter.

Site directed mutagenesis of Ets-1 binding sites at positions –213 to –204 and two at –176 to –163 was undertaken using the Stratagene QuickChange® XL Site Directed Mutagenesis Kit The same was also done at the PU 1 site at position –143 to –134 In each case the core AGGA binding sequence within the putative Ets and Pu1 sites (at positions was targeted for mutagenesis The QuickChange® XL Site Directed Mutagenesis Kit was used in preference to the Altered Sites Mutagenesis Kit®, which was used in constructing the CBF1 mutation in the *bfl-1* promoter The major advantage of this kit was that there was no need for a specialised mutagenesis vector such as pAlter

and no need for subsequent subcloning back into the reporter construct of choice In this system, mutagenesis was carried out in-situ in the-367/+81 bfl-1 Luc reporter construct (See Figure 3 41)

The QuickChange XL system was used to switch amino acids making up the Ets-1 and Pu 1 transcription factor binding sites and replace these with an alternative sequence of amino acids This completely obliterated any possible binding of these transcription factors The QuickChange XL method was performed using *Pfu Turbo*® DNA polymerase and a thermal cycler

The basic procedure utilises a supercoiled double-stranded (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation (Figure 3 41)

1 In this instance the double stranded vector was the –367/+81 bfl-1 Luc luciferase promoter reporter construct and the target sites for mutation were the ets-1 and PU 1 sites in the *bfl-1* promoter Initially each site had to be mutated individually

2 The *bfl-1* luciferase reporter plasmid (–367/+81 bfl-1 Luc) was denatured with high temperature, and oligonucleotide primers containing the desired mutations were annealed to the denatured plasmid The oligonucleotides selected to "knock out" the transcription factor binding sites had to be chosen carefully to ensure a number of things (i)the sequences selected could not encode any other relevant transcription factor binding sites (ii) the sequences selected had to encode a site recognised by a restrictrion endonuclease to allow identification of mutant clones In this case the PstI site was chosen to replace the GGAA core of the ets-1 and PU 1 transcription factor binding sites (iii) the sequences selected had to assist in ensuring a GC content >40% and a T_m >78°C The oligonucleotide primers designed to mutagenise each of the Ets-1 and PU 1 binding sites are shown in table 3 2 below along with the wild-type promoter sequence

3 The oligonucleotide primers, each complementary to opposite strands of the vector were extended during temperature cycling by using *Pfu Turbo*® DNA polymerase *Pfu Turbo*® DNA polymerase replicates both plasmid strands with high fidelity and without

displacing the mutant oligonucleotide primers. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks.

5. Following temperature cycling the product was treated with DpnI. The DpnI endonuclease is specific for methylated and hemi-methylated DNA and was used to digest the parental DNA template and to select for mutation-containing synthesised DNA The nicked vector DNA incorporating the desired mutations was then transformed into XL10-Gold® ultracompetent cells.

Table 3.2. Oligonucleotides Used to Mutagenise the Putative Ets1 and PU.1 Binding Sites.

Site Name and position	Sequence
Putative ets-1 binding site (-213 to –204). Wild type	5'-GGA-TTC-TAA-TTT-CTC-CAC-ATC-CTG-CAT-TTA-AGA-CTT-GCA-AAG-CTG-3'
Oligos for mutagenising site at (-213 to – 204)	-213 Fwd 5'-GGA-TTC-TAA-TTT-CTC-CAC-CTG-CAG-CAT-TTA-AGA-CTT-GCA-AAG-CTG-3' -213 Rev 5'- CAG-CTT-TGC-AAG-TCT-TAA-ATG-CTG-CAG-GTG-GAG-AAA-TTA-GAA-TCC-3'
Putative double ets-1 binding sites (-176 to –163).Wild type.	5'-GCA-AAG-CTG-AAT-TAA-TCA-CAG-GAT-GAG-GAA-GTG-GCT-TCT-CTG-3'
Oligos for mutagenising double site at (-176 to –163)	-177Fwd 5'-GCA-AAG-CTG-AAT-TAA -TCA-CAG-GCT-GCA-GAA-GTG-GCT-TCT-CTG-3' -177Rev 5'- CAG-AGA-AGC-CAC-TTC-TGC-AGC-CTG-TGA-TTA-ATT-CAG-CTT-TGC-3'
Putative PU.1 site (-143 to –134). Wild type	5'-GGA-AGT-GGC-TTC-TCT-GAA-ACA-TTT-TCC-TCT-TTC-ACA-TTT-T-3'
Oligos for mutagenising PU.1 site at (-143 to –134)	-146FWD 5'-GGA-AGT-GGC-TTC-TCT-GAA-ACA-TCT-GCA-GCT-TTC-ACA-TTT-T-3' -146Rev 5' –A-AAA-TGT-GAA-AGC-TGC-AGA-TGT-TTC-AGA-GAA-GCC-ACT-TCC-3'

Table 3.2 Quickchange Mutagenesis Reaction Reagents. This table shows the wild type sequences of the various portions of the bfl-1 promoter with the putative Ets1 and Pu1. sites indicated. The core AGGA binding sequence recognised by members of the Ets family (including Ets1 and Pu1) is shown in darker colour. In each case the oligonucleotides used in the muatgenesis reactions are shown. The change in the sequence introduced by the mutagenesis is indicated also.

Figure 3.41. Schematic Outline of Steps Involved in Quickchange Mutagenesis Reactions used to Generate the Ets1 and Pu1 *bfl 1* Mutant Promoter Reporter Constructs (-213mEts1 bfl1 Luc), (-176mEts1 bfl1 Luc) and (-143mPu1 bfl1 Luc) Respectively.

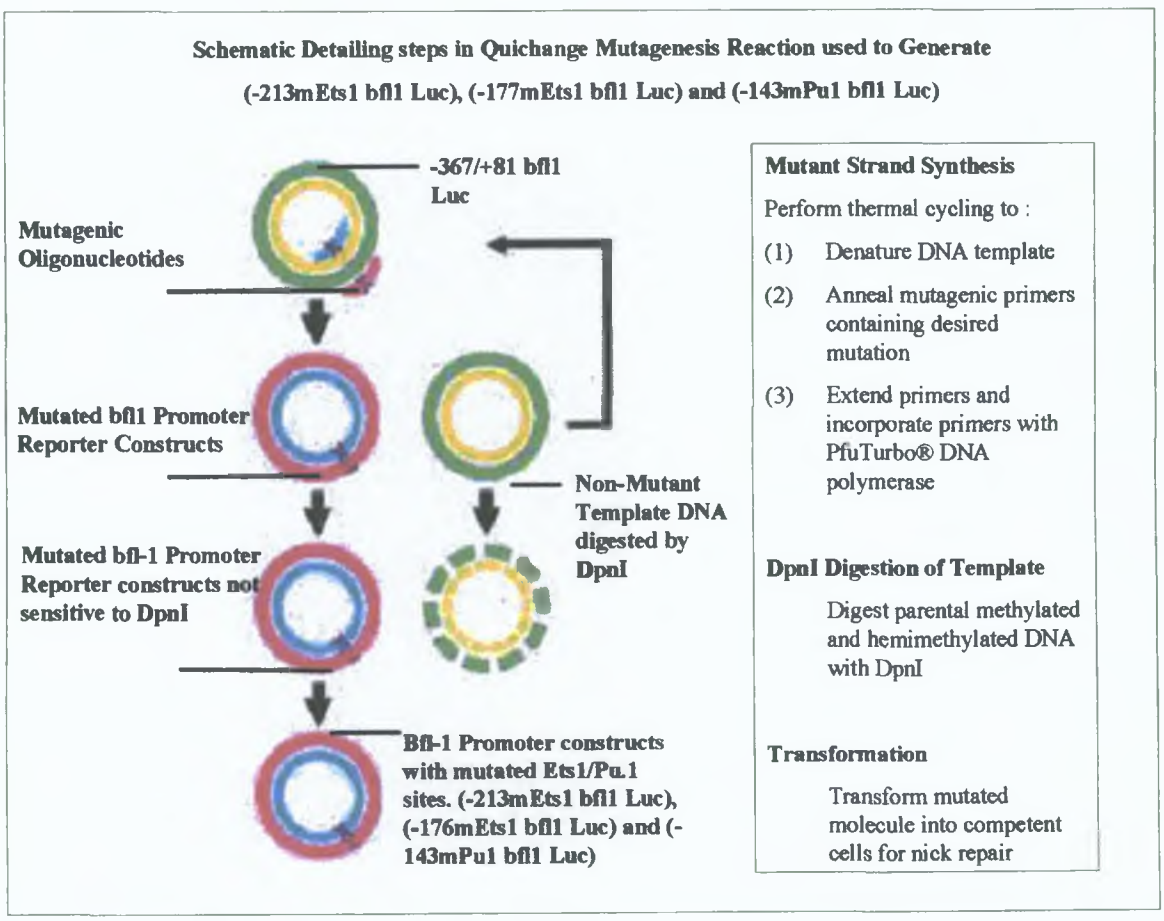


Figure 3.41. Schematic Outline of Steps involved in Quickchange Mutagenesis Reactions used to Generate the Ets1 and Pu1 *bfl 1* Mutant Promoter Reporter Constructs (-213mEts1 bfl1 Luc), (-176mEts1 bfl1 Luc) and (-143mPu1 bfl1 Luc) Respectively. The double stranded –367/+81 bfl1 Luc

promoter reporter plasmid was used as the template DNA This was denatured at high temperature and each of the oligonuleotides (See Table 3 2) were annealed to the denatured template and incorporated with Pfu Turbo DNA polymerase This results in the generation of the desired mutated vectors which contain nicked circular strands The methylated and hemimethylated template DNA (isolated from a dam+ E Coli strain Dh5α) was then susceptible to DpnI digestion while the mutated promoter reporter constructs are not The Mutant vectors are then transformed into the XL10Gold cells, which repair the nicks in the mutated plasmids

6 The transformed XL-10 gold cells were then grown and scaled up and the plasmid DNA harvested using the Qiagen® plasmid DNA extraction kit

The presence of the (putative) Ets-1 and PU 1 site mutations in the promoter were assayed by restriction analysis using the PstI restriction endonuclease, as a PstI site had been inserted into the possible Ets-1 and PU 1 sites respectively (Figure 3 42) Restriction with PstI, linearised the (–367/+81 bfl1 Luc) plasmid resulting in a band at ~5 8kb (Lane 2) The *bfl-1* luciferase reporter construct in which the Ets1 site at position –213 had been mutagenised, (-213mEts1 bfl1Luc), when digested with PstI yielded bands at 5 634kb and ~160bp (Lane 4) PstI Endonuclease restriction of the *bfl-1* luciferase reporter construct in which the double stes site at position –176 had been mutagenised (-176mEts1 bfll Luc) showed bands at 5 595kb and ~200bp (Lane 6) Digesting the *bfl-1* luciferase reporter construct in which the PU 1 site at position –143 had been mutated, (-143mutbfl-1luc) with PstI resulted in bands visible at 5 568kb and 230bp (Lane 8) These restriction patterns are clearly visible in Figure 3 42 below

Figure 3.42 Restriction Analysis of the (-213mEts1 bfl1 Luc), (-176mEts1 bfl1 Luc) and (-143mPu.1 bfl1 Luc).

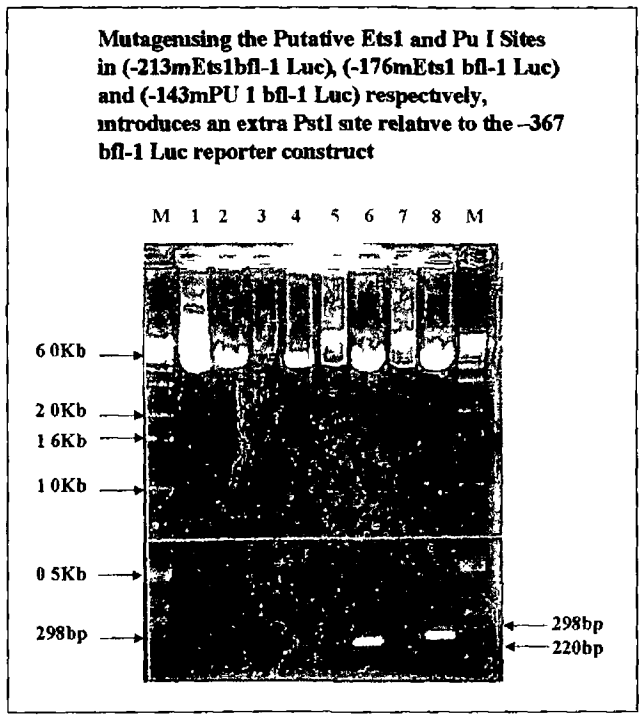


Figure 3 42. Restriction analysis of the (-213mEts1 bfl-1 Luc), (-176mEts1 bfl-1 Luc) and (-143mPu.1 bfl-1 Luc) M= Invitrogen 1Kb DNA Ladder Lanes 1 and 2 -367/+81bfl1 Luc Lanes 3 and 4 (-213mEts1 bfl-1 Luc) Lanes 5 and 6 (-177mEts1 bfl-1 Luc) lanes 7 and 8 (-143mPU 1 bfl-1 Luc) Undigested purified plasmid DNA (Lanes 1, 3, 5 and 7) PstI digested DNA (Lanes 2, 4, 6 and 8) Although the resolution at the top of the gel makes size determination difficult the gel had to be run this way in order to visualise the low molecular weight fragments For this reason also an excess of DNA was loaded (~500ng) Mutagenesis of -367/+81 using the Quickchange mutagenesis kit, introduced an extra PstI site in each of the three regions of interest resulting in fragments of the expected sizes

Below is a schematic of the resulting promoter constructs (Figure 3 43) These constructs were then co-transfected with pSG5EBNA2 in the DG75 cell line to assess the importance of the individual transcription factor binding sites in contributing to EBNA2 mediated trans-activation of the *bfl-1* promoter Also included was a transfection with the wild type (-1374/+81 wtbfl1 Luc) and the CBF1 mutant *bfl1* promoter reporter constructs (-1374/+81mCBF1bfl1 Luc)

Figure 3.43. Location of Putative Transcription Factor Binding Sites and Mutated Elements in the (-213mEts1 bfl1 Luc), (-176mEts1 bfl1 Luc) and (-143mPU.1 bfl1 Luc) Promoter Reporter Constructs.

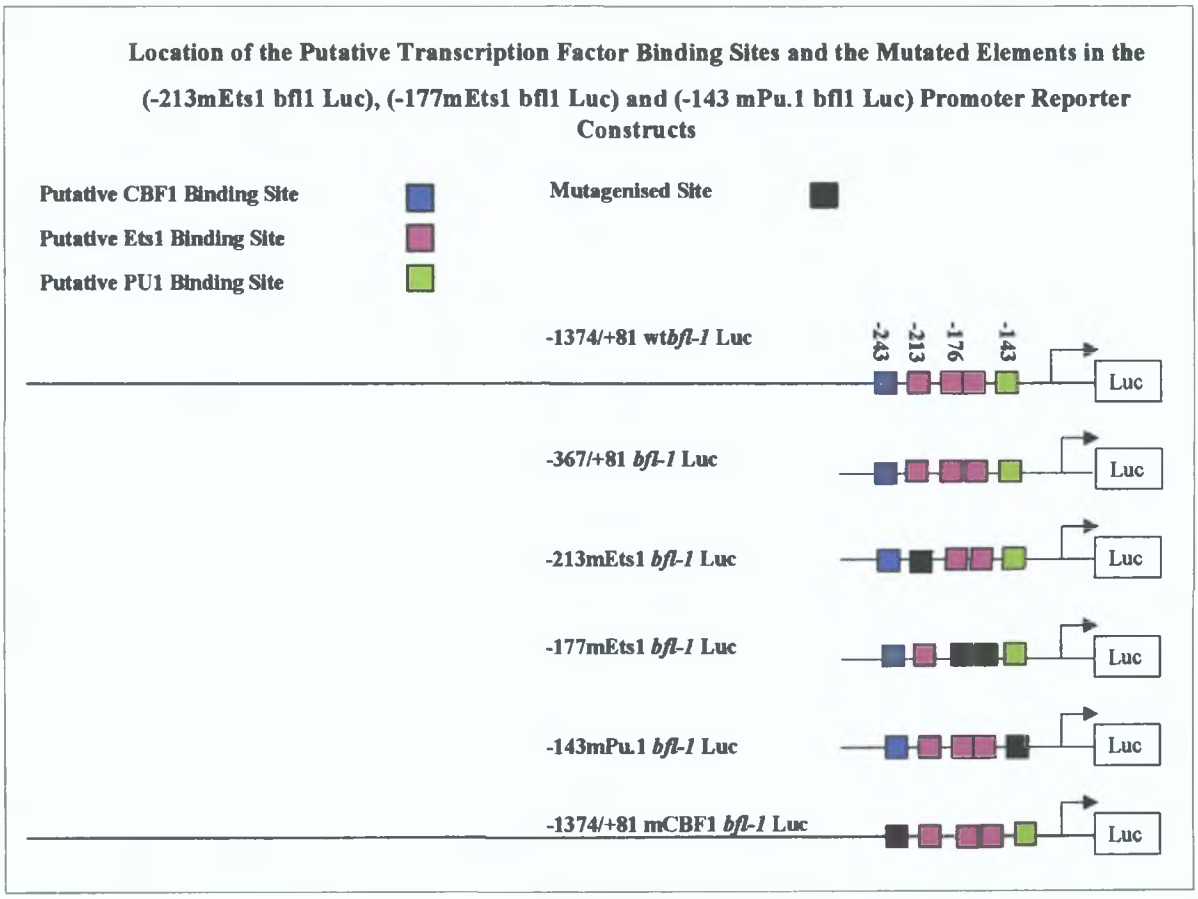


Figure 3.43. Schematic indicating the details of the mutated promoter constructs. The blue boxes represents the putative CBF1 binding site. The pink boxes represent possible Ets1 binding sites and the green boxes represent putative PU.1 binding sites. The positions of theses sites on the bfl-1 promoter is shown by the numbers above each of the respective boxes. The black boxes represent sites which have been abolished by mutagenesis.

Each of these promoters was cotransfected with either pSG5EBNA2 or pSG5EBNA2WW323SR in DG75 cells. The transfections were performed in the usual way by the DEAE dextran method in the optimised ratio of 1ug of promoter reporter construct and 7ug of EBNA2 expression plasmid (pSG5EBNA2). Trans-activation of the promoter was assessed using the luciferase assay. It can be seen from Figure 3.44 that

individually these Ets-1 and the PU 1 binding sites are crucial to EBNA2 mediated trans-activation of this portion of the bfl-1promoter

3.2.13 2. Mutation of the Putative Ets-1 and PU.1 Binding Sites Reduces EBNA2 Mediated Trans-activation of the *bfl-1* Promoter

It can be seen from Figure 3 44 that individual mutation of any of the transcription factor binding sites identified, results in a dramatic reduction in EBNA2 mediated trans-activation of the *bfl-1* promoter When the CBF1 binding site at –243 to –249 is destroyed, EBNA2 trans-activation drops from 6 fold down to less than two fold This demonstrates the importance of the CBF1 binding site for facilitating EBNA2 trans-activation of *bfl-1* However, when any of the Ets-1 or PU 1 binding sites are destroyed EBNA2 mediated trans-activation of *bfl-1* is also down-regulated Because these mutations were carried out in the truncated promoter construct –367/+81bfl1 Luc (See Figure 3 20), any trans-activation data has to be considered relative to trans-activation recorded in the –367/+81bfl1 Luc promoter and not the longer (-1374/+81 wtbfl1 Luc) promoter this was simply included here as a control for EBNA2 activity m the transfections EBNA2 trans-activated the truncated –367/+81bfl-1 Luc promoter an average of 5 5 fold Mutation of the putative Ets-1 binding site at –213 results in a decrease in fold transactivation of the promoter (by EBNA2) from 5 5 fold down to less than 3 fold This result suggests the Ets-1 site at position –213 on the *bfl-1* promoter is required for optimal EBNA2 mediated trans-activation of *bfl-1* Mutation of the double-Ets-1 transcription factor binding site at position –168 to –177 is more effective in further reducing EBNA2 driven trans-activation of the promoter, with fold activation for the full promoter falling from 5 5 fold to less than 2 fold after mutagenesis Levels of trans-activation are similar to those observed when the CBF1 binding site is obliterated It can be seen from this result that the transcription factor binding at this site (if there is one), either alone or in concert with the other transcription factors which bind to the promoter including CBF1, are required for EBNA2 responsivity in the bfl-1 promoter Similar results were obtained when the putative PU 1 binding site was mutated This resulted in a drop in EBNA2 mediated trans-activation of the promoter from 5 5 fold to below 2 fold

In total, the ensemble of these results show that these sites are crucial in facilitating EBNA2 mediated trans-activation of *bfl-1* One possibility is that these Ets family transcription factors complex with CBF1 itself to stabilize the EBNA2-CBF1 binding reaction, or conversely they may interact with the CBF1 repression complex thereby freeing CBF1 to bind EBNA2 and thus trans-activate the promoter Another possibility is that these transcription factor complexes interact with one and other and their action in concert with CBF1 is required for EBNA2 mediated transactivation of *bfl1* At present the mechanism of interaction of these sets of transcription factors is unknown, although their combined action is required to trans-activate another EBNA2 responsive promoter, LMP/TP2 (Johannsen *et al*, 1995)

Figure 3.44. The Putative Ets1 and Pu.1 Transcription Factor Binding Sites on the *bfl1* Promoter are Essential for EBNA2 Mediated Trans-activation of the *bfl1* Promoter in the DG75 Cell Line.

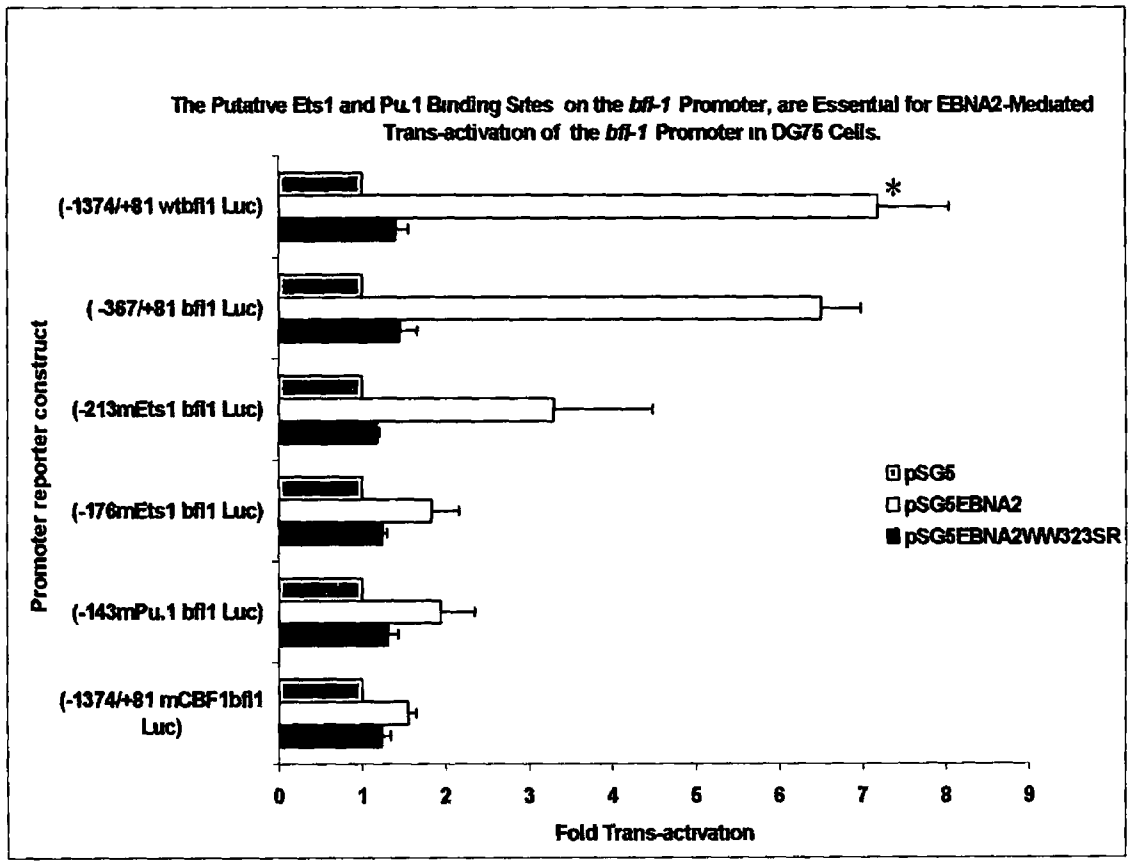


Figure 3 44. Putative Ets1 and Pu.1 sites are critical for EBNA2 mediated trans-activation of the bfl-1 promoter in DG75 cells. Transfections were carried out using the DEAE dextran method In each case 1ug of promoter reporter was co-transfected with the standard 7ug of expression plasmid Quantities of DNA transfected were normalized in each transfection by addition of pSG5 DNA Promoter activities were measured using the luciferase assay Luciferase values were equilibrated using the B gal assay in the usual manner * Transfections with the other -1374/+81 bfl1 promoter reporter construct (-1374/+81 bfl1 Luc) were also undertaken (not shown) and the level of trans-activation recorded was similar to that shown for the (1374/+81 wtbfl1 Luc) promoter reporter construct which is paired to the CBF1 mutant bfl1 reporter construct (-1374/+81 mCBF1bfl1 Luc).

It was also proposed to mutate all combinations of the possible Ets-1 and PU 1 binding sites where possible binding sites were knocked out together rather than just singly, however this was not undertaken as the dramatic effect of single mutation amply demonstrated the importance of each of the putative transcription factor binding sites

3.2 13.3. Further Evidence for the Importance of Ets-1 in EBNA2 Mediated Trans-activation of the *bfl-1* Promoter

If these Ets1 binding sites do play a part in conferring EBNA2 responsiveness to the *bfl-1* promoter, co-transfection of EBNA2 with an expression vector, which expresses a dominant negative form of Ets-1, would down-regulate EBNA2 trans-activation of the promoter To test this theory a dominant negative expression vector for Ets-1 (pCEP4-Ets-ND as described by (Nakada *et al*, 1999, Kim *et al*, 2000) was obtained, (H Sato Dept of Molecular Virology and Oncology Cancer Research Institute Kanazawa University) Also obtained was the empty pCEP4 (Invitrogen) vector into which the Ets-1 expression vector had been cloned The pSG5EBNA2 expression plasmid and various quantities of the Ets-1 dominant negative expression plasmid (pCep4eEtsDN) were co-transfected into DG75 cells and the resulting *bfl-1* promoter (-1374/+81 bfl1 Luc and –367/+81 bfl1 Luc) activity recorded in the usual way using the luciferase assay In all cases, the optimized ratio of 1ug of promoter reporter DNA to 7ug of EBNA2 expression plasmid was used In these co-transfection experiments the effect of the dominant negative expression plasmid on EBNA2 mediated trans-activation of the *bfl-1* promoter was assayed against both the–1374/+81bfl-1luc and the truncated promoter –367bfl-1luc reporter constructs respectively It can be seen from the results (Figure 3 45) that EBNA2 efficiently trans-activates both the full length and truncated promoter in the absence of the Ets dominant negative The promoters are trans-activated 5 fold and 3 9 fold respectively Addition of the dominant negative reduced EBNA2 mediated trans-activation of both promoters This reduction in trans-activation becomes more and more apparent as the quantity of dominant negative inputted is increased Trans-activation drops to 1 8 fold with respect to the longer promoter reporter over the range of 1ug to 7ug of dominant negative inputted Trans-activation of the truncated promoter also drops to 1 63 fold over a 1 to 5ug range of dominant negative inputted Strangely however, at the final point in each graph where 10ug of dominant negative was inputted, trans-activation of the promoters increased back up to 2 8 and 3 2 fold for the longer and truncated promoter respectively The quantity of DNA transfected was equalized in all cases by addition of the empty pCEP4 vector so this does not explain the result The results shown

are average values obtained from two independent experiments, but the trend of increasing trans-activation at the final time-point was consistent in all transfections and for both promoters

Figure 3.45.Transfection with an Ets1 Dominant Negative Confirms the Importance of Ets1 in Conferring EBNA2 Responsivity on the *bfl-1* Promoter.

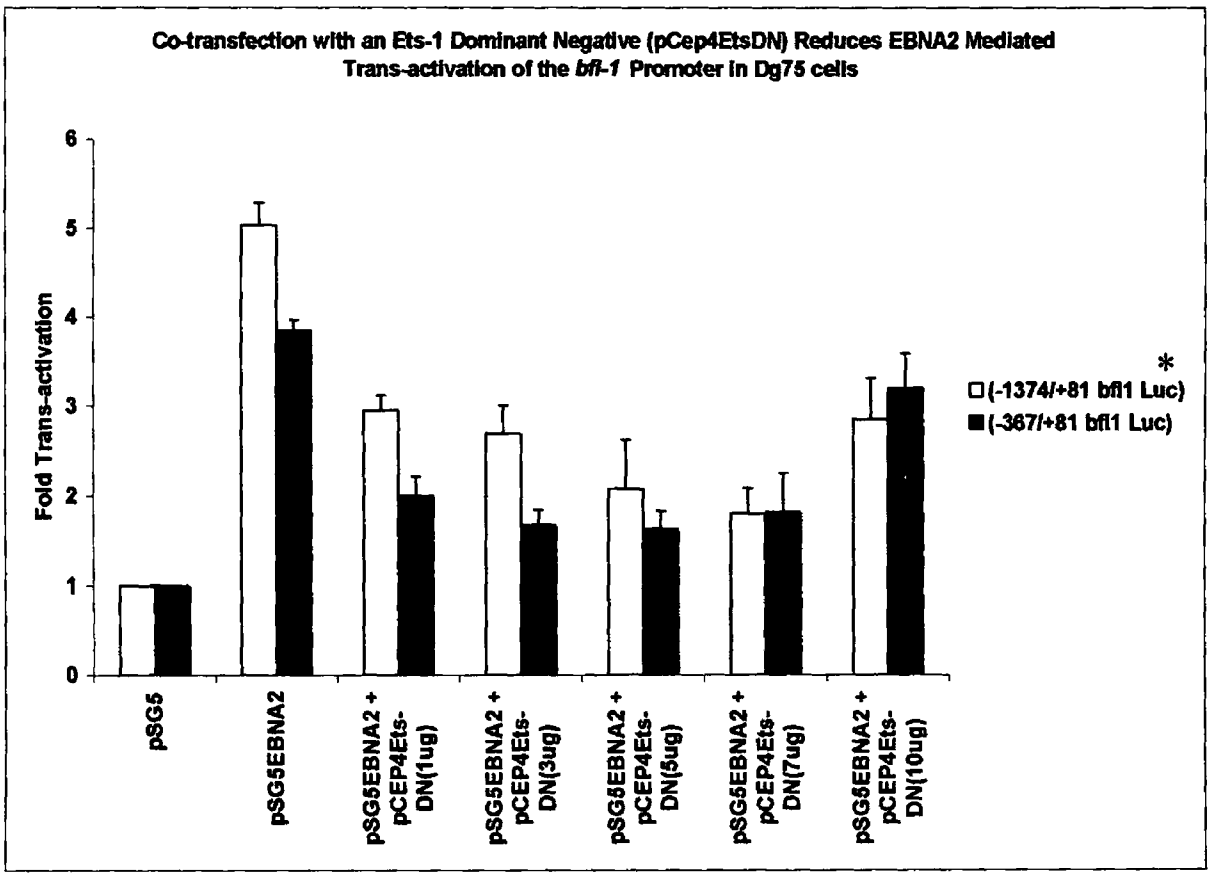


Figure 3.45. Transfection with an Ets1 Dominant Negative Confirms the Importance of Ets1 in conferring EBNA2 Responsivity on the *bfl-1* Promoter The Ets-1 dominant negative expression plasmid was titrated against the full length (-1374/+81bfl-1luc) and truncated promoter (-367/+81bfl-1) in co-transfection experiments Transfections were carried out in DG75 cells. The optimized ratio of 1ug of promoter reporter construct to 7ug of EBNA2 expression plasmid (pSG5EBNA2) was inputted into DG75 cells Various quantities of the Ets-1 dominant negative expression plasmid (pCEP4EtsDN) were used in the co-transfections, the amount of DNA transfected in each case is indicated below the x axis in the graph above * In this case trans-activation for the original *bfl1* promoter reporter construct (-1374/+81 bfl1 Luc)

is shown, again similar levels of trans-activation were seen for the (-1374/+81 wtbfl1 Luc) promoter reporter construct (not shown)

The fact that the dominant negative form of the Ets-1 expression plasmid reduces EBNA2 mediated trans-activation of the *bfl-1* promoter under the conditions tested is an indication that these sites and the binding of the relevant transcription factors are important features in conferring EBNA2 responsivity to the *bfl-1* promoter

3 2.14.0. The *bfl-1* Promoter is Not Trans-activated by Mouse Notch1-IC in BL-Derived Cell Lines.

EBNA2 is tethered to promoters by interacting with the cellular repressor CBF1 (Henkel *et al*, 1994, Grossman *et al*, 1994, Waltzer *et al*, 1994, Zimber-Strobl *et al*, 1994) This resembles the physiological activation of CBF1-repressed promoters by activated Notch receptors (Notch-IC) Since EBNA2 and Notch-IC have been shown to be partially interchangeable in regard to activation of target genes in B cell lines via CBF1 (Hofelmayr *et al*, 1999, Hofelmayr *et al*, 2001, Strobl *et al*, 2000, Gordadze *et al*, 2001), it was essential to investigate possible Notch responsivity of the *bfl-1* promoter in the context of B cell lines To this end, two mouse Notch1-IC expression plasmids were obtained (from Bettina Kempkes Laboratory of Molecular biology and Tumour Genetics, GSF National Research Centre for Environment and Health Munich Germany The vectors designated the names ED1 (an expression plasmid for mouse Notch1-IC), and ED4 (an expression plasmid for mouse NOTCH1-IC in which the RAM domain had been removed) were used in various transient transfection assays to determine if Notch1-IC could activate *bfl-1* promoter activity (See Appendix for plasmid maps) The removal of the RAM domain is important in that it contains a high-affinity binding site for transcription factors of the CSL group including CBF1 (Tamura *et al*, 1995) (See Figure 1 8 for schematic of Notch receptor) Also obtained was the "empty" vector for the Notch expression constructs pHACS1 into which the two Notch-IC sequences had been cloned It was sufficient to obtain plasmids expressing the Notch intracytoplasmic (Notch-IC) domain since several studies have demonstrated that truncated Notch molecules harboring only the IC domain behave as constitutive active forms of Notch (Fortini et al, 1993, Struhl and Adachi 1998) Thus expression of Notch-IC should result in effects comparable to those induced after activation of Notch by binding of one of it ligands.

Initially, EBNA2 and mouse NOTCH1-IC expression plasmids (pSG5 and pED1 respectively) were co-transfected with the pGa50-7 and pGa981-16 vectors (described in Figure 3 29) as a control to test for the functionality of both EBNA2 and mouse NOTCH1-IC (Figure3 46), in DG75 cells Both EBNA2 and NotchIC expression

plasmids (pSG5EBNA2 and pED1 respectively) were seen to trans-activate the synthetic pGa981-16 vector (relative to fold activation by their respective background vectors pSG5 and pHACS1 respectively) Thus both these constructs produce functionally active EBNA2 and Notch proteins Seven micrograms of the pSG5 EBNA2 expression plasmid was co-transfected with 1ug of the pGa981-16 promoter reporter plasmid and it can be seen that luciferase activity associated with the pGa9816 promoter reporter was increased 9,700 fold compared to transfection with the pSG5 vector alone Similarly, transfection with the mouse NOTCH1-IC expression plasmid pED1 (7ug) up-regulated promoter activity in the p981-16 vector by 10,000 fold compared to the control vector pHACS1 Transfection with 7ug of the ED4 expression plasmid resulted in an average 27 fold trans-activation of the multimerised promoter in the pGa981-16 vector Although this is a relatively high basal level of trans-activation by the RAM-deleted Notch-IC expression plasmid, the values are not significant when compared to the 10,000 fold trans-activation with Notch-IC expression plasmid The high level of background trans-activation, may be explained by the fact that another region of the activated Notch receptor, the ankyrin repeat region which is still intact in the ED4 expression plasmid has also been shown to be important for interaction between CBF1 and Notch-IC and may be enhancing trans-activation of the pGa981-6 reporter construct No trans-activation of the pGa50-7 reporter was reported relative to trans-activation by the empty pHACS1 or pSG5 vectors

Figure 3.46A. Both EBNA2 and Notch-IC are Functional in this Transient Transfection Assay and Trans-activate the pGa981-6 Reporter Construct in the Dg75 Cell Line.

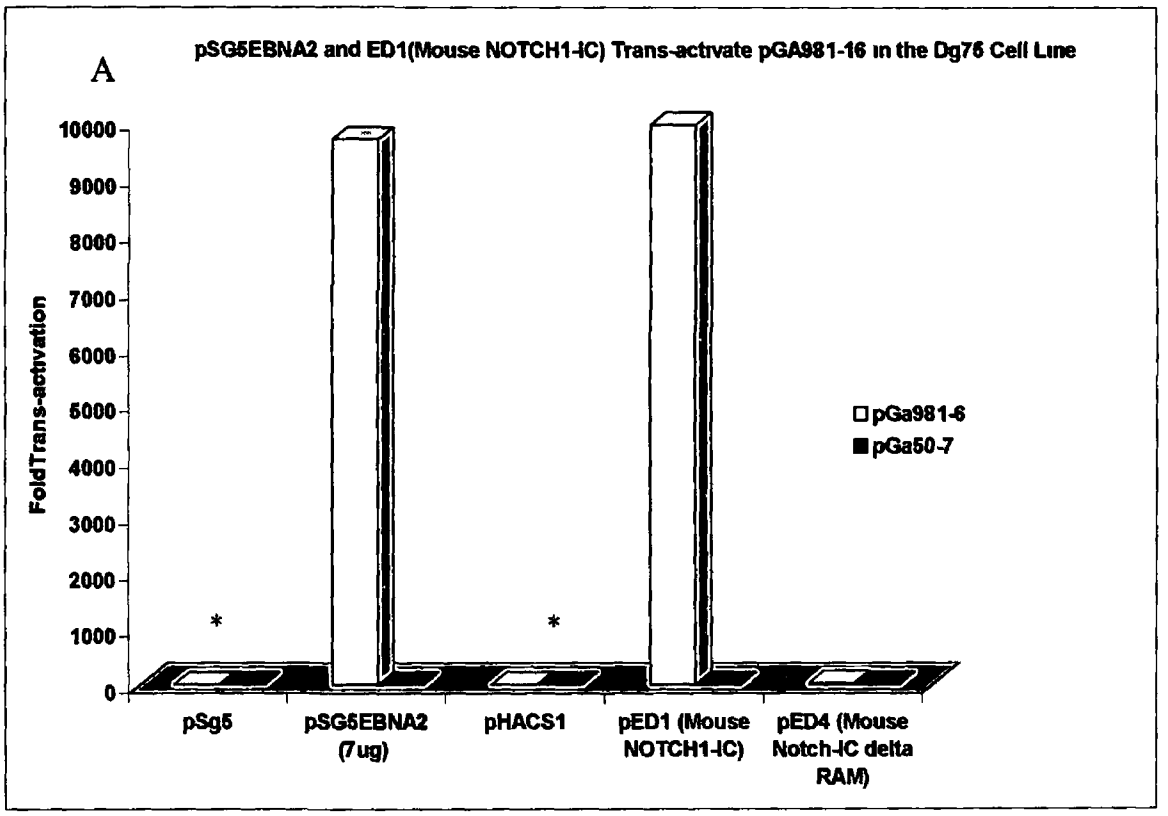


Figure 3.46(A). Both EBNA2 and Notch-IC are functional in this Transient Transfection Assay and Trans-activate the pGa981-6 Reporter Construct in the Dg75 Cell Line. Transfections were carried out in the DG75 cell line using the Deae dextran method As a control to assess the functionality of the expression vectors in the experiment, 7ug of pSG5EBNA2 and pED1 (Notch-IC) were cotransfected with 1ug of the pGa981-16 and pGa50-7 reporter constructs Reporter activity was measured using the luciferase assay and results were normalised as usual using Beta galactosidase data Total quantities of DNA transfected were equalised using the pSG5 or pHACS1 vectors as required. * Again fold activation by the pSG5 and pHACS1 vectors for each of the promoters was set to 1, fold activation recorded for each of the promoters was then relative to the appropriate background vector (pSG5 for EBNA2 expression lasmids and pHACS1 for NotchIC expression plsmids)

The ability of pSG5EBNA2 and pED1 to trans-activate the pGa981-16 promoter showed that both EBNA2 and Notch1-IC were functional in this experiment In this same experiment, the pSG5EBNA2 and pED1 mouse-Notch1-IC expression plasmids were co-transfected with the *bfl-1* promoter (-1374/+81 wtbfl1 Luc) and the CBF1mutant version

of the *bfl-1* promoter (-1374/+81 mCBF1 bfl1 Luc) These are shown on different graphs as the scale of trans-activation of the *bf-l1* promoter reporter constructs and the control plasmid pGa981-6 are vastly different

Figure 3.46(B). Mouse Notch-IC (pED1) Does Not Trans-activate the *bfl-1* Promoter Over a Range of Quantities Used.

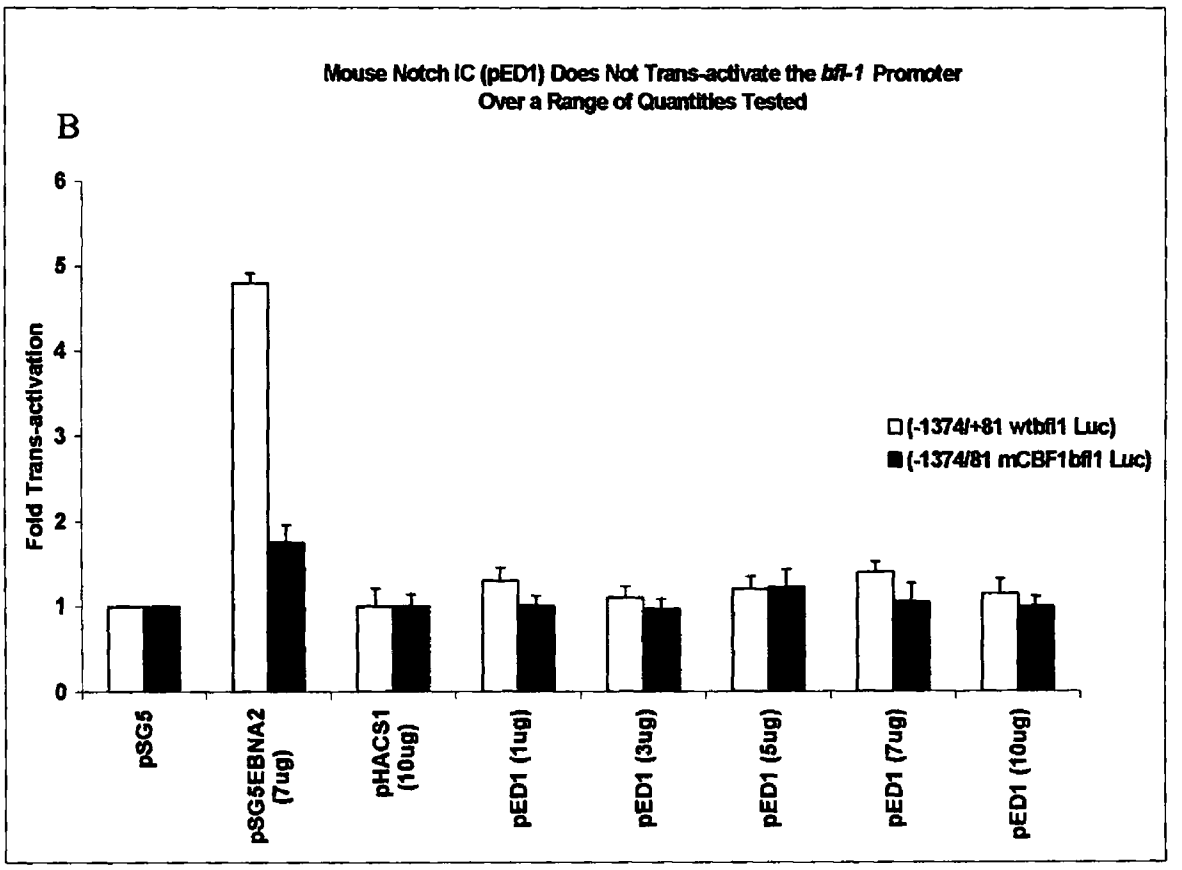


Figure 3.46(B) Mouse Notch-IC (pED1) Does Not Trans-activate the *bfl-1* Promoter Over a Range of Quantities Used In the same experiments as 3 46A above, the mouse notch expression plasmid pED1 was titrated against the *bfl-1* promoter reporter constructs (-1374/+81 wtbfl1 Luc) and (-1374/+81 mCBF1bfl1 Luc) Although both EBNA2 and Notch-IC are functionally active as demonstrated by their ability to trans-activate the pGa981-6 reporter construct (3 46A), only pSG5EBNA2 trans-activated the *bfl-1* promoter The Notch1-IC expression plasmid (pED1) failed to trans-activate either *bfl-1* promoter over the range of quantities used in this experiment.

Although both EBNA2 and Notch1-IC could trans-activate the CBF1 driven promoter in pGa981-6, only EBNA2 is seen to trans-activate the *bfl-1* promoter (Figure 3 46B)

EBNA2 trans-activated the *bfl-1* promoter (-1374/+81 wtbfl1 Luc) an average of 4 8 fold, however when increasing quantities of vector expressing mouse Notch-IC (pED1) was titrated against the *bfl-1* promoter (Figure 3 46B) it did not trans-activate the *bfl-1* promoter at any quantity over the 1 to 10ug range of expression plasmid tested Both EBNA2 and mouse Notch1-IC trans-activated the mutated bfl-1 promoter very weakly

ED4 was also titrated against the wild type *bfl-1* promoter (-1374/+81 wtbfl1 Luc) It did not trans-activate the promoter over the range (1-10ug) of expression plasmid (pED4) used, indeed higher levels of this expression plasmid appeared to repress basal levels of promoter activity (Figure 3 47) Thus, it can be concluded that in DG75 cells, under these conditions, the bfl-1 promoter is EBNA2, but not mouse Notch-IC responsive

Figure 3.47. The RAM Deleted Notch-IC Expression Plasmid (pED4) Does Not Trans-activate the *bfl-1* Promoter over a Range of Quantities Used, in the Dg75 Cell Line.

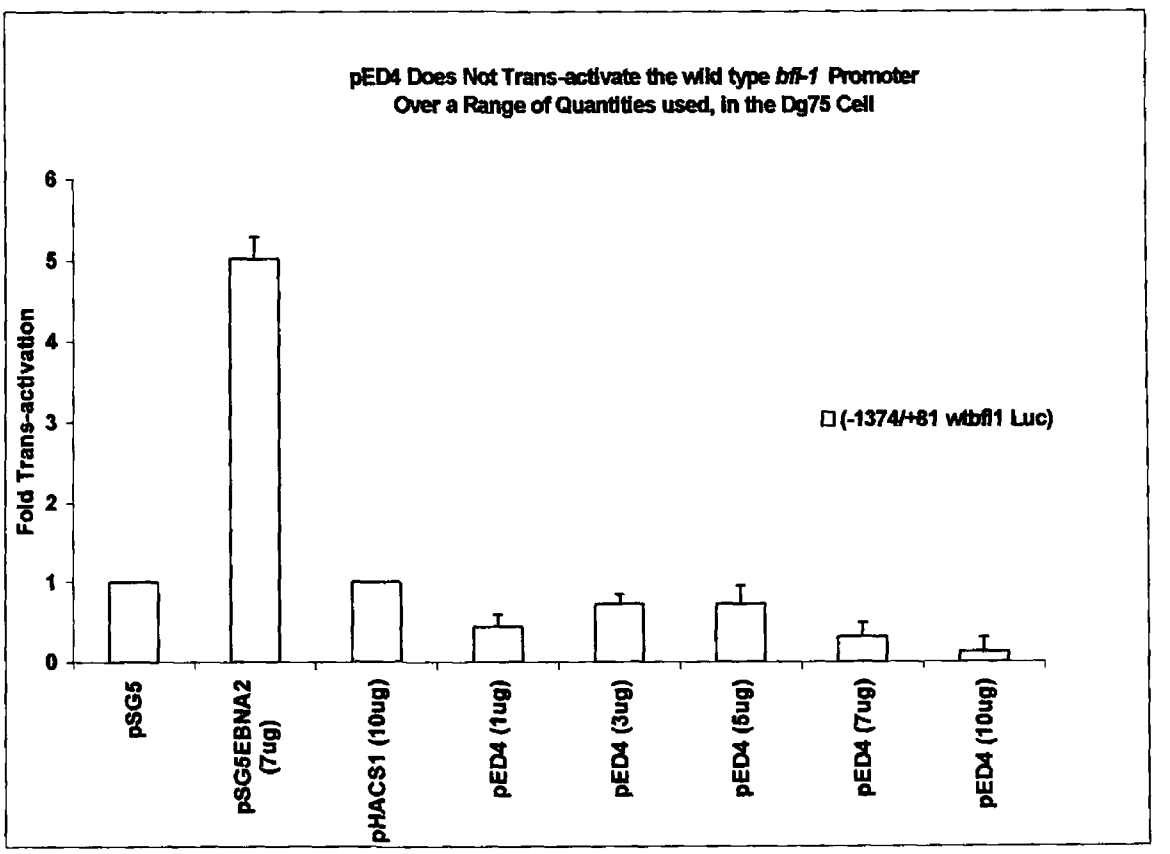


Figure 3 47 The RAM Deleted Notch-IC Expression Plasmid (pED4) Does Not Trans-activate the *bfl-1* Promoter over a Range of Quantities Used, in the Dg75 Cell Line. In the same manner as for pED1 before (Figure 3 46B), ED4 was titrated against the bfl-1 promoter 9-1374/+81 wtbfl1 Luc) pED4 did not trans-activate the promoter over the range of concentrations tested It is unsurprising that this expression plasmid did not trans-activate the promoter since the CBF1-binding RAM domain has been removed

In order to extend the analysis of *bfl-1* responsivity to mouse NOTCH1-IC in EBV negative BL cell lines, further transfections were carried out in another well-characterised EBV negative BL cell line, BJAB (Figure 3 48) The optimised ratio of expression plasmid (7ug) (pEd1/pED4/pSG5EBNA2) to 1ug of promoter reporter construct (pGa50-7/pGa981-6/-1374/+81 wtbfl1 Luc/ -1374/+81 mCBF1bfl1 Luc) was employed here as before Transfection of the pSG5EBNA2 expression plasmid activated promoter activity in the pGa981-16 construct an average of 49 fold over the course of three independent experiments (relative to fold transactivation by the pSG5 vector) In the same experiments, the Notch-IC expression plasmid pED1 trans-activated the pGa981-16 promoter reporter construct an average of 455 fold (relative to the pHACS1 vector) Neither pED4 nor the mutant pSG5EBNA2 expression plasmid (pSG5WW323SR, which cannot bind CBF1), trans-activated the pGa981-16 promoter in the Bjab cell line None of the expression constructs trans-activated the control pGa50-7 construct

Figure 3.48.(A). EBNA2 and mouseNotch-IC are Functionally Active and Trans-activate the pGa981-6 Promoter Reporter in the Bjab Cell Line.

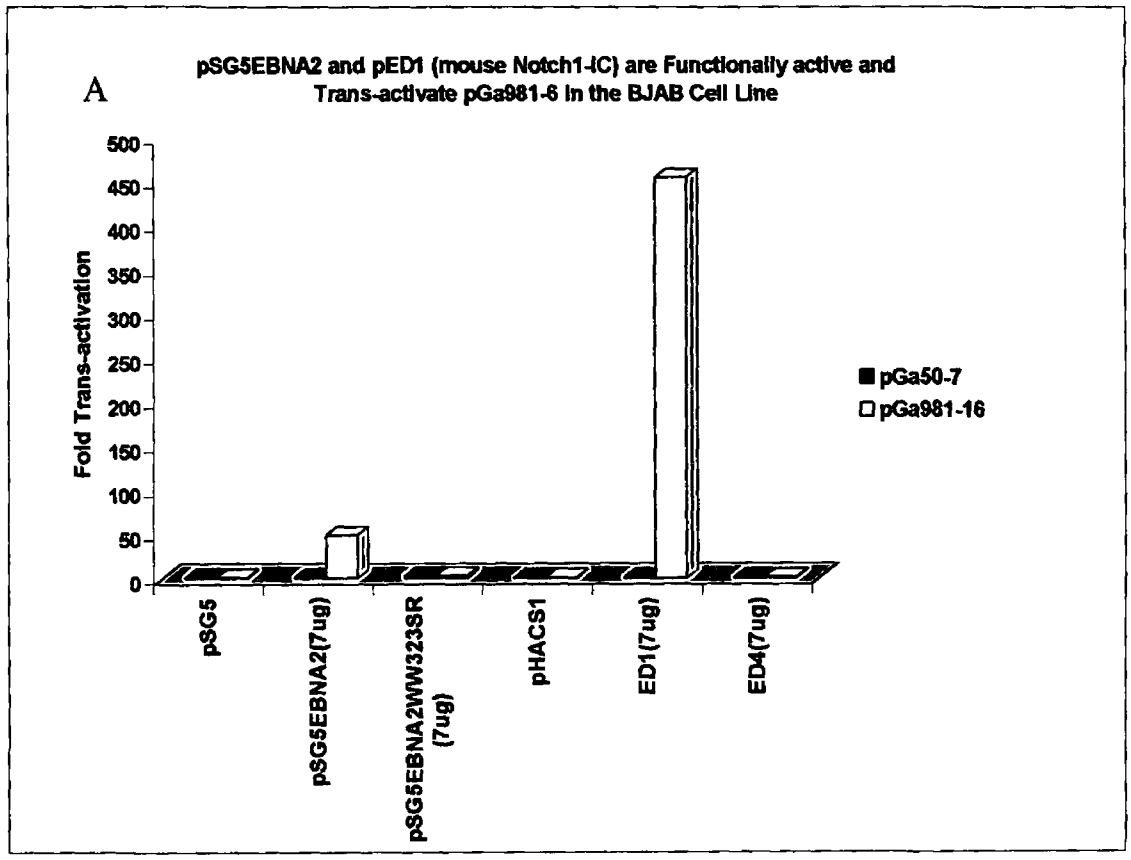


Figure 3.48 (A) EBNA2 and mouseNotch-IC are Functionally Active and Trans-activate the pGa981-6 Promoter Reporter in the Bjab Cell Line. Similar to Figure 3 46A, the functionality of the EBNA2 and NOTCh1-IC expression plasmids (pSG5EBNA2 and pED1 respectively) was checked by co-transfection with the pGa981-16 and pGa50-7 reporter constructs This experiment was carried out in the usual manner with transfection done by the DEAE Dextran method however these transfections were carried out in the Bjab cell line In this case again, both EBNA2 and NOTCH1-IC can trans-activate the multimerised promoter in pGA981-16 thus both pSG5EBNA2 and pED1 give rise to functionally active EBNA2 and NotchIC which can trans-activate the CBF1 driven pGA981-6 reporter construct

Despite both EBNA2 and NotchIC being functionally active as demonstrated by their ability to transactivate the pGA981-6 reporter construct, in the same experiment only EBNA2 could trans-activate the bfl-1 promoter in the Bjab cell line (Figure 3 48 B) As for Figures 3 46 A and B above the two lots of transfections (although done

simultaneously) are shown on separate graphs as the scale of trans-activation of the *bfl-1* promoter (-1374/+81 wtbfl1 Luc) and pGa981-6 promoters are so vastly different (Figures 3 48 A and 3 48 B) With respect to *bfl-1*, (-1374/+81 wtbfl1 Luc) it can be seen that only EBNA2 trans-activated the promoter (Figure 3 48B) In this instance trans-activation reached an average of about 4 fold compared to the empty vector pSG5 Neither pED1 nor pED4 trans-activated the *bfl-1* promoter (-1374/+81wtbfl1Luc) Possibly however titration of the Notch-IC expression plasmid (pED1) in this cell line may give different results No trans-activation above 1 5 fold was recorded with any of the expression plasmids when co-transfected with the *bfl-1* CBF1 mutant promoter (-1374/+81 mCBF1 bfl1 Luc)

Figure 3.48 B. The *bfl-1* Promoter (-1374/+81 wtbfl-1 Luc) is an EBNA2 but Not mouseNotch-IC Responsive Promoter in the Bjab Cell Line.

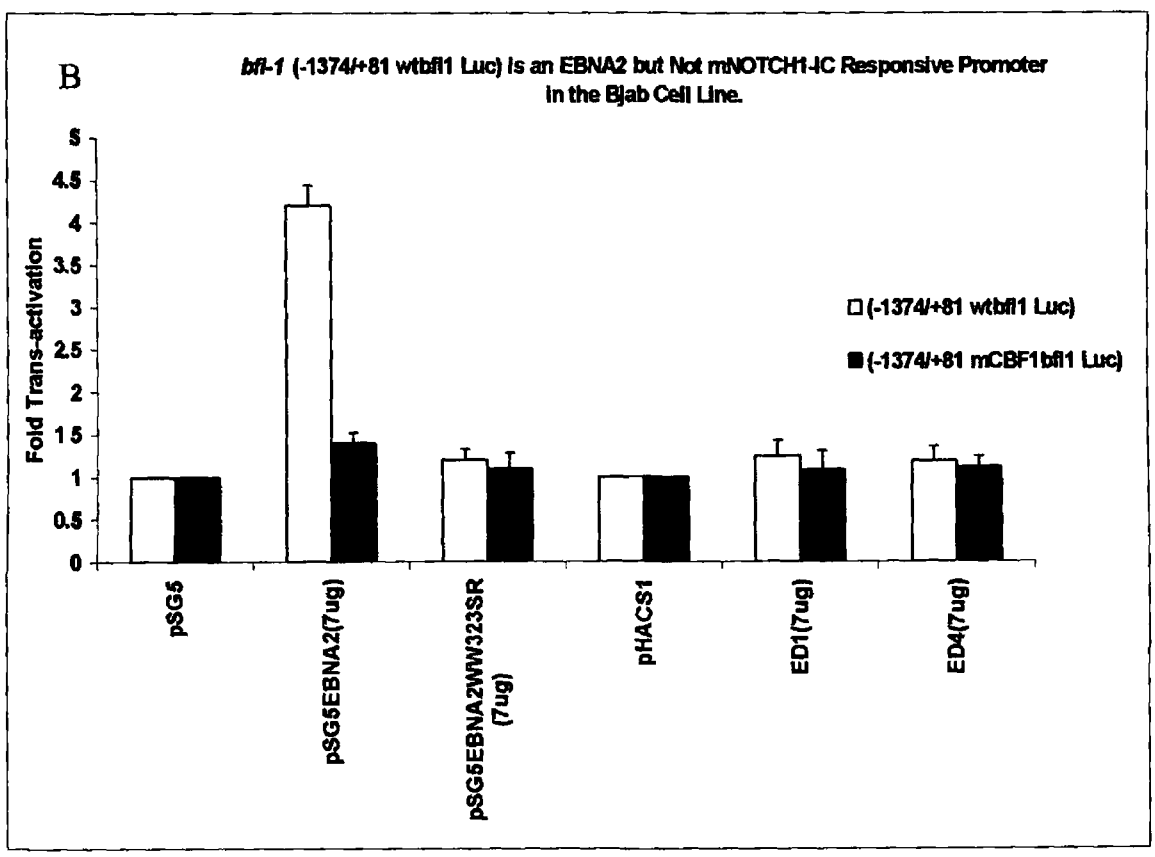


Figure 3.48 B. The bfl-1 Promoter (-1374/+81 wtbfl-1 Luc) is an EBNA2 but Not mouseNotch-IC Responsive Promoter in the Bjab Cell Line. In the same experiment as 3 48A above, the NotchIC and

Ram deleted Notch-IC expression plasmids (pED1 and pED4 respectively) were transfected with the wild type bfl1 promoter (-1374/+81 wtbfl1 Luc) and the CBF1 mutant bfl-1 promoter (-1374/+81 mCBF1bfl1 Luc) Despite the fact that Notch-IC appears to be functionally active in view of its ability to trans-activate the pGa981-6 reporter (3 48A), NotchIc does not trans-activate either bfl-1 promoter reporter constructs In the same experiment EBNA2 (psg5EBNA2) trans-activated the wild type *bfl-1* promoter (-1374/+81 wtbfl1 Luc) about 4 fold

These results in accordance with the results obtained in the DG75 cell line show that, like the CD23 promoter, *bfl-1* is responsive to EBNA2 but not NOTCHIC, at least in the BL-derived cell lines tested Since trans-activation of bfl-1 by LMP1 (D'Souza *et al*, 2000) and now EBNA2 appears to be a B cell specific effect, it is possible that the cell context is important in mediating possible *bfl-1* responsiveness to Notch-IC Further work could investigate this possibility using transient transfections with Notch-IC expression plasmids in a range of non B cell lines

3.2.15 0. Analysis of *bfl-1* Promoter Responsiveness in Cell Lines Expressing Estrogen-Activated EBNA2 or mouse Notch1IC

Transfections were also carried out in the BL41P3HR1mNotch-IC-ER (CL31) cell line in which (see materials and methods) mouse NOTCH-IC function is dependent on the presence of estrogen The two *bfl-1* promoter reporter constructs (-1374/+81wtbfl1 Luc and –1374/+81 mCBF1bfl1 Luc) and the two control reporter constructs (pGa50-7 and pGa981-16) were transfected into the (BL41P3HR1-ER/Notch-IC (CL31) cell line in the presence or absence of estrogen Thus the effect of activating mouse Notch1-IC on the various promoter constructs could be studied As shown in the figure below (Figure 3 49A) the promoter carrying the multimerized CBF1 binding sites (pGa981-6) could be trans-activated 250 fold after activation of the mNOTCH-IC-ER by addition of estrogen relative to transactivation prior to estrogen addition The negative control pGa50-7 promoter was trans-activated 31 fold in this cell line in response to estrogen addition This effect on the pGa50-7 reporter construct was not observed in either the K3 or 9A cell lines in response to estrogen addition and so it is not an artifact of the estrogen itself Since EBNA2 activation does not have this effect it may be a Notch specific effect on the minimal SV40 promoter in the pGa50-7 vector

These results (Figure 3 49A) indicate that the mNotch-IC-ER expressed in CL31 is functionally active and can be regulated by estrogen In the same experiment, the *bfl-1* promoter and its CBF1 mutant equivalent were also transiently transfected into the CL31 cell line (Figure 3 49B) The results showed no significant trans-activation of either the bfl-1 promoter (-1374/+81 wtbfl1 Luc) or its CBF1 mutated counterpart (-1374/+81 mCBF1bfl1 Luc) in the presence of estrogen suggesting that under these conditions *bfl-1* is not mNOTCH1-IC-responsive Again although these transfections were done simultaneously, the difference in the scale of activation of the bfl-1 promoter reporter constructs (-1374/+81 wtbfl1 Luc, -1374/+81 mCBF1bfl1 Luc) and the control reporter constructs (pGa981-6 and pGA50-7), meant the transfections for each pair of promoter reporter constructs were depicted on separate graphs

Figure 3.49A. Notch-IC is Functionally Active and Trans-activates the pGa981-6 Promoter Reporter Construct in the BL41P3HR1-ER/NotchIC (Cl31) Cell Line

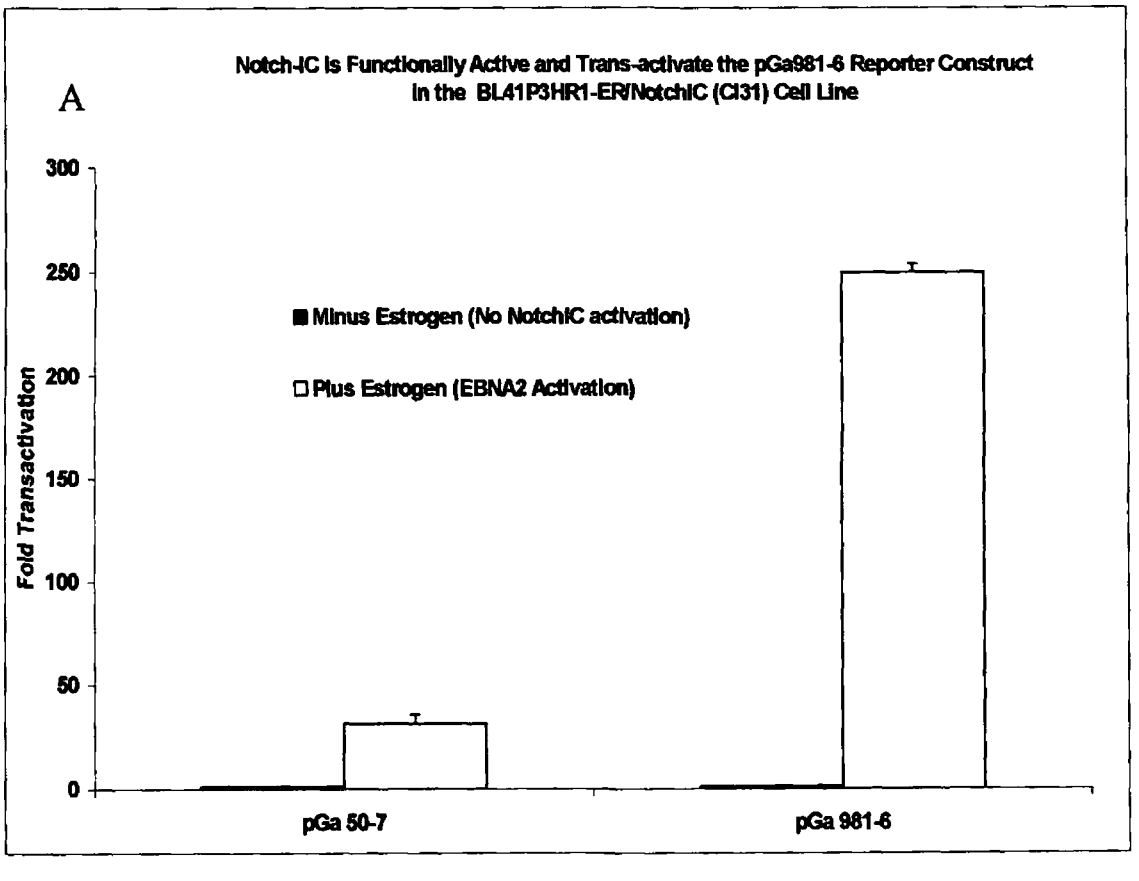


Figure 3 49A. Notch-IC is Functionally Active and Trans-activates the pGa981-6 Promoter Reporter Construct in the BL41P3HR1-ER/NotchIC (CL31) Cell Line. Transfections were carried out by electroporation Ten micrograms of promoter reporter DNA (pGa50-7/pGa981-6) were transfected into the CL31 cell line Promoter activity was measured before and after estrogen addition using the luciferase assay Transfection efficiency was corrected for using the β-gal assay In the case of each promoter pre estrogen luciferase values were assigned a fold transactivation of 1 and fold transactivation of each of the promoters in response to estrogen addition/(Notch-IC activation) were relative to this It can be seen that NotchIC activation trans-activates both pGa50-7 and pGa981-6 reporters in this experiment thus Notch-IC is functionally active One explanation as to why the "empty" pGa50-7 reporter is trans-activated may be that the Notch-IC or a downstream target of it trans-activate the minimal SV40 promoter in this construct.

Figure 3.49B Despite Notch-IC Being Functionally Active, the bfl-1 Promoter is not Notch-IC Responsive in the BL41P3HR1-ER/NotchIC (CL31 Cell Line).

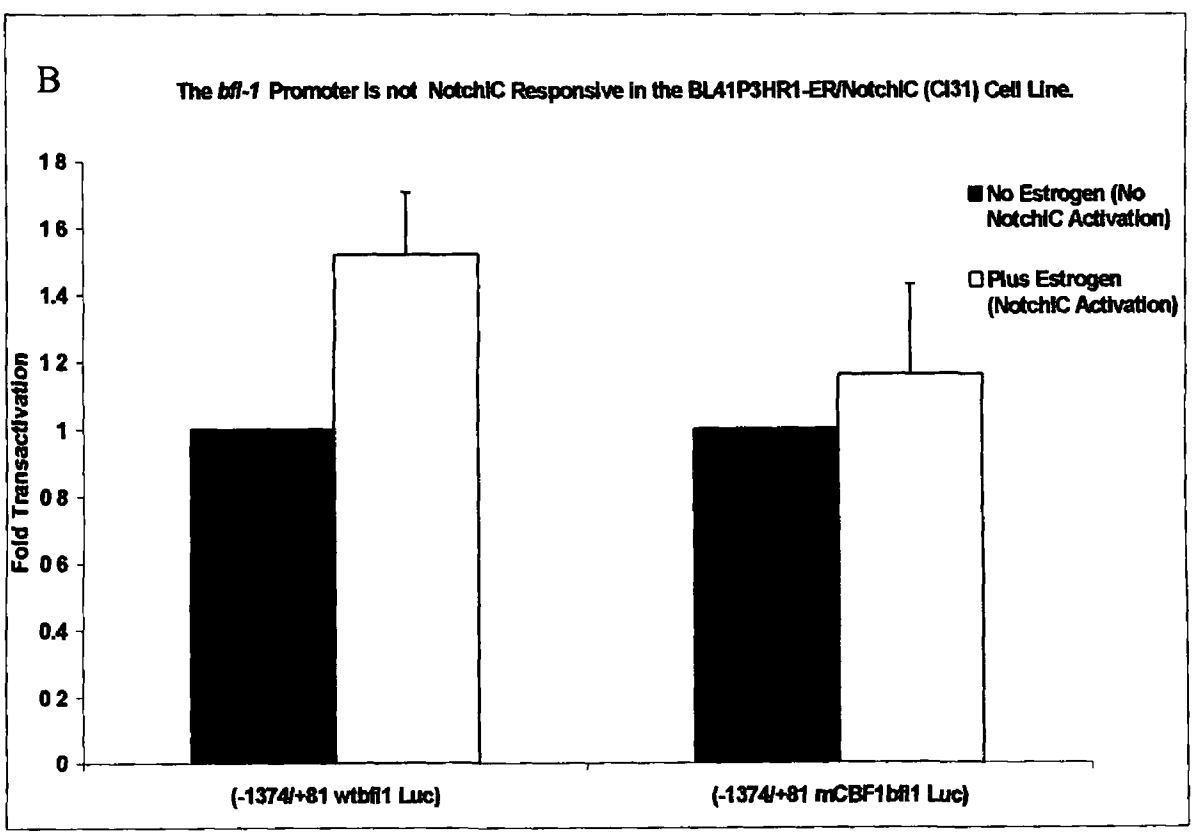


Figure 3.49B Despite Notch-IC Being Functionally Active, the *bfl-1* Promoter is not Notch-IC Responsive in the BL41P3HR1-ER/Notch-IC (CL31 Cell Line). Although Notch-IC is active as seen by its ability to transactivate the pGa981-6 reporter construct in 3 49A above, in the same experiment 3 49B it only weakly trans-activates the bfl-1 promoter reporter constructs (-1374/+81 wtbfl1 Luc and –1374/+81 mCBF1bfl1 Luc) in the CL31 Cell Line

3.2.16.0. Human NOTCH1-IC and Human NOTCH2-IC Do Not Trans-activate the *bfl-1* Promoter in B Cell Lines.

Human Notch1-IC and Notch2-IC expression plasmids were obtained (Paul Ling Baylor Medical school Baylor College Texas) The human NOTCH1-IC expression plasmid was named pJT111, similarly the human Notch2-IC expression plasmid was named pJT112 Briefly these consist of the human Notch1-IC cDNA and the Human Notch2-IC cDNA respectively, cloned into the EcoRI-BglII site of pSG5 upstream of the luciferase gene (Described by Gordadze *et al*, 2001) As before, EBNA2 and the Notch1-IC and Notch2-IC expression plasmids (pSG5EBNA2 and pJT111 and pJT112 respectively) were co-transfected in DG75 cells with the pGa50-7 and pGa981-16 vectors as a control to test for the functionality of EBNA2, humanNotch1-IC and humanNotch2-IC in DG75 cells Results are shown in Figure 3 50

Figure 3.50A. Human Notch1IC, (pJT111) Human Notch2Ic (pJT112) and EBNA2 (pSG5EBNA2) are Functionally Active in the DG75 Cell Line and Trans-activate the pGa981-6 Reporter Construct.

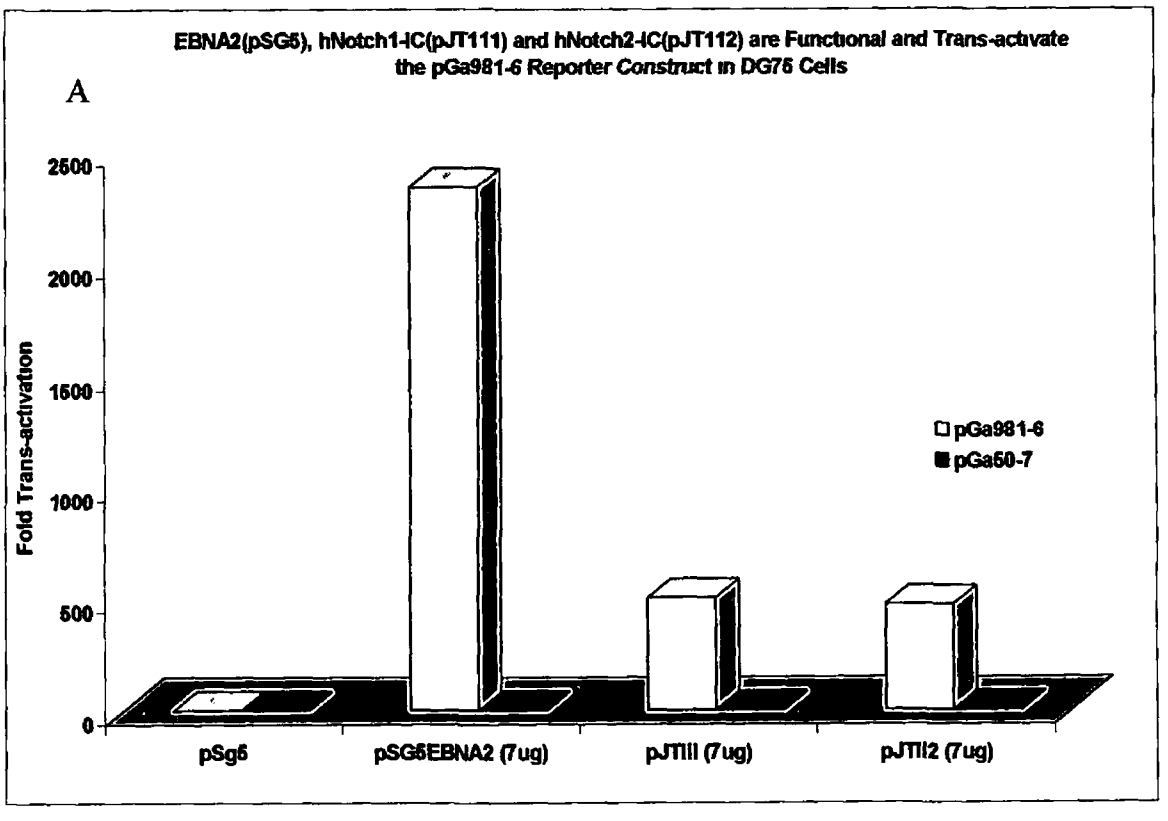


Figure 3.50A. Human Notch1-IC, (pJT111) Human Notch2-IC (pJT112) and EBNA2 are (pSG5EBNA2) Functionally Active in the DG75 Cell Line and Trans-activate the pGa981-6 Reporter Construct In order to test the functionality of the proteins expressed by the transfected expression plasmids, the human Notch1-IC and human Notch2-IC expression plasmids were cotransfected with the pGa981-16 and pGa50-7 reporter constructs This activity was compared to EBNA2 activity in the same experiment Transfections were carried out using the DEAE Dextran method and promoter activity was measured in the usual way using the luciferase assay again trans-activation by the pSG5 vector alone was assigned a fold activation of 1 for each of the promoter reporter constructs transfected Again Fold activation by the pSG5 related human notch1IC (pJT111), Notch2IC (pJT112) and EBNA2 (pSG5EBNA2) expression vectors were relative to this

In all cases 7ug of expression plasmid to 1ug of promoter reporter, was transfected into DG75 cells using the DEAE dextran method The results showed that the transiently transfected pJT111 transacivated the pGa981-16 promoter reporter vector (containing the multimerized CBF1 sites) an average of 505 fold relative to the empty pSG5 vector Promoter activity, again relative to the pSG5 vector was increased an average of 475 fold by transfection with the pJT112 expression plasmid In the same experiment EBNA2 (pSG5EBNa2) trans-activated the promoter in the pGa981-16 vector an average of 2341 fold These results show, that both human Notch1-IC and humanNotch2-IC as well as EBNA2 are functionally active in these experiments in the DG75 cell line

Knowing that both pJT111 and pJT112 were expressing functional forms of NOTCH 1 and 2 respectively, their effects on the *bfl-1* promoter were then investigated and so both pJT111 and pJT112 were titrated against the *bfl-1* promoter It can be seen from Figure 3 50 that HumanNotch1-IC did not trans-activate the *bfl-1* promoter (-1374/+81 wtbfl-1 Luc) In fact, transfection of the human Notch1-IC expression plasmid resulted in a reduction in basal trans-activation of the *bfl-1* promoter compared to co-transfection with pSG5 vector alone As a control for transfection efficiency pSG5EBNA2 was also transfected and, as before transfection with EBNA2 increased *bfl-1* promoter activity an average of 7 fold The same titration experiment was carried out using the pJT112 humanNOTCH2-IC expression plasmid Results from these transfections showed that *bfl-1* is not human Notch1-IC or human Notch2-IC responsive in this cell line since transfection with varying quantities of Notch2-IC/Notch1IC expression plasmid did not

result in any up-regulation of bfl-1 promoter activity (-1374/+81 wtbfl1 Luc) As for human Notch1, transfection with increasing quantities of human Notch2 expression plasmid resulted in a dose dependent reduction in basal levels of promoter activity (See Figures 3 50B and 3 50C) Here, promoter activity was reduced below basal trans-activation levels recorded for transfections involving the empty pSG5 vector alone

Taken together the results for the transfections in DG75, BL41 and Bjab cell lines show that although in each case the Notch expression plasmids transfected are producing functional proteins, they do not lead to promoter trans-activation Thus, in the BL cell lines examined, the *bfl-1* promoter is responsive to EBNA2 but not mouse Notch1-IC or human Notch1-IC or Notch2-IC

Figure 3.50B. Human Notch1-IC Does Not Transactivate the *bfl-1* Promoter in the DG75 Cell Line.

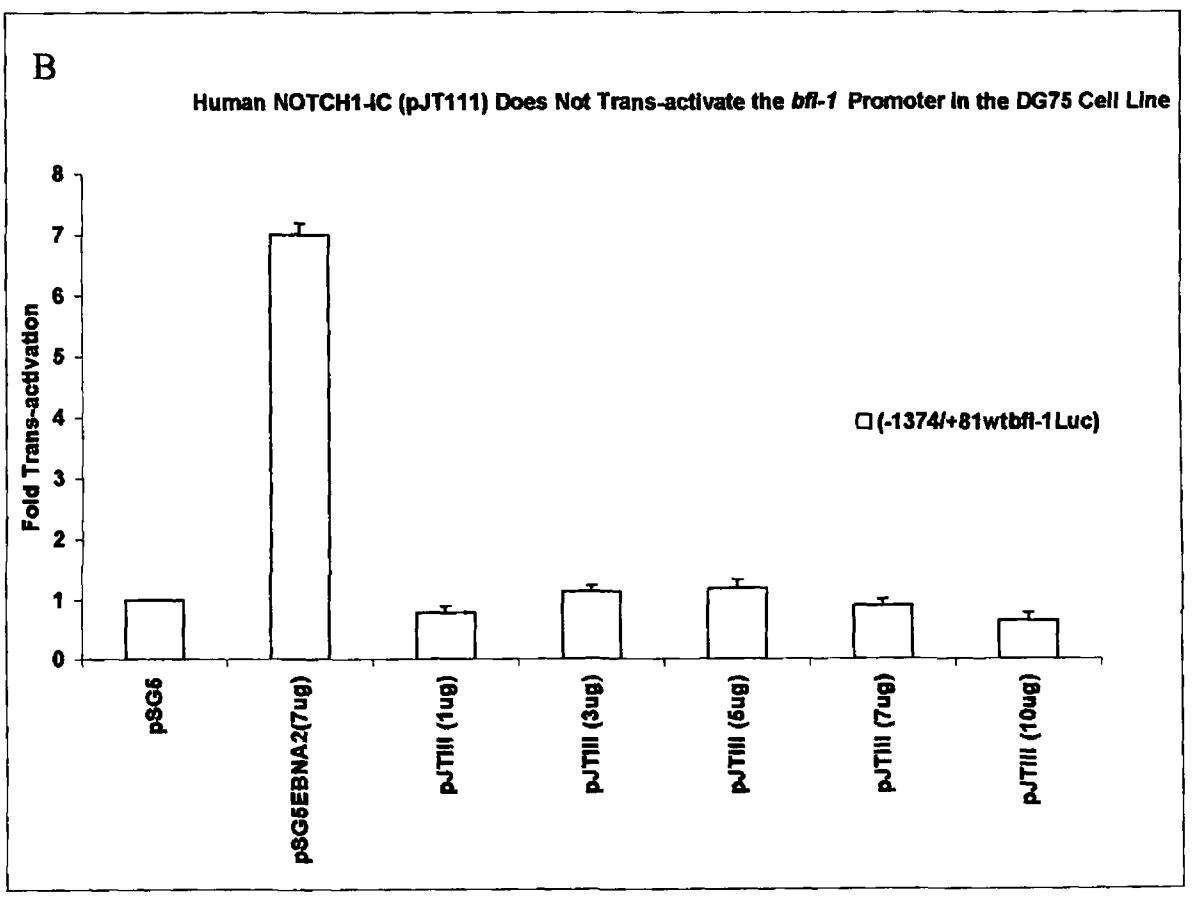


Figure 3.50B. Human Notch1-Ic Does Not Transactivate the *bfl-1* Promoter in the DG75 Cell Line. Although the human Notch1-IC produces functional Notch-IC in the DG75 cell line as demonstrated by its

ability to transactivate the pGa981-6 promoter reporter construct in 3 50A above, the bfl-1 promoter (-1374/+81 wtbfl1 Luc) is not transactivated in co-transfections with pJT111 in the DG75 cell line The human Notch1-IC expression plasmid (pJT111) was titrated against the *bfl-1* promoter over a range of concentrations however it failed to transactivate the bfl1 promoter (-1364/+81 wtbfl1) over the range of quantities used Thus *bfl-1* may be EBNA2 but not human Notch-IC responsive in the DG75 cell line

Figure 3.50C. Human Notch2-IC Does Not Transactivate the *bfl-1* Promoter in the DG75 Cell Line.

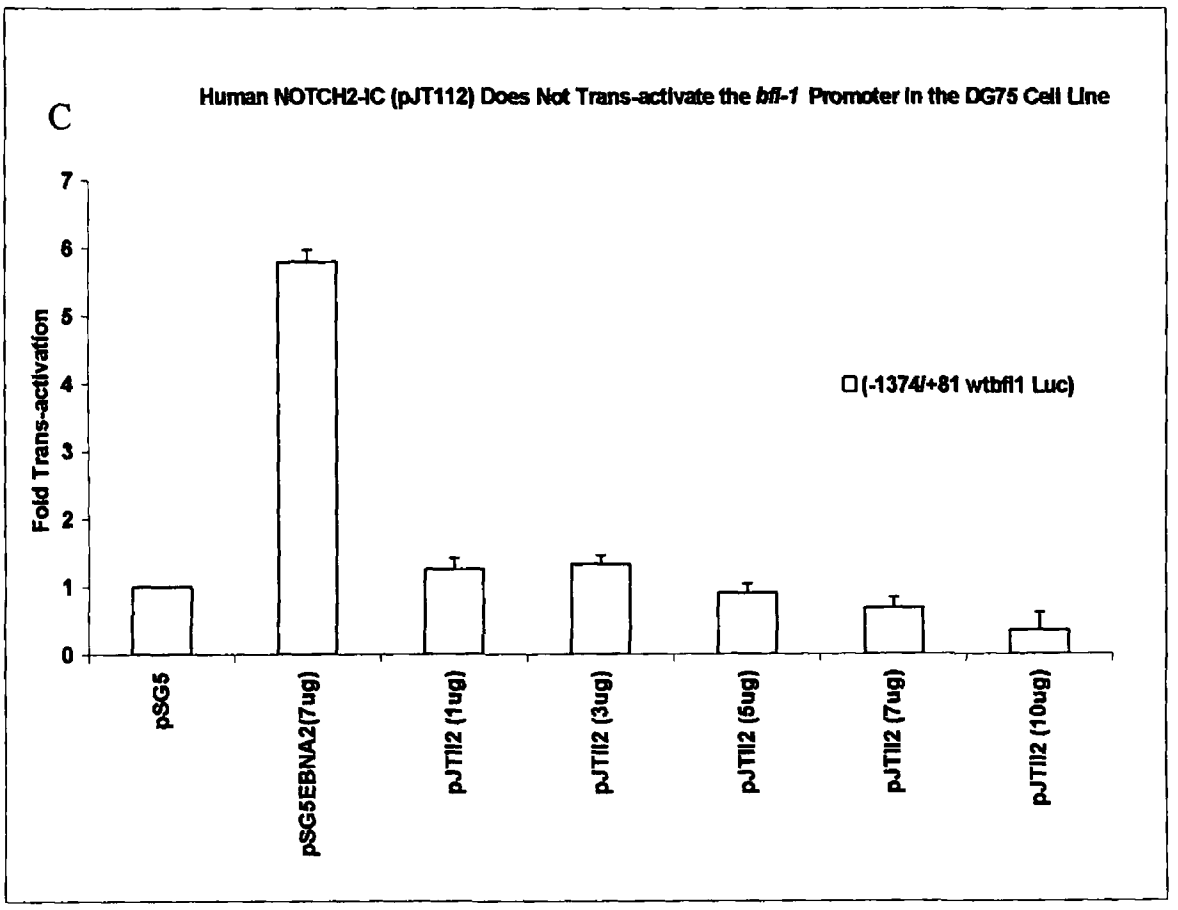


Figure 3.50C. Human Notch2-IC Does Not Transactivate the *bfl-1* Promoter in the DG75 Cell Line. Although the human Notch2-IC produces functional Notch2-IC in the DG75 cell line as demonstrated by its ability to transactivate the pGa981-6 promoter reporter construct in 3 50A above, the *bfl-1* promoter (-1374/+81 wtbfl1 Luc) is not trans-activated in co-transfections with pJT112 in the DG75 cell line The human Notch2-IC expression plasmid (pJT112) was titrated against the *bfl-1* promoter over a range of concentrations as for pJT111 (Figure 3 50B) Like human Notch1-IC, human Notch2-IC also failed to

trans-activate the *bfl-1* promoter (-1364/+81 wtbfl1) over the range of quantities used Thus *bfl-1* may be EBNA2 but not human Notch-IC or Notch2IC responsive in the DG75 cell line

3.2.15.1. Human Notch 2 and mouse Notch 1 Repress EBNA2-Mediated Trans-activation of *bfl-1*.

Since both EBNA2 and Notch-IC proteins share affinities for some of the same nuclear proteins including CBF1 it is possible that Notch is capable of interacting with CBF1 when the latter is bound to the *bfl-1* promoter, even though significant trans-activation is not detectable Its inability to trans-activate the promoter may indicate (i) that the Notch-IC trans-activation domain is much weaker than that of EBNA2, or (ii) that trans-activation via the CBF1 site requires interaction with other additional factors, something achieved by EBNA2 but not Notch-IC, or (iii) Notch may not bind to or have reduced affinity for the CBF1-corepressor complex formed at the –243/-249 site on the *bfl-1* promoter, if, for example, the RBP-J isoform present has weak affinity for Notch proteins but not EBNA2 One way to test possible Notch-CBF1 interaction on the *bfl-1* promoter was to co-transfect EBNA2 and Notchexpression plasmids to see if co-expression of Notch and EBNA2 would result in decreased EBNA2-mediated trans-activation This competition experiment was carried out for the mouse and human Notch1-IC expression plasmids (pED1, pJT111) and the human Notch2-IC expression plasmid (pJT112) Results shown m Figures 3 51-53 Increasing quantities of pJT111 were co-transfected with pSG5EBNA2 and the subsequent trans-activation of the *bfl-1* promoter analysed using the luciferase assay Figure 3 51

Figure 3.51. Co-transfection with Human Notch1-IC (pJT111) does not Affect pSG5EBNA2-Mediated Trans-activation of the *bfl-1* Promoter in the DG75 Cell Line.

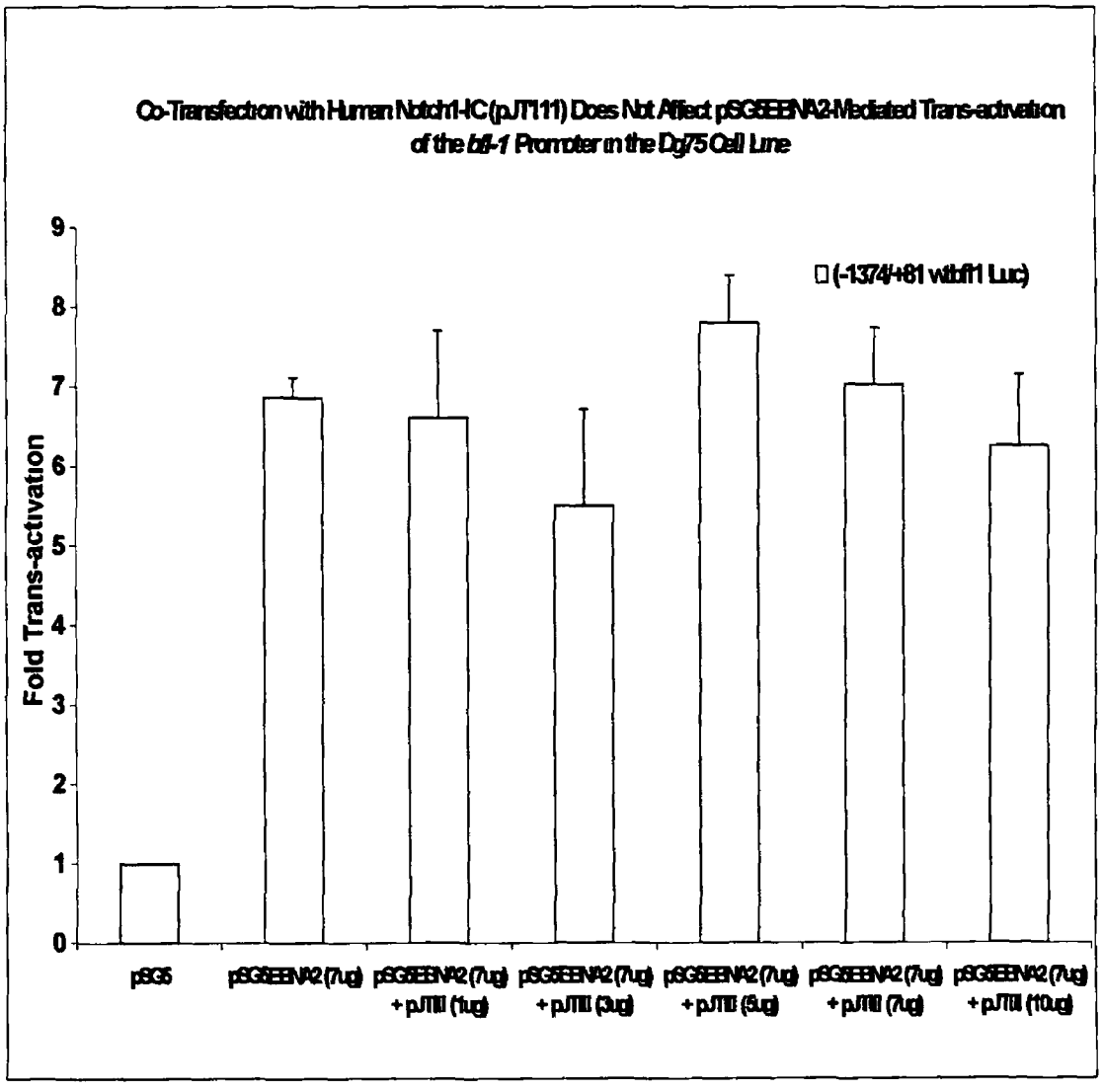


Figure3.51. Co-transfection with Human Notch1-IC (pJT111) does not Affect pSG5EBNA2-Mediated Trans-activation of the *bfl-1* Promoter in the DG75 Cell Line. The EBNA2 Expression plasmid (pSG5EBNA2) was co-transfected with increasing quantities of the human Notch1-IC expression plasmid (pJT111) and the transactivational affect on the wild typr bfl1 promoter reporter construct was recorded (-1374/+81 wtbfl1 Luc) No affect adverse or otherwise on EBNA2-mediated transactivation of the bfl1 promoter were seen over the range of quantities of pJT111 used

The results again show that pSG5EBNA2 trans-activates the bfl1 promoter (-1374/+81 wtbfl1 Luc) an average of 6 8 fold when transfected alone with the promoter (Figure 3 51) As increasing quantities of pJT111 (from 1 to 10μg) were cotransfected with pSG5EBNA2, promoter activity was neither increased nor decreased Overall co-transfection with the pJT111 vector expressing humanNotch1-IC did not affect EBNA2 mediated trans-activation of the *bfl-1* promoter (-1374/+81 wtbfl1 Luc) The same titration experiment was carried out with the pJT112 construct expressing human notch2-IC Interestingly, cotransfection with human Notch 2 decreased pSG5EBNA2 driven activation of *bfl-1* by 50% Promoter activity was impeded even when only 1μg of Notch2-IC expression plasmid (pJT112) was added Addition of increasing amounts of Notch 2 expression plasmid, up to 10μg did not further reduce EBNA2 mediated promoter activity

Figure 3.52. Co-transfection with Human Notch2-IC (pJT112), Down-Regulates pSG5EBNA2 Mediated Trans-activation of the *bfl-1* Promoter in DG75 Cells.

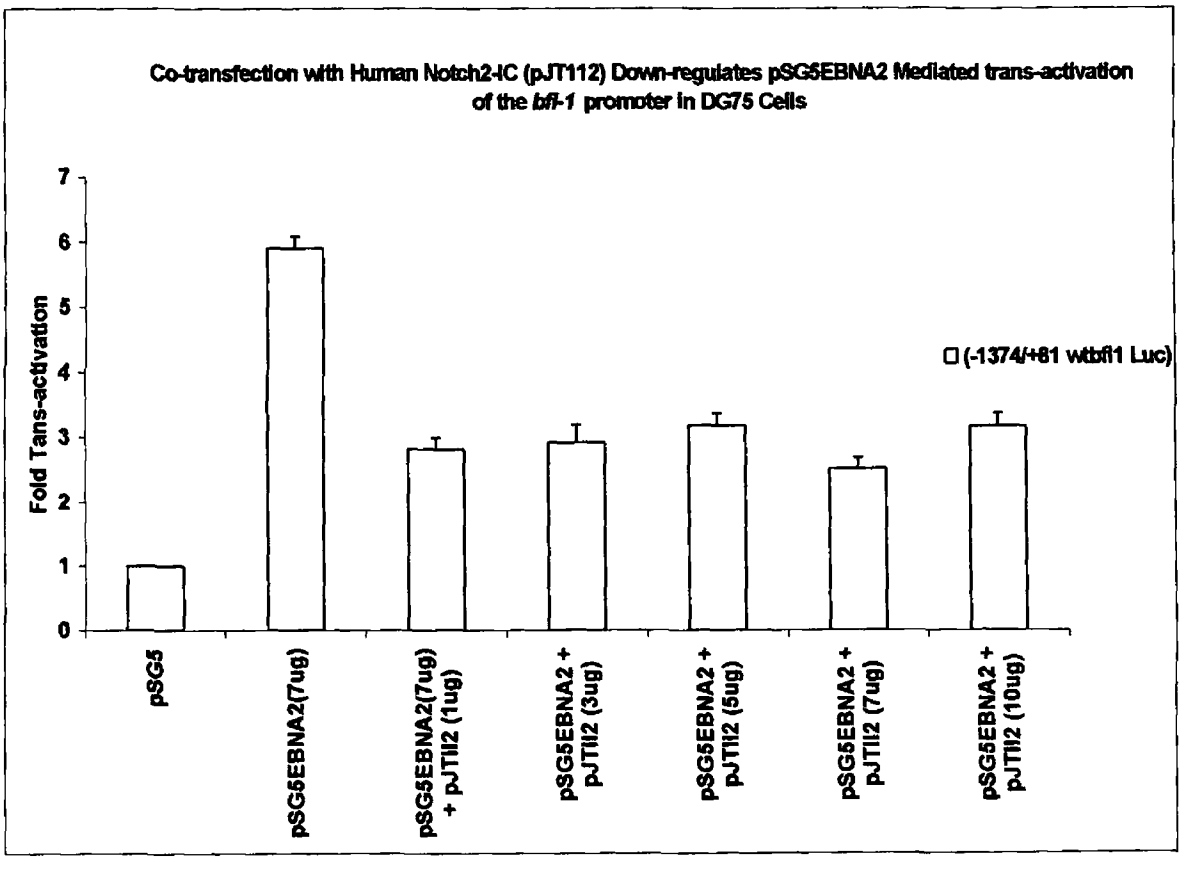


Figure 3 52. Co-transfection with Human Notch2-IC (pJT112), Down Regulates pSG5EBNA2 Mediated Trans-activation of the *bfl-1* Promoter in DG75 Cells. The EBNA2 Expression plasmid (pSG5EBNA2) was co-transfected with increasing quantities of the human Notch2-IC expression plasmid (pJT112) and the affect on EBNA2 mediated transactivation of wild type bfl1 promoter reporter construct was recorded (-1374/+81 wtbfl1 Luc) Addition of even 1ug of Notch2-IC expression plasmid (pJT112) resulted in a 50% decrease in pSG5EBNA2-mediated trans-activation of the bfl-1 promoter (-1374/+81 wtbfl1 Luc) Increasing the quantity of pJT112 did not increase the reduction in EBNA2 mediated transactivation of the bfl 1 promoter In this case the ability orf Notch2 IC to reduce EBNA2 transactivation of the promoter may imply that Notch2-IC interacts with either CBF1, a member of the repression complex and so competes with EBNA2 to bind CBF1 thereby repressing EBNA2 mediated tactivation of the promoter Alternatively it may not bind to bfl-1 via CBF1 at all instead Notch2-IC may just stearically hinder EBNA2 interaction with CBF1

One final set of transfections was undertaken comparing the reductive effect Notch co-transfection had on EBNA2 mediated trans-activation of the bfl-1 promoter In this experiment 3μg of each of the Notch expression plasmids were co-transfected with 7μg of the EBNA2 expression plasmid pSG5EBNA2 and *bfl-1* promoter activity was assessed using the luciferase assay Included in this experiment was the mouse NOTCH1-IC expression plasmid ED1 Surprisingly, as for the human notch2 expression plasmid, co-transfection of this expression vector (ED1) reduced EBNA2 mediated trans-activation of the promoter from an average of 6 fold to 1 6 fold Because of the high degree of homology between murine and human species of Notch1 it was unexpected that the two did not behave similarly in this experiment, however over two independent experiments, mouse and not human Notch-IC reduced EBNA2 mediated trans-activation of the *bfl-1* promoter

Figure 3.53 Co-transfetcion with Human Notch2-IC (pJT112) and mouse Notch1-IC (pED1) but Not Human Notch1-IC (pJT111), Represses EBNA2-Mediated Trans-activation of the *bfl-1* Promoter in the DG75 Cell Line.

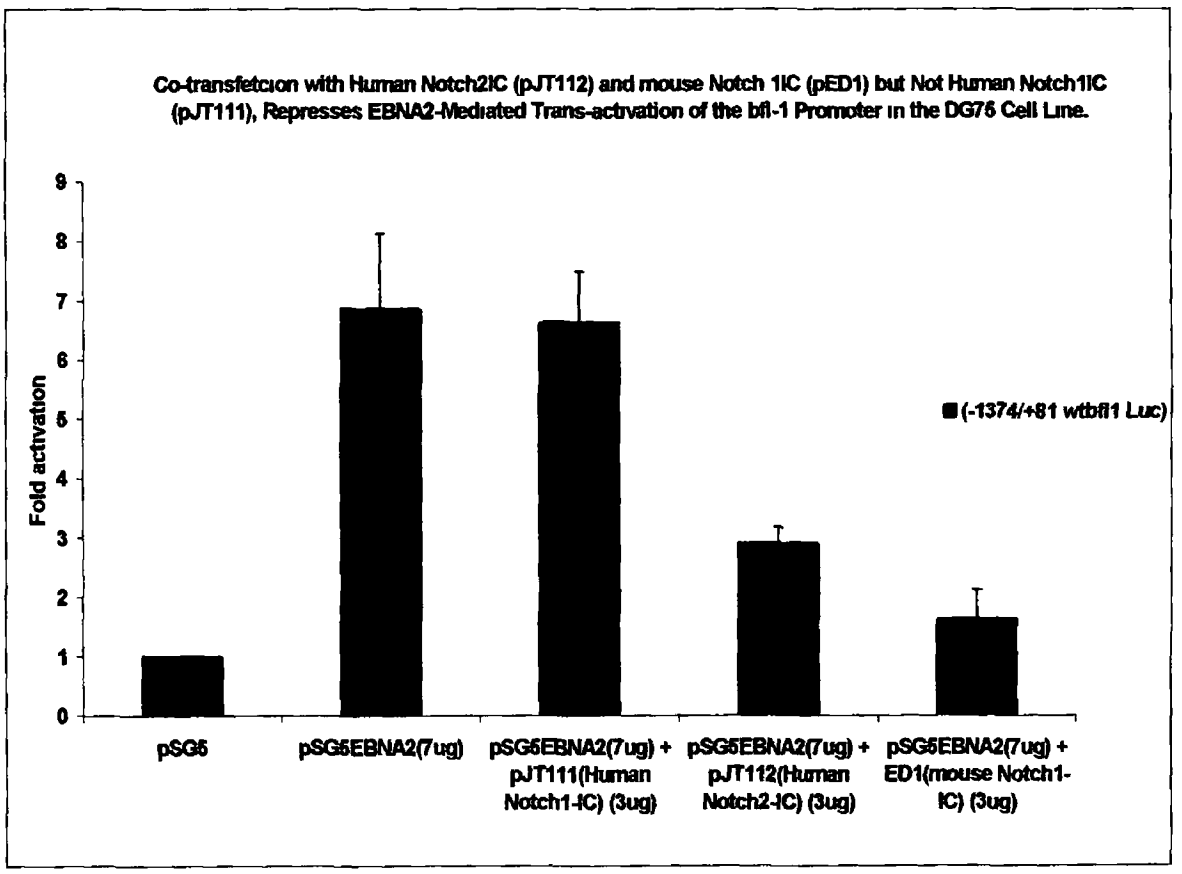


Figure 3.53 Co-transfetcion with Human Notch2IC (pJT112) and mouse Notch 1IC (pED1) but Not Human Notch1IC (pJT111), Represses EBNA2-Mediated Trans-activation of the bfl-1 Promoter in the DG75 Cell Line. As a final experiment a nominal amount (3ug) of human Notch1IC expression plasmid (pJT111), human Notch2IC expression plasmid (pJT112) and mouse notch*IC* expression plasmid (pED1) were cotransfected with pSG5EBNA2 and the affect on pSG5EBNA2 mediated trans-activation of the bfl1 promoter (-1374/+81 wtbfl1 luc) was assessed Strangely despite the large degree of homology and the shared functions of human and murine notc1IC, only the mouse notch1 expression plasmid affects pSG5EBNA2 mediated transactivation of the bfl1 promoter reducing it by ~50%. Similarly the human notch2IC expression plasmid reduced EBAN2 mediated transactivation of the bfl1 promoter by 50%.

Overall these results showed that co-expression of either human Notch2-IC or mouse Notch1-IC leads to a reduction in pSG5EBNA2-mediated *bfl-1* promoter activity The fact that Notch cannot trans-activate the promoter itself but can impair EBNA2 mediated

trans-activation, may indicate their shared ability to bind to CBF1 and/or other component(s) of the Notch nuclear complex on the promoter The ensemble of the results to date explicitly shows the importance of the CBF1 binding site in conferring EBNA2 responsiveness to the promoter Knowing that Notch can bind to CBF1 suggests that Notch2IC/mouseNotch1IC may indeed be binding to CBF1 thereby hindering the EBNA2-CBF1 mediated trans-activation of the bfl-1 promoter In order to address protein-DNA interactions if Notch is binding CBF1, electrophoretic mobility shift assays (EMSA) could be employed However if Notch is binding it is not transactivating the bfl1 promoter in the BL cell lines tested, possibly other transcriptional equipment required by NotchIC to transactivate the bfl1 promoter is not available in BL cell lines, one possible avenue of investigation to explain this would be to attempt to trans-activate the bfl-1 promoter with Notch expression plasmids in a different cellular background This was attempted in vascular smooth muscle cells with available notch expression plasmids however no trans-activational effect was observed in this cell line either (not shown) Further experiments could possibly be attempted in T cell lines such as the jurkat cell line for example

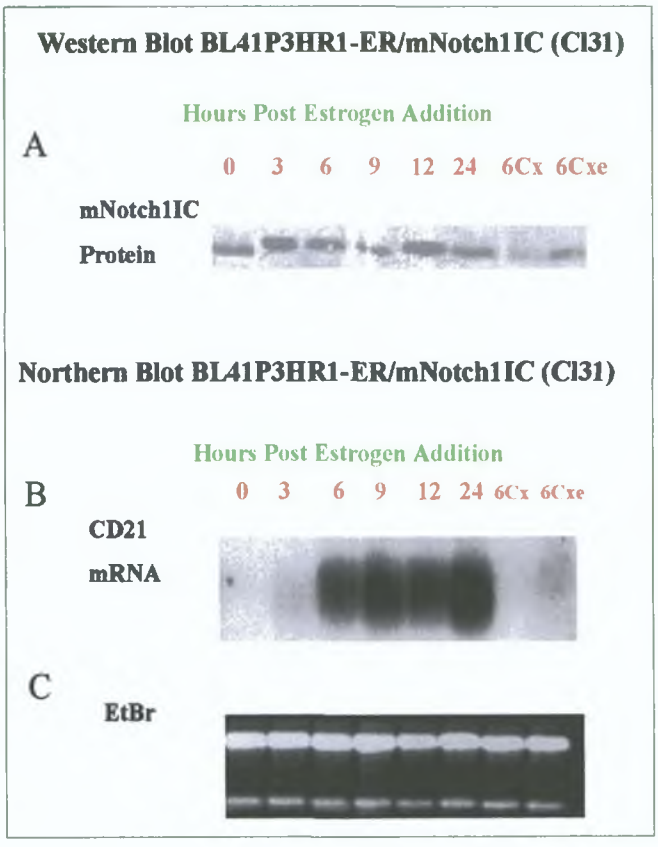
3.3.0. *bfl-1* mRNA levels do Not Change in Response to Activation of mNotch1-IC in BL41P3HR1mNotch1-IC-ER (Cl31).

Both EBNA2 and activated NOTCH-IC trans-activate genes by interacting with transcription factors including CBF1 The viral protein EBNA2 may hence be regarded as a functional equivalent of an activated Notch receptor Studies by (Strobl et al , 2000) showed that activated Notch1 modulates gene expression in B cells similarly to EBNA2 The aim of this part of this study was to directly investigate the role of Notch in regulating the EBNA2 responsive *bfl-1* gene, at the RNA level To this end, the BL41P3HR1-ER/mNotchIC (CL31) cell line was employed, Notch1 was activated in the presence of estrogen similar to that described earlier Functionality of the expressed Notch was then checked by probing for the presence of a mRNA from a target gene of notch namely CD21 (Figure 3 54) Functional mouse Notch1-IC, was activated by estrogen withdrawl over a 24-hour period and CD21 and *bfl-1* mRNA levels were

detected over the same time course by northern blotting (Figures 3 54 and 3 55) The CD21 and *bfl-1* probes used were identical to those used in the blots in Figures 3 5-3 9 Also included in the *bfl1* Northern blot (Figure 3 55) were samples from LCLs and type III BLs as positive controls for cell lines in which high levels of *bfl-1* mRNA is known to be present It can be seen from the results that levels of CD21 mRNA increased in response to notch activation over the 24 hour period of estrogen addition (3 54B) Panel C of figure 3 54 shows the ethidium bromide stained gel used in the Northern blot and was used as a control for loading Despite the fact that the Notch-Ic in this cell line (CL31) is functionally active and up-regulates CD21 mRNA levels (Figure 3 54B), in Figure 3 55A/B it can be seen that *bfl-1* mRNA levels are only transiently up-regulated at 3 hours post Notch activation the lane for the 6 hour time point is underloaded so this effect may still be present at this stage but it cannot be seen from the blot below (if there is an effect its over by 9 hours) Bfl-1 levels then fall again to basal levels It can also be seen from the results that *bfl-1* mRNA was detected in the control cell lines IARC171 IARC290B AG876 and DG75LMP1 (post LMP1 induction) and at very low levels in the Dg75LMP1 (pre LMP1 induction)- cell lines (3 55A/B) The fragment size, matches that previously reported as for *bfl-1* mRNA On addition of cyclohexamide *bfl-1* mRNA levels are up-regulated both in the presence and absence of activated Notch though activation of Notch results in slightly higher levels of *bfl-1* mRNA (1 6 fold) The high levels of *bfl-1* mRNA in the presence of cyclohexamide suggest that this chemical which inhibits protein sythesis may be stabilising any *bfl-1* that is already there however since this is not seen in the E2/ER cell lines (k3/9A), it is possible that this effect is a specific effect of the background in this cell line

Overall even though Notch is present and functioning (since CD21 mRNA levels are elevated) and *bfl-1* mRNA is detectable by the probe in use, Notch activation only transiently up-regulates *bfl-1* mRNA levels in the CL31 cell line

Figure 3.54. Notch1-IC is Present and Functionally Active in the BL41P3HR1-ER/mNotch1IC (Cl31) Cell Line.



NotchIC is present and and functionally active in the CL31 cell line.

Western Blotting

Panels A shows the NotchIC protein detected before and after estrogen addition.

Northern Blotting

Panels B and C show that the NotchIC present after addition of Estrogen is functional as CD21 mRNA is up-regulated in response to NotchIC Activation.

Figure 3.54. Notch1-IC is Present and Functionally Active in the BL41P3HR1-ER/mNotch1IC (Cl31) Cell Line. mNotch1-IC was activated in the BL41P3HR1-ER/mNotch1IC (CL31) cell line by addition of estrogen to the culture media. Total RNA and protein was harvested at 0, 3, 6, 9 12 and 24 hours post estrogen addition (mNotchIC activation) and after six hours in the presence of cyclohexamide (Cx) before and after Notch activation. Panel A Western blot showing mNotch1-IC expression before and after induction over the experimental timecourse. In order to verify that the (mNoptch1-IC) protein expressed in the cells was functional in response to estrogen addition, transcription from a known target gene of notch namely CD21 was analysed by Northern blotting. Panel B. Northern blot showing the up-regulation of CD21 in response to mNotch1-IC activation over the 24-hour time course. Panel C shows the ethidium bromide staining of the gel used for blotting in panel C. In the same experiment, the affect of mNotch-IC activation on *bfl-1* mRNA was also assessed (Figure 3.55).

Figure 3.55. *Bfl-1* mRNA and Protein is Only Transiently Up-regulated in the BL41P3HR1-ER/mNotch1-IC (CL31) Cell Line in Response to mNotch1-IC Activation.

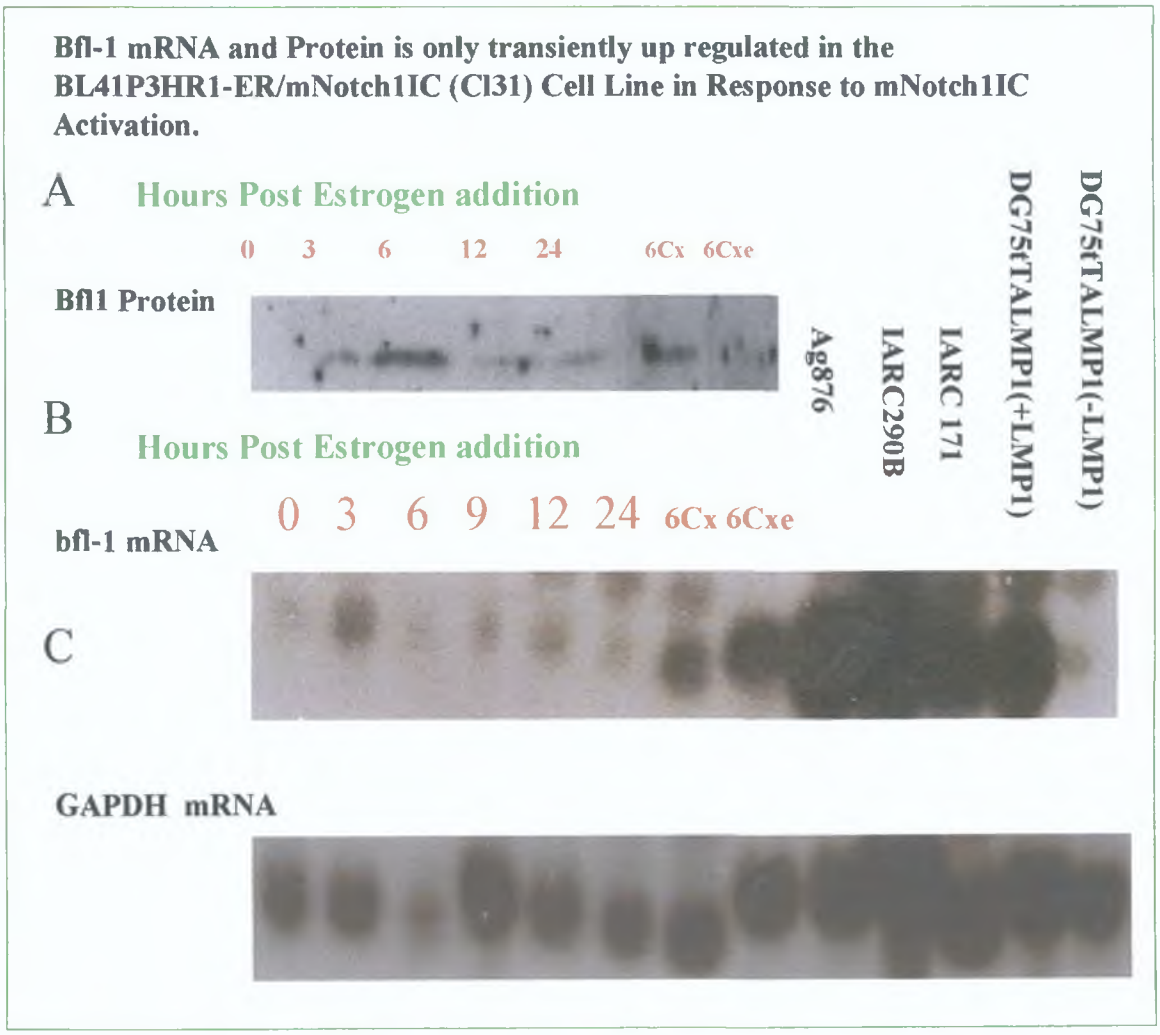


Figure 3.55. *Bfl-1* mRNA is only transiently up regulated in the BL41P3HR1-ER/mNotch1-IC (Cl31) Cell Line in Response to mNotch1-IC Activation. Panel A shows *bfl-1* mRNa levels detcected in the CL31 cell line over a 24 hour period in response to mNotchIIC activation. Also shown in this panel is the bfl-1 transcript detected in a number of LCLs and type III BLs in which *bfl-1* is known to be present. The effect of LMP1 induction on *bfl-1* mRNA can also be seen in the last two lanes as bfl-1 mRNA levels are dramatically higher in the case where LMP1 has been induced. Panel B shows the GAPDH mRNA levels detected in the lanes in panel A, which were reprobed for GAPDH mRNA after blotting for *bfl-1*. Despite mNotch1-IC being functionally active in the CL31 cell line as demonstrated by the upregulation of CD21 mRNA in Figure 3.54 above, bfl-1 mRNA is only transiently up-regulated over a 9 hour period in response to mNotch1-IC.

3.4.0. PHYSIOLOGICAL RELEVANCE OF EBNA2 INDUCED EXPRESSION OF *BFL-1* IN DG75 CELLS.

Type III BL cell lines expressing the full compliment of EBV latent genes have been shown to have a higher apoptotic threshold than their type I counterparts It has been shown that LMP1-induced expression of *bfl-1* mRNA protected Bl cells against apoptosis induced by growth factor-deprivation (D'Souza *et al*, 2000) The novel findings in this study, which identify *bfl-1* as a target gene of the EBV latent protein EBNA2, independent of LMP1 expression, suggest regulation of the *bfl-1* gene may be an important feature of EBV/EBNA2 induced resistance to apoptosis The results presented in this study showed a definitive link between EBNA2 expression and the up-regulation of *bfl-1* at the mRNA and protein levels as well as activation of the *bfl-1* promoter The physiological relevance of EBNA2 induction of *bfl-1* in terms of its involvement in protection against apoptosis and regulation of the apoptotic response in EBV infected cells, thus had to be investigated

3.4 1. DG75 CELLS ARE SUSCEPTIBLE TO APOPTOSIS ONLY UNDER CONDITIONS OF EXTREME SERUM DEPLETION.

Initially susceptibility of the Dg75 cell line to apoptosis was first analysed Apoptosis was induced by two methods (i) growth factor withdrawal (serum starvation) and (ii) exposure to toxin (ionomycin) Dg75 cells were cultured in media containing 10% (normal supplement) 5%, 1%, 0 5%, and 0 1% foetal bovine serum Cell viability was monitored every 24 hours over a seven-day period using trypan blue exclusion (Figure 3 56) The mechanism of cell death was confirmed as apoptosis by fluorescence microscopy after acridine orange staining, designed to specifically detect cell death as a result of apoptosis (Figure3 57)

It can be seen from the results obtained that cell viability remained close to 100% when the serum concentration was at 10% and 5% Cell viability decreased to just less than 90% after 7 days incubation in media supplemented with 1% serum at both cell densities

When serum concentration in the growth media was further reduced to 0 5%, cell viability was also reduced with the percentage of viable cells after 7 days falling to 64% again at both cell densities Results using 0 1% serum supplemented growth media showed cell viability at 42% and 46% after 7 days in the flasks seeded at $1x10^5$ cells/ml and $5x10^5$ cells/ml respectively Thus growth factor withdrawal resulted in increased cell death as represented by the reduction in the number of viable cells after serum starvation Cells were seeded at two different densities however both cell concentrations reacted similarly to serum starvation conditions suggesting seeding Dg75 cells at the lower cell density did not adversely affect cell viability Trypan blue exclusion was used to assess cell viability, with live cells appearing colourless Dead cells stain blue due to the uptake of the stain as a result of membrane damage Although trypan blue exclusion gives a clear picture that cell death is occurring, the actual method of cell death be it necrosis or apoptosis is not distinguished

FIGURE 3.56. DG75 CELL VIABILITY IS REDUCED BY SERUM STARVATION.

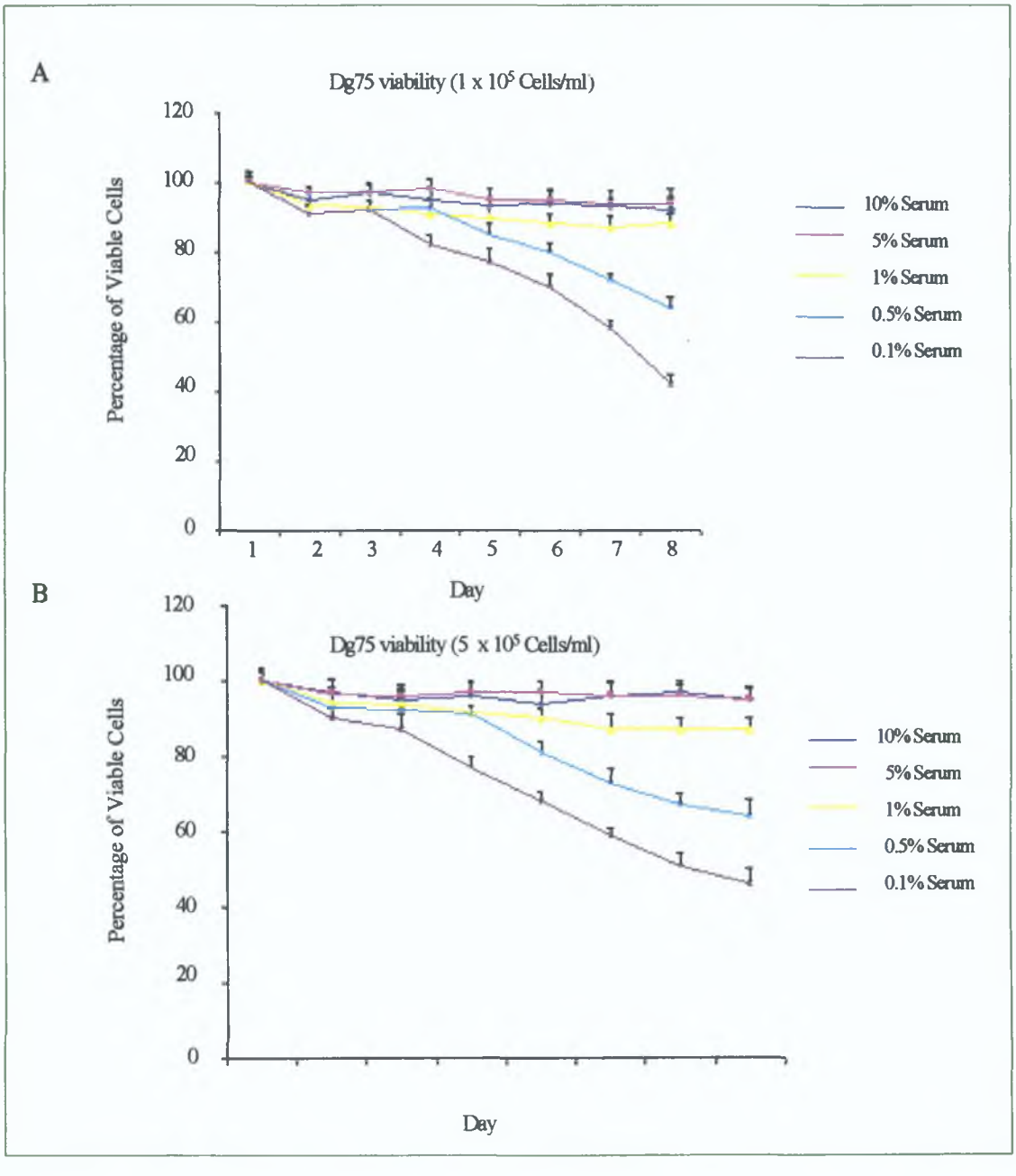


Figure 3.56. Dg75 Viability is Reduced by Serum Starvation. Dg75 cells were seeded at a density of 1 x 10^5 cells/ml and 5 x10^5 cells/ml in A and B above, respectively. Cells were then cultured over a seven day period in RPMI1640 supplemented with Pen/Strep and L-glutamine, however, each of the five culture flasks (at each cell density) were additionally supplemented with 10% (the usual supplement), 5% 1% 0.5% and 0.1% foetal bovine serum. It can be seen that reduction in Growth factor from 10% to 1% had little effect however in 0.5% serum cell viability was reduced to 64% (for both cell densities) and in 0.1% serum cell viability was reduced to 42% and 46% in the flasks seeded at 1 x 10^5 cells/ml and 5 x10^5 cells/ml respectively.

In order to determine if the cell death occurring as a result of serum starvation was apoptotic, acridine orange staining was employed. Acridine orange is a metachromatic dye which differentially stains double stranded (ds) and single stranded (ss) nucleic acids. When acridine orange intercalates into dsDNA it emits green fluorescence upon excitation at 480-490nm. Conversely, it emits orange fluorescence when it interacts with ssDNA or RNA. Samples from flasks containing the 10% serum supplemented media were stained as a positive control to show the staining and morphology of healthy cells. A cell sample from the 0.1% serum supplemented flask after 7 days was also examined to determine if the dead cells observed by trypan blue exclusion were dying by apoptosis. Acridine orange staining was carried out as described in the materials and methods section. An aliquot of suspension cells were fixed to microscope slides using ice-cold acetone. Cells were then stained with acridine orange at a concentration of 10ug/ml. Slides were viewed directly with a fluorescence ultraviolet light microscope (Zeiss Axiskop: camera JVC 3CCd-Cmount); (Figure 3.57).

FIGURE 3.57. DG75 CELLS DIE BY APOPTOSIS UNDER CONDITIONS OF GROWTH FACTOR WITHDRAWL/SERUM STARVATION.

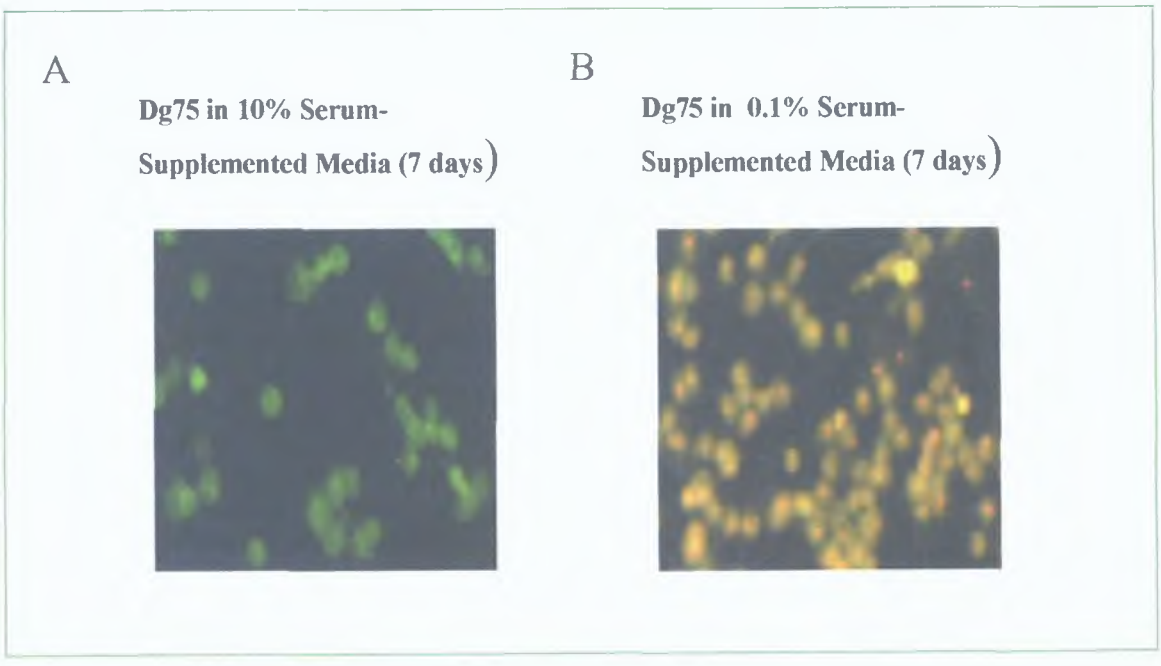


Figure 3 57. Dg75 Cells die by Apoptosis Under Conditions of Growth Factor Withdrawl/Serum Starvation. (B) In order to asses the mode of cell death in 0 1% serum supplemented media, cells were fixed and stained using Acridine Orange (10ug/ml) Cell morphology and staining pattern were then viwed using a KodakX Flouresence Microscope **(A)** Cells cultured under normal conditions in 10% supplemented media were also stained and visualised in the same way The Diffuse staining pattern of the healthy cells emitting a green fluorescence is in contrast to the serum starved cells whose condensed nuclei stain a bright orange colour, characteristic of apoptotic cells

It can be seen from the results that those cells cultured in serum supplemented with 10% serum fluoresced green after staining with acridine orange Thus nuclear DNA is intact in double stranded form In contrast those cells cultured under conditions of serum starvation show tightly stained orange "apoptotic bodies" Thus the nuclear DNA of these cells must be single stranded and so apoptosis is occurring There is a noticeable difference between the staining pattern of the nuclei of the healthy and apoptotic cells In picture (a) nuclei are diffusely stained and appear as large crenated structures with moderate green fluorescence while in picture (b) where cells are dying by apoptosis, nuclei appear as smaller rounded structures brightly fluorescing (orange) as a result of chromatin condensation

Dg75 and B cells in general have a relatively high apoptotic threshold and thus the method used to induce apoptosis and the cell line in which to induce apoptosis had to be considered carefully Studies by Brimmel et al (1998) used the calcium ionophore ionomycin in conjunction with serum starvation to induce apoptosis in the Dg75 cell line In an attempt to increase cell death this more stringent assay was utilised In this case cells were set up in 0 1% serum supplemented media containing 2ug/ml of ionomycm As per the serum starvation experiment, cell death was monitored by trypan blue exclusion and the mode of cell death confirmed as apoptosis using acridine orange staining

FIGURE 3.58. TREATMENT OF DG75 CELLS WITH IONOMYCIN AND SERUM STARVATION PRODUCES RAPID CELL DEATH AT LOW SERUM CONCENTRATIONS.

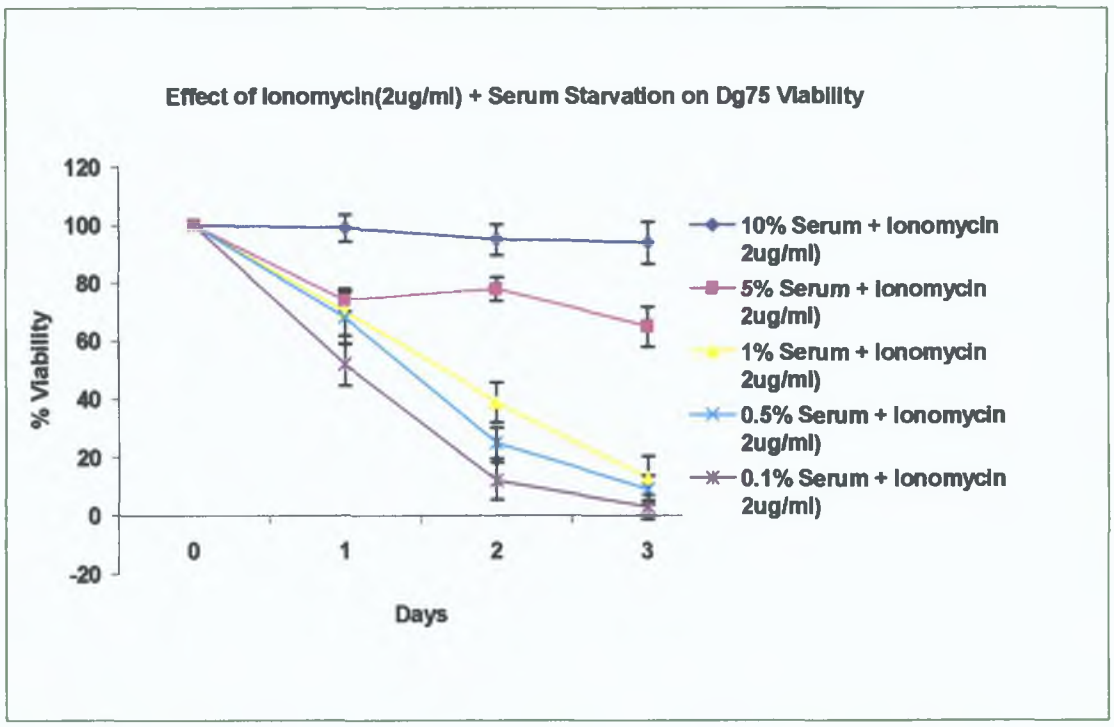


Figure 3.58. Treatment of Dg75 Cells with Ionomycin and Serum Starvation Produces Rapid Cell Death at Low Serum Concentrations. Dg75 cells were cultured as before in RPMI 1640 medium supplemented with Pen/Strep and L-glutamine and 10%, 5% 1% 0.5% and 0.1% foetal Bovine serum. Since cell death using Growth factor withdrawl alone took 7 days it was decided to add 2ug/ml to each of the flasks to increase the rate and or the quantity of cell death. In 10% supplemented media cell viability was reduced to 94% over a three day period and in 5% serum supplemented media cell viability was reduced to 65% over the same period. Almost total cell death had occurred at the lower serum concentrations in the presence of ionomycin.

As shown in Figure 3.58 cells cultured in normal supplemented media containing 10% serum treated with ionomycin alone reduced in viability to only 94% over 3 days of assessment. Thus at this concentration in the absence of serum starvation ionomycin is not particularly efficient in inducing cell death in this cell line. In media supplemented with 5% serum, the addition of ionomycin resulted in cell viability dropping to around 65% after three days. At lower serum concentrations, the addition of ionomycin resulted in rapid and almost total cell death (Figure 3.58). The mode of cell death was assessed using acridine orange staining (Figure 3.59).

For comparison, cells were also cultured in 0.1% serum supplemented media and assessed for 6 days prior to acridine orange staining. As per the previous experiment, (Figure3.59B) cells cultured in 0.1% serum in the absence of ionomycin reduced in viability to around 48%. As a positive control, cells were also cultured in 10 % supplemented media in the absence of ionomycin and visualised using acridine orange staining.(Figure 3.59A) It can be seen from the photographic results below that the positive control cells in standard supplemented media containing 10% serum in the absence of ionomycin grew normally fluorescing green after acridine orange, also cellular content is diffusely stained. Cells were monitored every 24 hours and those cells which had been both serum starved and treated with ionomycin were visualised with acridine orange staining, after 3 days of incubation. (Figure 3.59C). The morphology of these cells corresponded to apoptotic cells where cellular dehydration, cell shrinkage is clearly visible also, and nuclear condensation and the formation of resulting in bright orange flouresence after acridine orange staining.

FIGURE 3.59. DG75 CELLS ARE SUSCEPTIBLE TO APOPTOSIS BY TREATMENT WITH IONOMYCIN AND SERUM STARVATION.

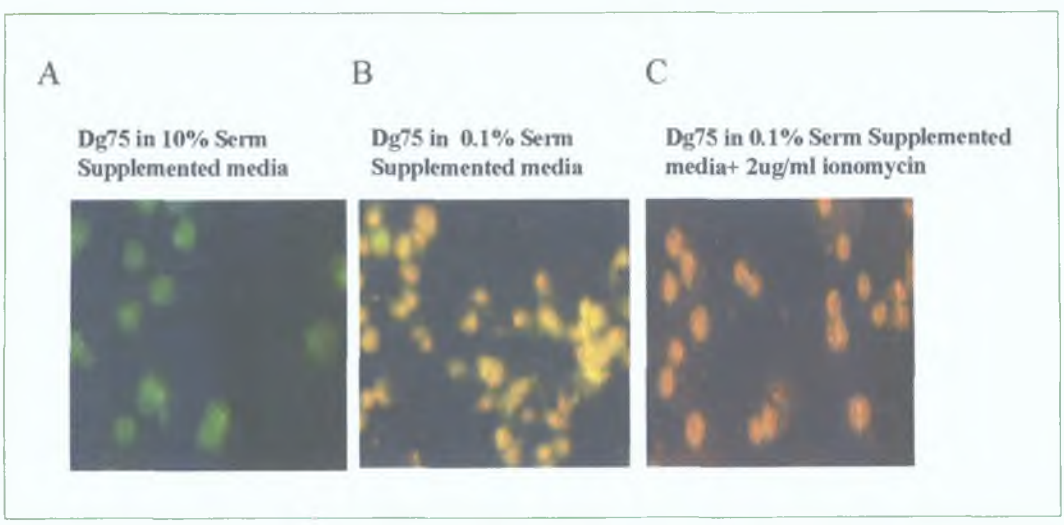


Figure 3.59. Dg75 Cells are Susceptible to Apoptosis by Treatment with Ionomycin and Serum Starvation . (C) In order to determine the mode of cell death as a result or treatment with ionomycin and serum starvation, cells were stained using Acridine orange as before and visualised as before. (A) as a control cells cultured in media supplemented with 10% serum were also stained and compared to the dual treated cells in panel (C). Also included were cells cultured under conditions of serum starvation alone.

Having determined that Dg75 cells are susceptible to apoptosis induced by both serum starvation and exposure to ionomycin, it then had to be determined if EBNA2 expression in this cell line could protect the cells from apoptosis under these apoptosis-inducing conditions It was decided to investigate if EBNA2 expression could protect serum starved cells from apoptosis, since exposing the cells to ionomycin and serum starvation resulted in such catastrophic and rapid cell death that any protective effect, exerted by EBNA2 might not be sufficient to maintain or rescue cell viability

3.4.2. Selection of an Appropriate Concentration of Puromycin to Select Transfected/Untransfected Dg75 Cells.

In order to assess possible EBNA2 protection of B cells from apoptosis, as a result of *bfl-1* induction, and to assess the role of EBNA2/CBF1, in this effect, Dg75 cells were stably transfected with the pSG5, pSG5EBNA2 and the pSG5EBNA2WW323SR expression plasmids The EBNA2 expression plasmids were co-transfected into Dg75 cells with the pGK3puro plasmid which expresses the *pac* gene The expression of the *pac* gene confers puromycin resistance to transfected mammalian cells expressing it Puromycin is particularly useful for the selection as it quickly kills eukaryotic cells that do not contain the *pac* gene Thus puromycin was used to select cell clones expressing the *pac* gene in the hope that those cells would also express the co-transfected EBNA2 expression plasmids Subsequent expression of EBNA2 in these cell lines was assessed by Western blotting (Figure3 61) below

Initially a "drug curve" had to be established in order to identify that concentration of puromycin required to select transfected clones A number of controls were also used in this experiment, pSG5 was co-transfected with the pGK3puro plasmid to provide information on any basal effect of transfecting DG75 cells with this vector, thus any data collected from transfections with the EBNA2 clones would represent true effects of EBNA2 As a control to assist in the selection of an appropriate concentration for the drug curve, pSG5 vector was also transfected alone in the absence of the puromycin resistance plasmid Thus cells surviving longer than this cell pool should do so due to expression of the *pac* gene in the pGK3puro plasmid

The drug curve was initially undertaken with untransfected DG75 cells, which were grown in RPMI 1640 with 10% FCS until just sub-confluent Cells were then plated in 24 well culture plates at a concentration of ~ 1x 10^3 cells/ml and grown overnight before addition of drug (puromycin) As cells can divide once or twice in selective media which can kill them, it was important to seed cells at low density to ensure that cells did not reach confluency before selection could take effect After 24 hours puromycin was added at various concentrations as follows, 0, 0 25, 0 5, 1 0, 2 0, 5 0, and 10 0 ug/ml and cells were grown for 10 days Media was changed every 4 days and cells were observed for decline of cell numbers under the inverted microscope after 24 hours and every day thereafter It can be seen from the results that treatment with 0 25ug/ml and 0 5 ug/ml only reduces viability to 75% and 51% respectively over the time course examined while treatment with 10ug/ml, 5ug/ml and 2ug/ml rapidly kills off the *pac* negative cells in 2, 3 and 4 days respectively (Figure 3 60) Treatment with 1ug/ml gradually kills off all cells over a seven-day period Since treatment with 1ug/ml is less harsh and more gradual than that of the higher concentrations this concentration of puromycin was chosen to select the stably transfected pools of cells (Figure 3 60)

Figure 3.60. Puromycin at a Concentration of 1μg/ml optimally Kills *Pac* negative-DG75 cells over a seven day Period

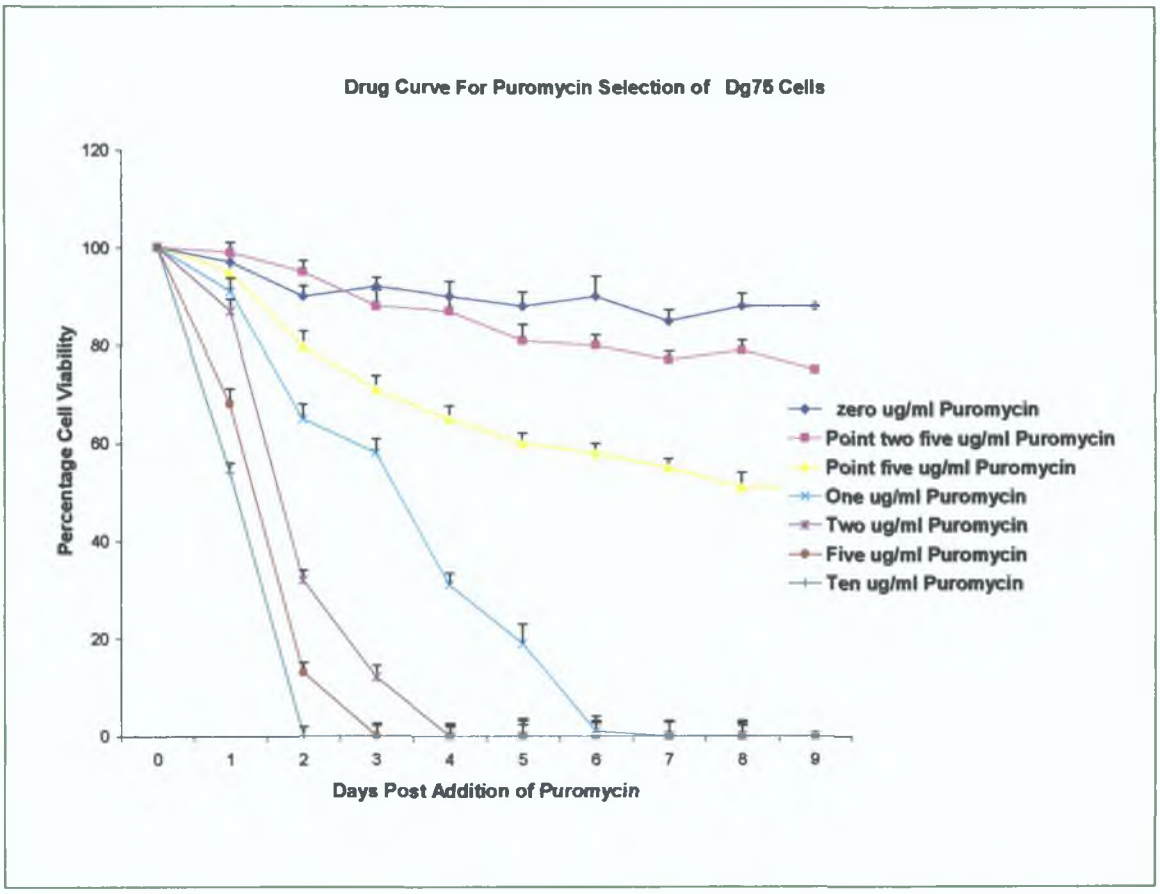


Figure 3.60. Puromycin at a Concentration of 1ug/ml optimally Kills *Pac* negative-DG75 cells over a seven day Period. Dg75 cells were cultured in Supplemented RPMI 1640 medium and treated with 0ug/ml, 0.25ug/ml, 0.5ug/ml,1ug/ml, 2ug/ml, 5ug/ml and 10ug/ml puromycin and their viability measured by trypan blue exclusion over a 10 day period. Treatment with 10ug/ml, 5ug/ml and 2ug/ml completely kills off the Dg75 cells in 2 3 and 4 days respectively. While treatment with 1ug/ml of puromycin completely kills cells after 7 days of exposure, treatment with 0.5ug/ml ionomycin reduces viability to 51% over the same period.. Treatment with puromycin at 0.25ug/ml reduces viability to 75% over the ten day period. Treatment with 0.25ug/ml and 0.5ug/ml is not effective in killing the Pac negative cells in this time frame. While treatment with 5 ug/ml and 10ug/ml is effective in killing the cells it kills the cells very rapidly. Treatment with 1ug/ml of puromycin kills the cells gradually over a seven day period thus if cells are transfected with a *pac* expressing vector they will have time to divide and express the gene and thus be protected from the effects of this antibiotic.

3.4.3. Stable Transfection of the Dg75 cell line with EBNA2 Expression Plasmids pSG5EBNA2 and pSG5EBNA2WW323SR

Dg75 cells were transfected by electroporation using the Biorad x409 as described in the materials and methods section The combinations transfected are shown in the table below The ratio of expression plasmid to selection marker transfected was 1 5 Three micrograms of expression plasmid (pSG5/pSG5EBNA2/pSG5EBNA2WW323SR) and fifteen micrograms of *Pac* expressing pGK3-Puro were transfected in each case Twenty -four hours after transfection, puromycin was added to a final concentration of 1μg/ml (The concentration of puromycin, which just killed all *pac* negative Dg75 cells Figure 3 60) After 7 days the wells containing the cells transfected in the absence of the puromycin resistance expression plasmid, were dead as confirmed by trypan blue staining In theory thus the remaining cells should contain the transfected pGK3 puro and also the EBNA2 expression plasmids Every 24 hours, trypan blue exclusion was used to evaluate cellular viability in wells, which still contained cells Cells were then cultured under continuous selection for two weeks In order to test for the presence of EBNA2 in the stably transfected cell lines, western blotting was undertaken with lysates extracted from the transfected cells Although the PE2 antibody did not detect EBNA2 in transiently transfected cells, the antibody did detect the expression of the EBNA2 and EBNA2WW323SR protein in the stably transfected cells and the protein appears to be expressed at similar levels in both cases, no EBNA2 was detected in the Dg75-pSG5 cell line (Figure 3 61 below) The protein may be detected in the stably transfected cells but not in the transiently transfected cells due to the fact that a higher number of cells in the stably transfected cell line express the EBNA2 protein as transfected pools of cells have been specifically selected under optimised antibiotic selection conditions

Figure 3.61. EBNA2 is Expressed at a similar levels in the two Pools of Stably Transfected Cells -Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR.

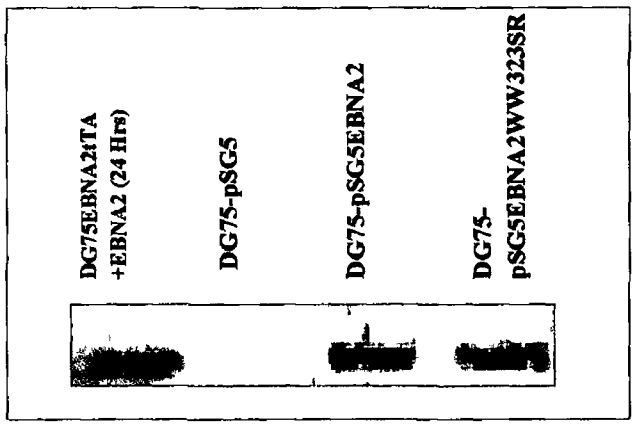


Figure 3 61. EBNA2 is Expressed at a similar levels in the two Pools of Stably Transfected Cells-Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR. Western Blotting using the PE2 anti EBNA2 antibody, was carried out on total protein extracts from each of the cell pools Dg75-pSG5, DSg75-pSG5EBNA2 and DG75-pSG5EBNA2WW323SR. EBNA2 was detected in both Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR but not in the Dg75-pSG5 transfectants As a control for EBNA2 expression, blotting was also carried out on the DG75EBNA2tTA cell line 24 hours post removal of tetracycline

Mutiprobe RPA analysis was then carried out with extracts from the stably transfected cell lines and the effect of EBNA2 expression on *bfl-1* mRNA in this system was analysed Additionally a number of transient transfections were carried out with these cell lines and the *bfl-1* promoter reporter constructs as well as the control pGa50-7 and pGa981-6 reporter constructs It can be seen from the RPA analysis (Figure 3 62) that as before, *bfl-1* mRNA is induced 3 1 fold with the expression of EBNA2 in the DG75-pSG5EBNA2 cell line (in this case 3 1 fold relative to Dg75-pSG5) This is not the case in the cells transfected with the pSG5 vector alone Further, *bfl-1* mRNA levels, are not induced (1 6 fold) by expression of the CBF1 mutant EBNA2 (EBNA2WW323SR) in the Dg75-pSG5EBNA2WW323SR cell pool These results again comply with the previous findings that EBNA2 mdependantly up-regulates *bfl-1* mRNA in this cell line The finding that a cells expressing the CBF1 mutant EBNA2 does not up-regulate *bfl-1* mRNA indicates that CBF1 may be important at a transcriptional level in mediating EBNA2 resposivity to *bfl-1* and complies with the promoter study data which strongly

implicates a role for CBF1 binding in regulating EBNA2 mediated activation of the *bfl-1* promoter In addition, RNA from the MutuI and MutuIII cell lines was also included in this RPA and the dramatic increase in *bfl-1* mRNA due to expression of the full range of EBV latent proteins is evident The up-regulation of *bfl-1* mRNA in response to expression of the full spectrum of EBV latent proteins (characteristic of type III cell lines) is much higher than that seen by expression of EBNA2 alone thus again implicating a major role for other EBV protein(s) in regulating the expression of this cellular gene (D'Souza *et al*, 2000)

FIGURE 3.62. *BFL-1* MRNA IS INDUCED BY WILD-TYPE EBNA2 EXPRESSION IN THE DG75-PSG5EBNA2 CELL LINE BUT NOT IN THE DG75-PSG5EBNA2WW323SR CELL LINE

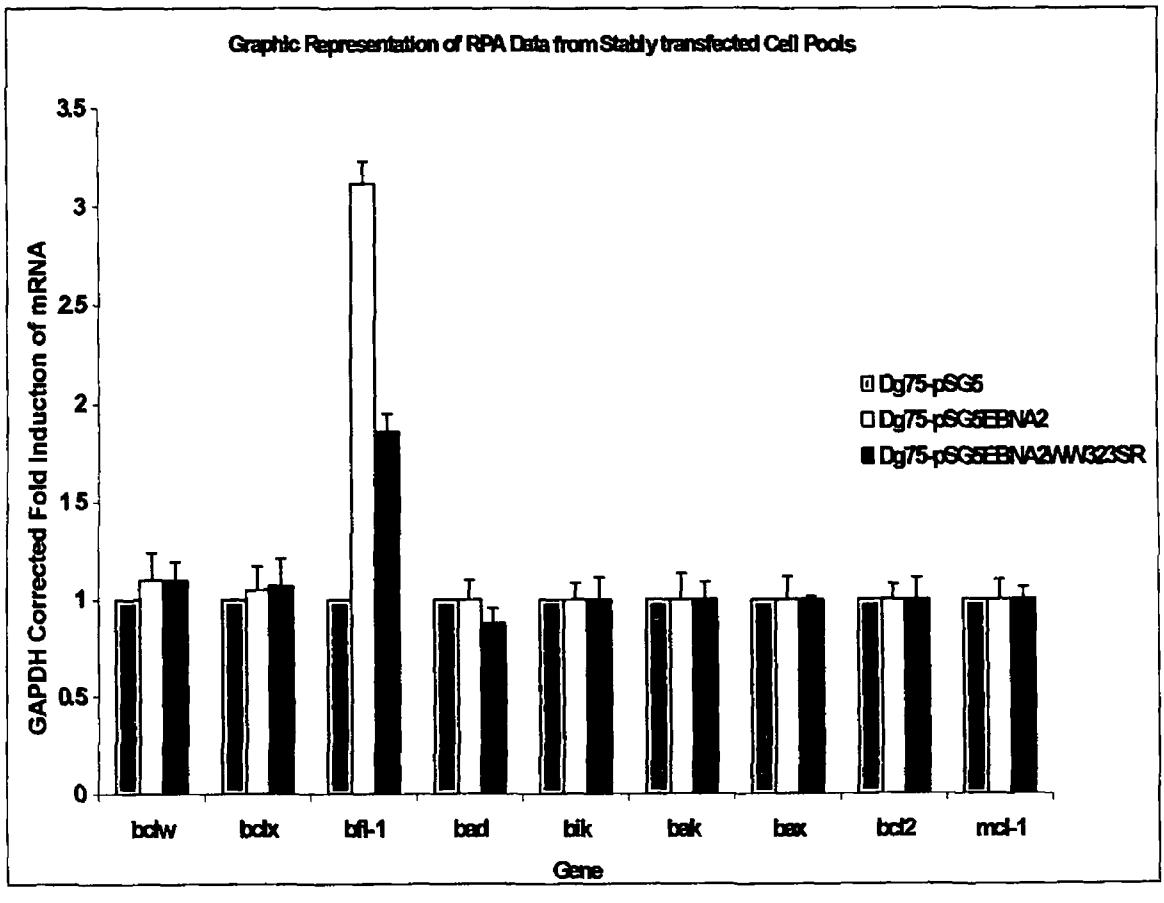


Figure 3 62 *Bfl-1* mRNA is induced by wild type EBNA2 expression in the Dg75-pSG5EBNA2 cell line *but not* in the Dg75-pSG5EBNA2WW323SR Cell Line. mRNA levels from the apoptosis related genes bclx l/s, *bfl-1*, bad, bik, bak, bax, bcl2, and mcl-1 were analysed in each of the cell lines indicated. bfl-1mRNA is clearly up-regulated in the Dg75-pSg5EBNA2 cell line relative to the Dg75-pSG5 cell lines This is not the case in the cell line expressing EBNA2WW323SR in which CBF1 binding has been abolished No up-regulation of the mRNA species from any of the other apoptosis related genes was recorded

Functionality of the EBNA2 proteins was also examined by co-transfecting each of the pools of cells with the wild type and CBf1 mutated *bfl-1* reporter constructs and the pGa50-7 and pGa981-6 reporter vectors The results obtained (Figure 3 63) mirrored those of the transient transfections using the pSG5 and pSG5EBNA2WW323SR plasmids In the case of the stably transfected cell pools only the cells expressing wild type EBNA2 (Dg75-pSG5EBNA2) trans-activated the wild type *bfl-1* promoter and the pGA981-6 promoter reporter The pool of cells expressing the EBNA2WW323SR protein (Dg75-pSG5EBNA2WW323SR) failed to trans-activate the wild type *bfl-1* promoter or the pGa981-6 reporter (Figure 3 63) Although fold trans-activation by EBNA2 and EBNA2WW323Sr were made relative to trans-activation by pSG5, no increase in basal transcription was recorded in the cell line transfected with pSG5 relative to untransfected Dg75 Neither the CBF1 mutated promoter nor the pGa50-7 reporter was activated in these transfections

FIGURE 3.63. THE *bfl-1* PROMOTER IS TRANS-ACTIVATED WHEN TRANSFECTED INTO THE DG75-PSG5EBNA2 CELL POOL, BUT NOT IN THE DG75-PSG5EBNA2WW323SR CELL POOL.

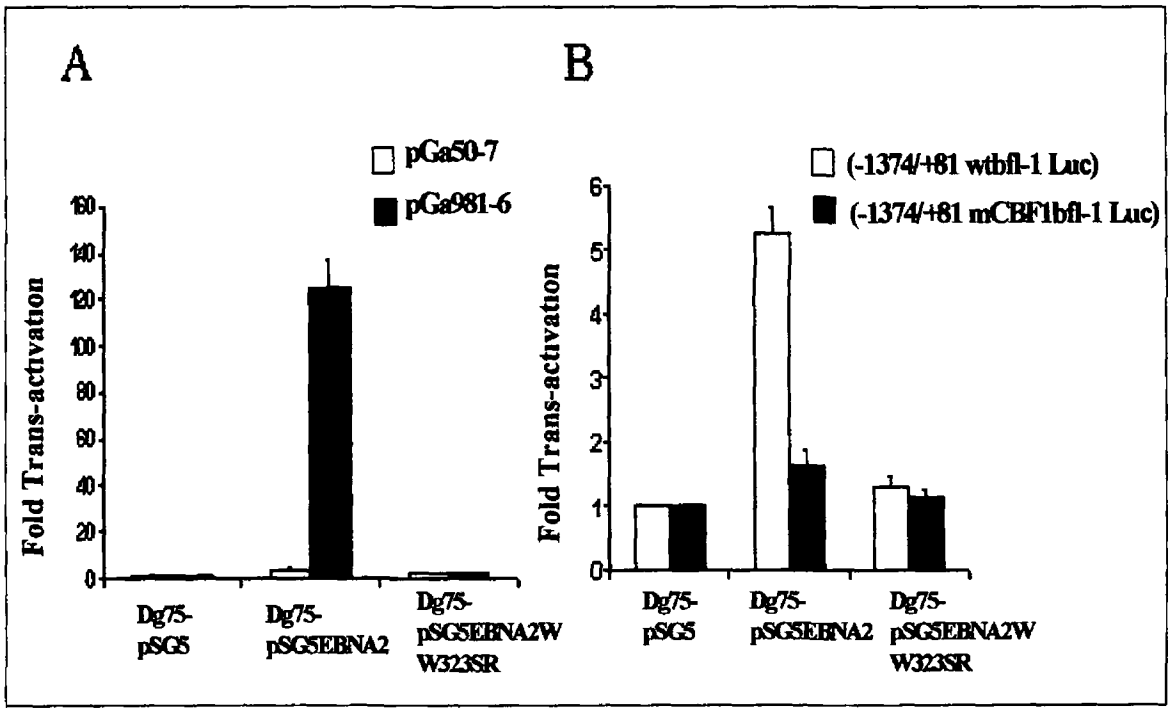


Figure 3.63. The *bfl-1* promoter is trans-activated when Transfected into the Dg75-pSG5EBNA2 Cell pool, but Not in the Dg75-pSG5EBNA2WW323Srexpressing cells. The stably transfected cell pools were then transfected with the *bfl-1* promoter reporter construct (-1374/+81 wt*bfl-1* Luc) and its CBF1 mutated partner (-1374/+81 mCBF1*bfl-1* Luc) and activity of the promoter was analysed using the luciferase assay In the same experiment, the three indicated pools of cells were also transfected with the pGa50-7 and pGa981-6 reporter constructs. In all cases transfections were carried out using the DEAE dextran method and 10ug of promoter reporter construct was transfected Fold activation represent the normalized (for transfection efficiency based on β-galactisidase activity measured from co-transfected pCMVlacZ reporter included in all transfections) luciferase activity due to expression of EBNA2 and EBNA2WW323SR in the Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR cell pools respectively All data is an average of three independent experiments and fold activations are relative to trans-activation by transfection in the Dg75-pSG5 cell line, which has been adjusted to 1 fold

3.4.4. EBNA2 Expression May Provide Some Protection from Apoptosis Induced by Serum Starvation in the Dg75 Cell Line.

Untransfected Dg75 cells and cells stably transfected with pSG5EBNA2, pSG5EBNA2WW323SR and the pSG5 vector alone were then serum starved in 0.1% serum and their viability compared after a 6-day period. The mode of cell death was then assessed using acridine orange and propidium iodide staining.

Figure 3.64. Expression of EBNA2 Provides a Measure of Protection to Dg75 Cells from Apoptosis Induced by Serum Starvation. (Acridine Orange)

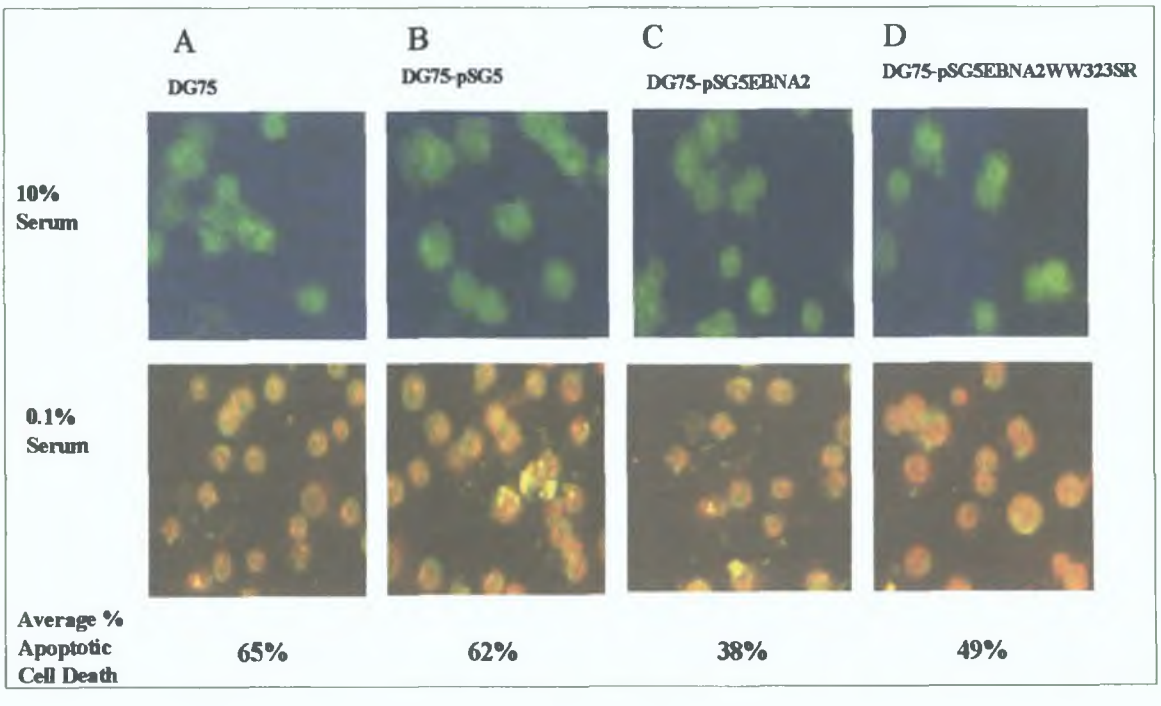


Figure 3.64. Stable Expression of EBNA2 in the Dg75 Cell Line Protects these Cells from Apoptosis Induced by Serum Starvation. Cells were cultured for 6 days in RPMI 1640 Cell culture media supplemented with either 10% or 0.1% foetal bovine serum. Culture media was changed every 3 days. Cell viability was monitored every 24 hours and the mode of cell death after 7 days was analysed by acridine orange staining as before. Column A shows the results for the untransfected Dg75 cell line. Column B shows the results for the DG75-pSG5 cell pool and Columns C and D show the results for the DG75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR cell pools respectively. Although apoptosis is occurring in all cases in cultures supplemented with 0.1% serum, the average percentage of cell death was consistently lower in the case of both cell pools expressing EBNA2. (Dg75-pSG5EBNA2 and Dg75-

pSG5EBNA2WW323SR) Further the EBNA2 CBF1 site may be involved in conferring some of this protective effect as the percentage cell death in the Dg75-pSG5EBNA2WW323SR cell pool in which EBNA2 cannot bind CBF1, consistently showed a relatively higher level of cell death compared to its counterpart cell line expressing wild type EBNA2

It can be seen from the results (Figure 3 64) that apoptosis is occurring in all cell lines after seven days in 0 1% supplemented media The mode of death can be characterized as apoptosis by acridine orange staining which clearly shows the formation of apoptotic bodies stained as bright orange punctate dots in the nuclei of the apoptotic cells, also the intense orange staining of the apoptotic cells is in marked contrast to the diffusely stained green fluorescence emitted by the healthy cells in 10% supplemented media Cell counting was performed and the percentage of apoptotic cells was determined from counts on at least 150 cells per individual culture and all cultures were set up in triplicate A representative section from a field for each cell line is displayed above After seven days under conditions of serum starvation the percentage of apoptotic cells was calculated at an average of 65% and 62% in the untransfected cell line Dg75 and the cell pool expressing the empty pSG5 vector, Dg75-pSG5 respectively Apoptotic cell death was consistently lower in the two cell pools expressing EBNA2, Dg75-pSG5EBNA2 and DG75-pSG5EBNA2WW323SR, suggesting EBNA2 confers a measure of protection from apoptosis on these pools of cells in vitro A slight increase in the anti-apoptotic threshold was consistently recorded for the Dg75-pSG5EBNA2 cell pool relative to the Dg75-pSG5EBNA2WW323SR cell pool suggesting CBF1 binding, or a downstream consequence of this, may be involved in this protective effect In the case of this study EBNA2 has been shown to up-regulate the anti-apoptotic *Bfl-1* protein in this cell line and it is therefore likely that EBNA2 induced up-regulation of *bfl-1* may be involved in the EBNA2 mediated resistance to apoptosis here

In order to better quantitate the number of cells apoptosing, Flourescence Activated Cell Sorting (FACS) analysis was undertaken The results obtained using the acridine orange analyses are mirrored by the results obtained from the same cell lines stained with propidium iodide and studied using FACS analysis (Figure 3 65) Initially measurements made in these analyses were *forward light scatter* and *side light scatter*, respectively

Forward light scatter provides some information on the *relative size* of individual cells, whereas side light scatter provides some information on the *relative granularity* of individual cells In this case these two are combined to identify the light scatter pattern emitted by a healthy population of cells and this is compared to the characteristic light scatter pattern emitted by condensed, shrunken apoptotic cells The sets of data are presented as dot plots In each of the cell lines Dg75, Dg75-pSG5, Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR, a $1^* 10^7$ sample of cells in 10% serum was analysed by trypan blue and FACS, the trypan blue analyses showed over 95% cell viability for each of the cell lines and correlates with the healthy diffusely stained cells stained by acridine orange in Figure 3 64 above Using FACS the light scatter pattern of 10,000 cells from each of the cell lines was then analysed after staining with Propidium Iodide It can be seen that the cell populations for the healthy cells are predominant in the upper right quandrants for the dot plots for each cell line, represented in Figure 3 65 below AI, BI, CI and DI Over 96%, 94%, 94% and 91% of the cell populations are present in the upper right quadrant for the healthy Dg75, DG75-pSG5, Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR cell lines respectively These data show a relatively high level of forward light scatter indicating relatively large healthy cells After six days in media supplemented with only 0 1% serum, the population of cells for each of the cell lines shifts to the left towards the origin suggesting a decrease in size, characteristic of cells undergoing the latter stages of apoptosis Cells in the lower right quandrant are also apoptotic indicated by their reduction in forward scatter (size) however they have not reached the same level of gross nuclear condensation indicative of the latter stages of apoptosis characterised by the cells shown in the lower left quadrant The side scatter parameter on the Y-axis indicates the relative granularity of the cells In the case of each of the cell lines serum starvation results in a reduction in granularity indicated by the shift in the population of cells vertically downwards In the early stages of apoptosis, apoptotic cells should increase in granularity due to the formation of apoptotic bodies, and thus an increase in light scattered in the right angle (Side) direction However in the latter stages of apoptosis, the intensity of light scattered at both forward and right angle (side) directions is decreased Although this reduction in light scattered at both forward and right angles may be characteristic of necrosis this is not the likely cause of cell death here

as the mode of cell death was determined by acridine orange staining which specifically detects apoptosis Due to the high anti-apoptotic threshold of this cell line, especially stringent conditions were required to induce apoptosis, namely serum starvation for six days after which point a large population of the cells are in the late stages of apoptosis The mode of cell death was verified as apoptosis by acridine orange staining and the number of cells apoptosing was quantified by FACS analysis It can be seen after serum starvation that over 77% and over 83% of Dg75 and Dg75-pSG5 cells respectively have shifted into the lower left quadrant indicative of undergoing the latter stages of apoptosis (Figure 3 65) In the two cell lines expressing EBNA2, the percentage of cells in this quadrant is increased from less than 1% in 10% supplemented media to over 39% and 58% in the Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR cell lines respectively Thus the two cell pools expressing EBNA2 have a lower number of cells apoptosing relative to the two Dg75 cell pools not expressing EBNA2 These results show therefore that expression of EBNA2 in the Dg75 cell line correlates with a measure of increased resistance to apoptosis as induced by serum starvation Additionally, in the cell pool in which EBNA2-CBF1 mutant is expressed this protective effect is consistently somewhat reduced since ~58% of these cells display the characteristic apoptotic light scatter profile relative to ~39% of the cells expressing wild type EBNA2 (Dg75-pSG5EBNA2)

FIGURE 3.65. EXPRESSION OF EBNA2 PROVIDES A MEASURE OF PROTECTION FROM APOPTOSIS INDUCED BY SERUM STARVATION IN DG75 CELLS, BY A MECHANISM WHICH MAY INVOLVE CBF1. (PROPIDIUM IODIDE).

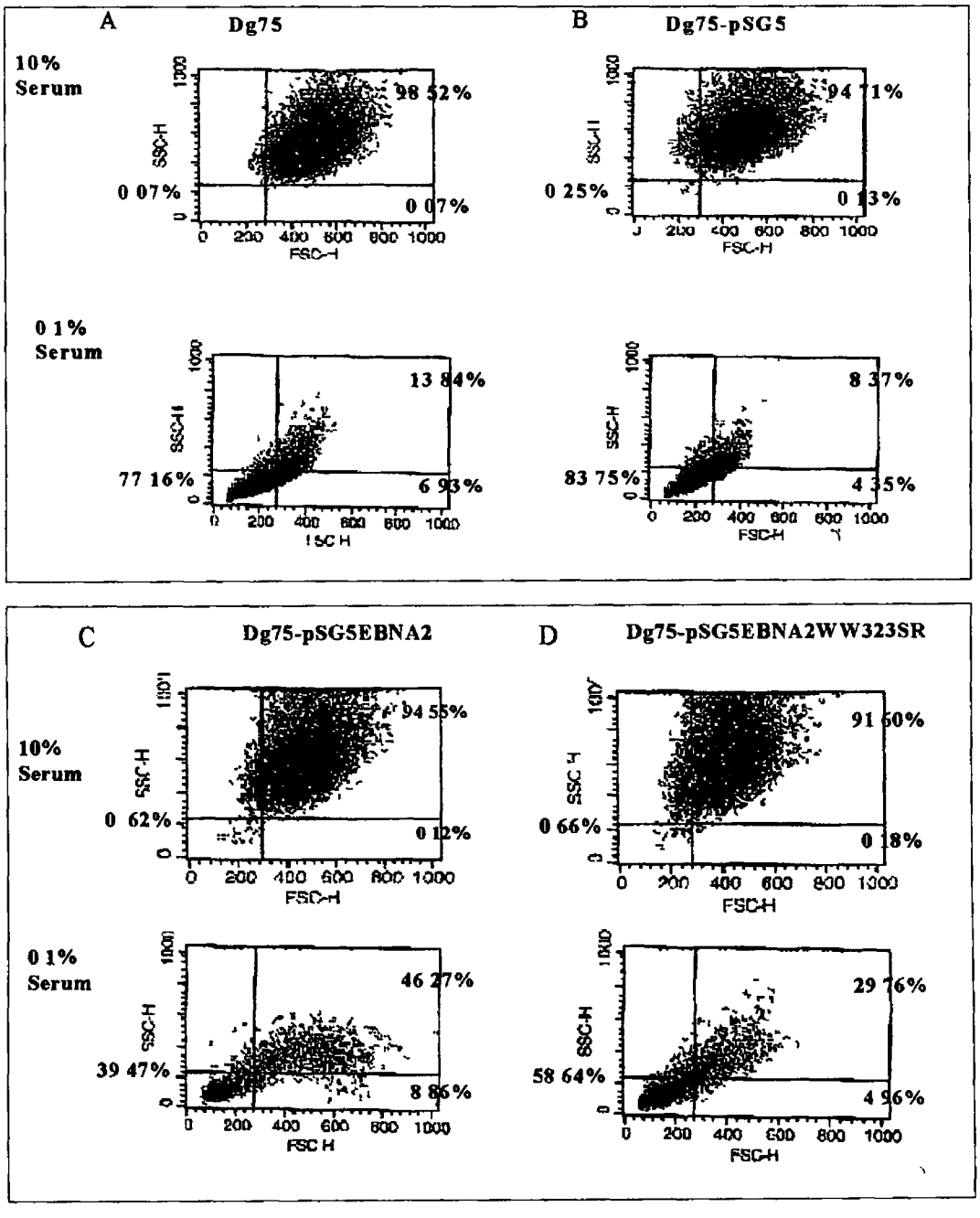


Figure 3 65. Expression of EBNA2 Provides a measure of Protection from Apoptosis Induced by Serum Starvation in Dg75 Cells by a Mechanism which may Involve CBF1. (Propidium Iodide). Using FACS analysis with the BD FACsCAlibur, light scatter in the forward and right angle (Side) directions was measured and representative dot plots were generated with CellQuest software (BD) The indicated cell lines were cultured in 10% and 0 1% serum supplemented RMPI1640 media and the light

scatter patterns analysed where forward scatter (FSC-H) indicates the relative size of the cells and side scatter (SSC-H) indicates the granularity of the cells Quadrants were subjectively placed on each of the dot plots and the percentage of cells in each quadrant deconvoluted by CellQuest software(BD) The shift of the cell population vertically downwards and towards the origin is representative of cells in the late stages of apoptosis Apoptosis was verified by Acridine orange staining (Figure 3 64) as this scatter pattern may also be elicited from necrotic cell populations

3.4 5. EBNA2 EXPRESSION INCREASES THE POPULATION OF DG75CELLS IN THE G0/G1 PHASE OF THE CELL CYCLE.

Further to the previous results, after propidium iodide staining, the percentage of cells in G1, S phase and G2/M of the cell cycle was calculated by subjectively applying markers which provide a measurement of the percentage of cells in each phase Cell samples from the cells cultured in 10% serum supplemented media 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 The percentage of cells in each phase of the cell cycle was calculated by CellQuest software and is shown with its corresponding histogram It can be seen that in the two cell lines which do not express EBNA2, Dg75 and Dg75-pSG5 that the percentage of cells in the G0/G1 phase of the cell cycle is 60% and 62% respectively with 12 8 and 12 3% of cells in the S phase In the two cell lines expressing EBNA2, Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR, the number of cells in the G0/G1 phase is just over 72% in each case, also the percentage of cells in the S phase is reduced to 6 7 and 6 6% respectively These data show that expression of EBNA2 in the Dg75 cell line causes some accumulation of cells in the G0/G1 phase The physiological antiproliferative effect of this has been noted in lymphocytes elsewhere and may be due to EBNA2 up-regulation of c-myc (Kempkes *et al*, 1995b) The percentage of cells in the G2/M, or pre-G1/apoptotic phase, were not altered

FIGURE 3.66 EBNA2 EXPRESSION INCREASES THE POPULATION OF DG75CELLS IN THE G0/G1 PHASE OF THE CELL CYCLE.

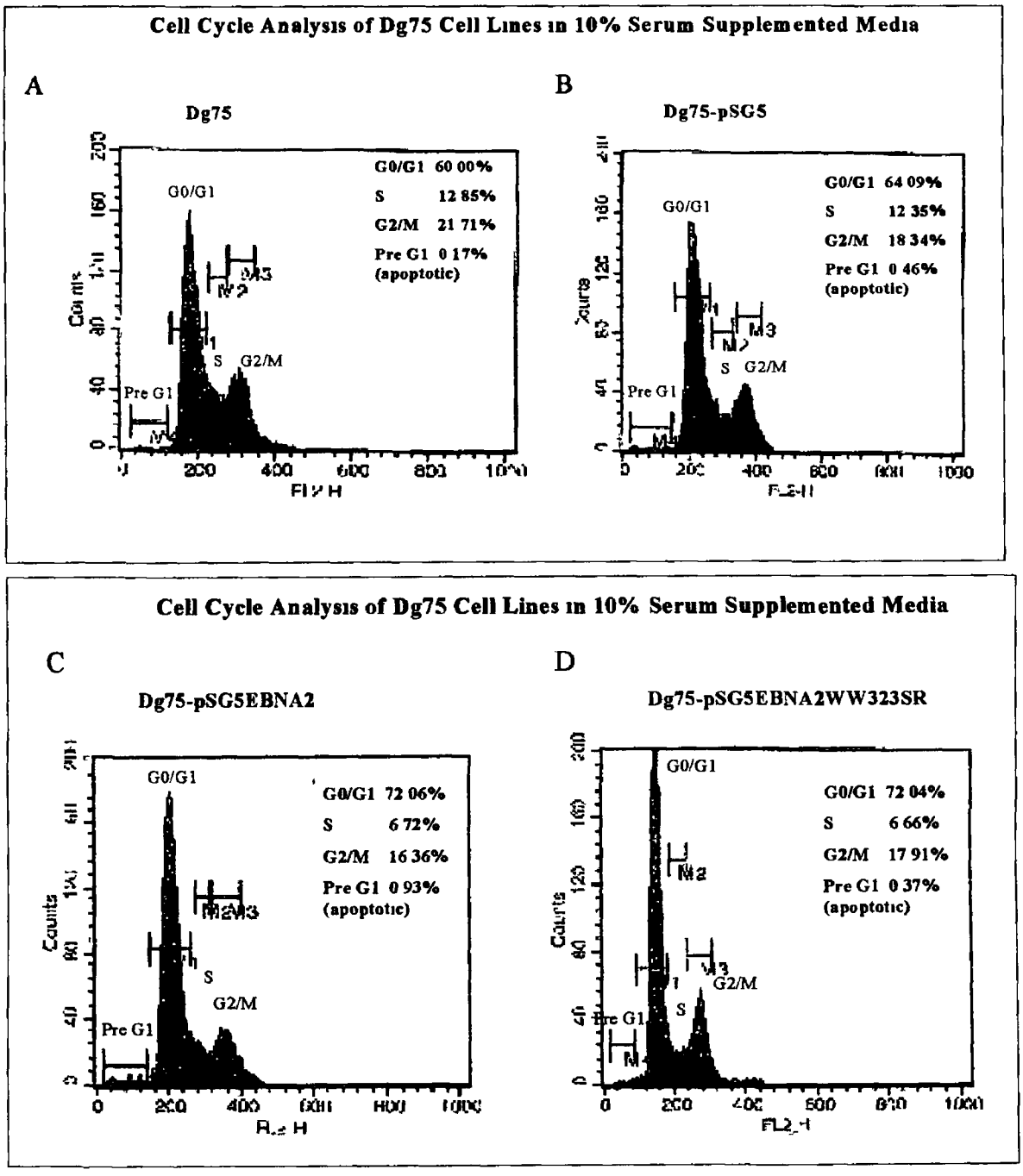


Figure 3 66 EBNA2 EXPRESSION INCREASES THE POPULATION OF DG75CELLS IN THE G0/G1 PHASE OF THE CELL CYCLE. Flourecence from the PI stained cells is used to generate histograms depicting the DNA content of the cells representative of the passage of cells through the cell cycle The peaks representing each phase of the cell cycle and the percentage of cells in each phase is shown It can be seen that in the cell pools expressing EBNA2 Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR (Panels C and D

respectively), that there is an increase (~10%) in the number of cells in the G0/G1 phase of the cell cycle and a 50% reduction in the number of cells in the S phase of the cell cycle relative to the cells in which EBNA2 is not expressed, Dg75 and Dg75-pSG5 (Panels A and B respectively) Thus EBNA2 expression causes cells to accumulate in the G0/G1 phase of the cell cycle

3.4.6. EBNA2 EXPRESSION REDUCES THE NUMBER OF PRE-G1/APOPTOTIC CELLS UNDER CONDITIONS OF SERUM STARVATION - FURTHER EVIDENCE FOR THE PROTECTIVE EFFECT AFFORDED BY EBNA2 EXPRESSION IN DG75 CELLS.

The cellular DNA content can be measured following cell fixation that 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, as cells with low DNA stamability (sub G0-G1 peak) It can be seen from the results (Figure 3 67), that after serum starvation, the percentage of apoptotic cells in the EBNA2 positive cell line Dg75-pSG5EBNA2 is ~48% Expression of EBNA2 results in a diminished sub G0-G1 peak in the EBNA2 positive cell line Dg75-pSg5EBNA2 relative to the EBNA2 negative DG75-pSG5 cell pool which shows ~70% apoptotic cells Thus a lower proportion of cells are apoptotic in the EBNA2 expressing Dg75-pSG5EBNA2 cell line than the EBNA2 negative DG75-pSG5 cell line these results comply with the light scatter data, which also suggest a higher proportion of apoptotic cells in the EBNA2 negative cell lines relative to the EBNA2 positive cell lines These data further imply a role for EBNA2 and thus possibly *bfl-1* in the protection of Dg75 cells from apoptosis induced by growth factor withdrawal

Figure 3.67 EBNA2 EXPRESSION REDUCES THE NUMBER OF PRE G1/APOPTOTIC CELLS UNDER CONDITIONS OF SERUM STARVATION

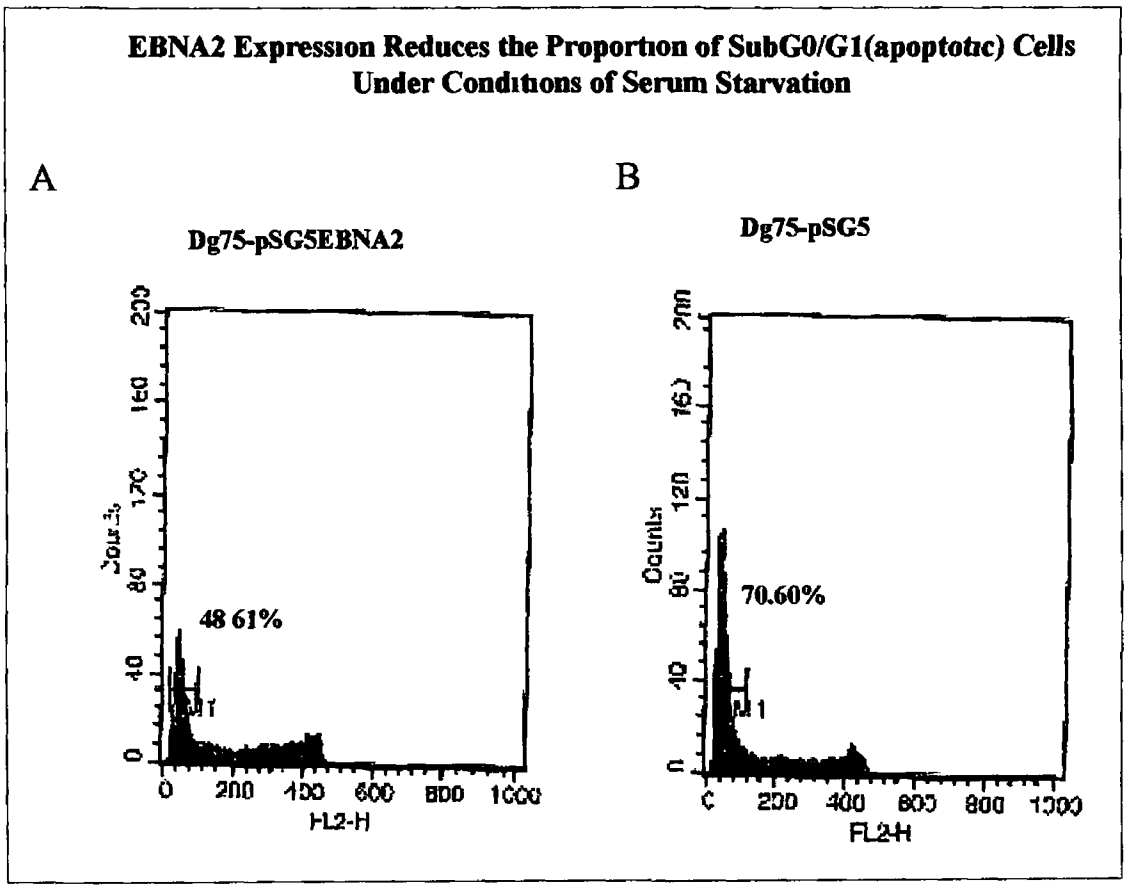


Figure 3.67 EBNA2 EXPRESSION REDUCES THE NUMBER OF PRE G1/APOPTOTIC CELLS UNDER CONDITIONS OF SERUM STARVATION. Dg75 cells were serum starved by culturing in 0.1% serum supplemented media, and fixed and stained as before using PI. Cell fluorescence was then measured by FACS analysis (FacsCalibur, BD) and the resulting histograms generated using CellQuest software. It can be seen under these conditions that expression of EBNA2 in panel (A) above offers some protection from apoptosis indicated by the reduction in the pre G1 peak to ~48%, relative to the cell line in which EBNA2 is not expressed (Panel B) where over 70% of cells are in this region.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)/BANDSHIFT ANALYSIS.

3.5.0. INTRODUCTION

EMSA is widely used in studying the sequence specific binding of nuclear proteins such as transcription factors and is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free double stranded DNA fragments or oligonucleotides. The gel shift assay is performed by incubating a purified protein or a complex mixture of proteins (such as nuclear extracts) with a ^{32}P labelled DNA fragment containing the putative protein-binding site, followed by analysis on a non denaturing ploy acrylamide gel. The specificity of the DNA binding protein for the putative binding site is established by competition experiments using unlabeled DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences. Supershift analysis can also be performed by preincubating the nuclear extract with an antibody to a protein suspected to be present in the complex formed with the labelled oligonucleotide. If the antibody specific protein is present, the radiolabeled protein-DNA complex may be diminished, or "shifted" up the gel due to the increase in size as a result of antibody binding, depending on whether the antibody binds to the DNA binding region of the protein or not.

The results presented so far indicate that increased transcriptional activity of the *bfl-1* gene partly accounts for the EBNA2-mediated up-regulation of steady state levels of *bfl-1* mRNA and Bfl-1 protein, in B cells. The finding that the transactivation of the *bfl-1* promoter is dependent on EBNA2 binding CBF1 suggests that this transcription factor may be involved in mediating EBNA2 responsivity on the *bfl-1* promoter (Figure 3.16). Furthermore, deletion analysis of the 5' region of the gene suggests that the major elements mediating transcriptional activation of the *bfl-1* gene by EBNA2 are located between positions –367 and –129. (Figure 3.21) (positions are relative to the transcriptional start site proposed by Zong et al 1999). A transcription factor search of the *bfl-1* promoter using Transcription Element Search Software (www.cbil.upenn.edu/tess) as well as Transfac/Mat Inspector (MatInspector *V2.2*) and Alibaba (http://www.alibaba2.com) software had revealed the presence of a potential CBF1

binding site at position –243 (GTGGGAA) in the reverse orientation (bp –243 to –249) See Figure 3 22

The potential involvement of the CBF1 site at position –243 of *bfl-1* in the EBNA2 – induced enhancement of *bfl-1* promoter activity was investigated by EMSA using nuclear extracts prepared from DG75-tTA-EBNA2, BL41-ER/EBNA2 and BL41P3HR1-ER/EBNA2 cells before and after induction or activation of EBNA2 Since the studies of the kinetics of induction of *bfl-1*mRNA by EBNA2 in these cells indicated that the magnitude of induction was maximal by 24 hours post induction, (Figures 3 3 3 11 and 3 12), nuclear extracts were prepared from each of the cell lines at the earlier time point of EBNA2 induction or activation of 20hr, when *bfl-1* mRNA levels are still increasing

3.5.2.0. Lack of Demonstrable Binding of an EBNA2-Activated CBF1 Complex to the CBF1 site on the Cp Promoter and the Putative CBF1 site at Position –243 to –249 of the *BFL-1* Promoter Region, in DG75-tTA-EBNA2 Cells.

As a preliminary experiment, the effect of EBNA2 induction on CBF1 binding activity in DG75-tTA-EBNA2 cells was assessed To this end EMSA was performed with nuclear extracts prepared from DG75-tTA-EBNA2 cells before and 20hr after induction of EBNA2 and a pair of 30bp oligonucleotides containing the consensus CBF1 binding site along with the natural flanking sequence from the Cp promoter corresponding to bp-359 to –388 (Ling *et al*, 1994) The same study also showed that EBNA2 can form a stable complex with this Cp-bound CBF1 in an EMSA This pair of oligonucleotides will henceforth be referred to as the Cp probe for the purposes of this thesis A titration experiment of the amount of nuclear protein used in the binding reaction with the Cp probe was first conducted to establish optimal binding conditions Four protein DNA complexes were formed designated C1 C2 C3 and C4 (Figure 3 68) A faint band designated C1a was also observed To determine if these complexes were EBNA2 specific, the binding experiment was repeated but with pre incubation of the nuclear extract with an EBNA2 specific antibody R3 This antibody does not bind to the EBNA2 CBF1 binding domain thus binding of EBNA2 to the Antibody does not interefere with

EBNA2 binding to the radiolabeled Cp probe containing the consensus CBF1 site (Kempkes *et al*, 1995, Zimber Strobl *et al*, 1994) If the complexes are EBNA2 specific binding of the antibody to the complex will increase the size of the complex and retard the electrophoretic mobility of the complex, in other words "shift" the complex up the gel None of these complexes were "shifted" by the addition of an EBNA2 specific antibody thus these complexes do not contain EBNA2 As EMSA analyses have been published elsewhere using this probe, it had been expected that a high molecular weight EBNA2 specific complex would have been visulalised as published previously Despite the fact that this experiment failed to show the anticiated complexes, further EMSA were undertaken with nuclear extracts from the Dg75-tTA-EBNA2 cell line before and after EBNA2 induction to determine whether nuclear proteins could bind to the putative CBF1 site at position –243 to –249 of the *bfl-1* promoter EMSA was performed using a 36bp pair of oligonucleotides derived from the *bfl-1* promoter sequence that includes the putative CBF1 site at position –243 to –249 This oligonucleotide shall heneceforth be referred to as the *Bfl-1* probe for the purposes of this thesis Again three complexes were visualised after separation by non-denaturing plyacrylamide gel (5%) electrophoresis, however, again these three complexes failed to "shift" in response to preincubation with an EBNA2 specific antibody Similar results were obtained with both the Cp and *bfl-1* probes using extracts from the K3 and 9A cell lines before and after activation of EBNA2 Thus no direct interaction between EBNA2 and CBF1 could be shown in this study Shown below is a representative gel using the Cp probe and extracts from the Dg75-tTA cell line The four non-specific complexes are complexes are labeled (Figure 3 68)

Although EBNA2 has been shown to bind to the Cp CBF1 site in EMSA analyses by others the experiments presented here failed to detect any interaction between EBNA2 and the Cp CBF1 binding site or EBNA2 and the putative CBF1 binding site Since the "control" experiment involving the Cp probe failed to bind CBF1 in the cell extracts used it may be that binding conditions in this system have not been optimised In the case of the putative CBF1 binding site it is possible that EBNA2 affinity for the *Bfl-1* CBF1 site is too weak to bind or be detected by these analyses If other transcription factors are involved in the transactivational effect of EBNA2 on the *bfl-1* promoter it may be

beneficial to use a longer oligonucleotide spanning a larger section of the *bfl-1* promoter to include the putative sites for the PU 1 /Ets1 factors Although no direct interaction between EBNA2 and the putative CBF1 binding site could be detected by EMSA analyses, transient transfection data and promoter deletion data already presented indicates that CBF1 binding has a key role in the EBNA2 mediated up-regulation of *bfl-1*

FIGURE 3.68. LACK OF DEMONSTRABLE BINDING OF AN EBNA2-ACTIVATED CBF1 COMPLEX TO THE CBF1 SITE ON THE CP PROMOTER REGION IN DG75-TTA-EBNA2 CELLS.

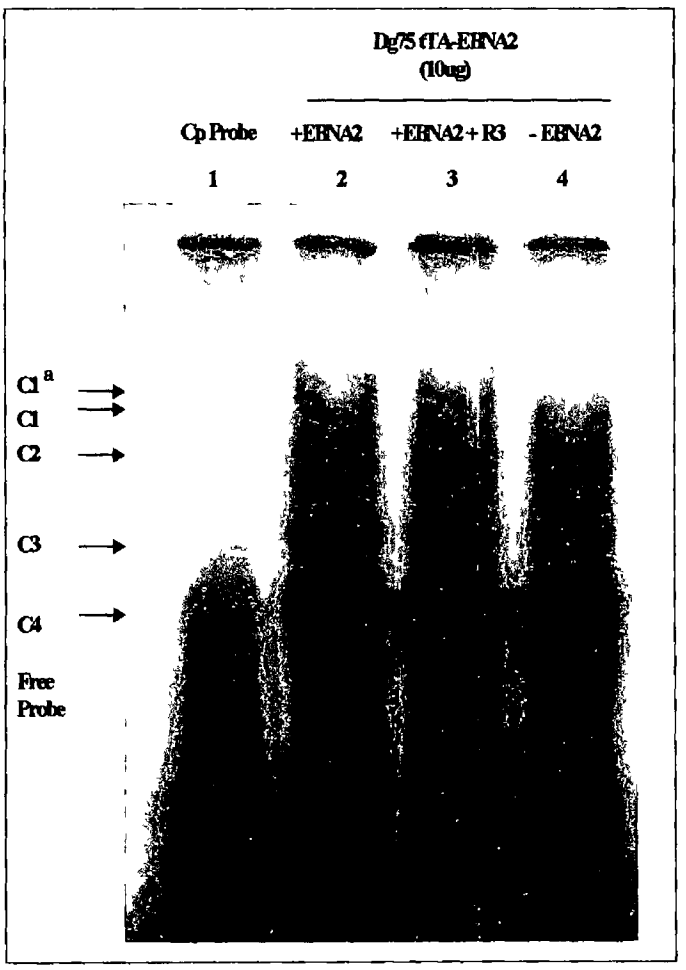


Figure 3.68. EMSA Analysis with a 30bp Oligonucleotide Spanning the CBF1 site Present in the Cp Promoter for Nuclear Protein CBF1 Binding Activity 10 0ug of nuclear extracts prepared from uninduced DG75-tTA-EBNA2 cells or from cells 20hr post induction of EBNA2 were incubated with the

^{32}P labelled Bfl-1 probe After separating the protein-DNA complexes by non-denaturing plyacrylamide gel (5%) electrophoresis (140V 3-4Hrs), the shifted bands were visualized using autoradiography No EBNA2-induction-specific complexes were formed however four protein-DNA complexes designated C1, C2 C3 and C4 were visualised pre and post EBNA2 induction in the Dg75-tTA-EBNA2 cells

CONCLUSION

The ability of EBNA2 to provide cell survival signals by up-regulating the expression of the anti-apoptotic protein Bfl-1 is very significant from the standpoint of the mechamsm of EBV persistence in the memory B cell in peripheral blood In lymphoid follicles, *bfl-1* transcript has been detected in germinal centres, which are the sites for B cell proliferation and differentiation Indeed long-lived B cells are distinguished by elevated expression of *bfl-1/A1* In this regard, Bfl-1 might be viewed as a key determinant of cell fate for immature peripheral B cells The strong induction of *bfl-1* transcripts by proinflammatory cytokines in endothelial leukemic and hemopoietic cells is consistent with a role for protecting them from apoptotic extinction (Choi *et al*, 1995, Karsan *et al*, 1996, Lin *et al*, 1993, Moreb and Schweder 1997) In vivo, 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 and Thorley-Lawson 2000) Thus EBNA2 associated induction of the antiapoptotic Bfl-1 may be important in terms of the EBV associated malignancies in which EBNA2 is expressed

CHAPTER 4

DISCUSSION

DISCUSSION

A major component of the infection strategy of EBV is its suppression of the cellular apoptotic programme (Allday 1996, Klein *et al*, 1994) which functions primarily to ensure persistence in a latent state in the memory B cell compartment of infected individuals (Reviewed by Gregory *et al*, 1995) In vitro, type I BL cell lines (expressing only EBNA1) and various EBV-negative BL cell lines can readily be induced to apoptose by a variety of triggers including growth factor withdrawal, Ca+ ionophore treatment and over-expression of the p53 tumour suppressor gene, (Henderson *et al*, 1991, Okan *et al*, 1995) however type III BL cell lines expressing the full compliment of EBV latent genes have been shown to have a higher apoptotic threshold than their type I counterparts Similarly EBV negative cell lines converted to the type III phenotype after infection with the B95-8 strain of the virus showed increased resistance to apoptosis (Gregory *et al*, 1991) Thus expression of the full spectrum of EBV latent genes reduces susceptibility to apoptosis This increased anti-apoptotic threshold has been partly attributed to the up-regulation of Bcl2 in infected BL cells A number of EBV latent genes have been shown to regulate the expression of Bcl2 including LMP1 EBNA2 and EBNA3B (Henderson *et al*, 1991, Liu *et al*, 1991, Rowe *et al*, 1994, Finke *et al*, 1992, Silins and Sculley 1995) Although Bcl2 is important in conferring the anti-apoptotic effects of EBV infection, numerous other studies implicate Bcl2-independent mechanisms, that may afford infected cells increased resistance to apoptosis (Milner *et al*, 1992, Pezzella *et al*, 1990, Rowe *et al*, 1994)

Recent studies in our laboratory showed that another anti-apoptotic member of the Bcl-2 family, *bfl-1*, is expressed at high levels in LCLs and type III cell lines relative to type I cell lines (D'Souza *et al*, 2000) The physiological or functional relevance of *bfl-1* expression was shown in the same study using transfection analysis, which showed expression of *bfl-1* in a type I (Mutul) cell line protected these cells from apoptosis under sub-optimal growth conditions Thus *bfl-1* must also be considered as an important element in conferring increased resistance to apoptosis on EBV infected cells LMP1 has been shown to up-regulate a number of anti-apoptotic proteins including Bcl-2, A20 and

Mcl-1 (Rowe *et al*, 1994, Fries *et al*, 1996, Laherty *et al*, 1992, Wang *et al*, 1996) and now LMP1 has been shown to up-regulate *bfl-1* expression in the EBV negative BL cell line Dg75 (D'Souza *et al*, 2000) Nonetheless the same experiments also suggest LMP1 independent mechanisms may regulate *bfl-1* expression in EBV infected cells as although *bfl-1*mRNA levels are increased in response to LMP1 induction in Dg75-tTA-LMP1, the levels of *bfl-1* mRNA do not reach the same levels as those in a typical type III BL (MutuIII) expressing the complete set of EBV latent proteins This left the possibility that other EBV latent proteins may stimulate or co-stimulate *bfl-1* up-regulation (D'Souza *et al*, 2000) Further to this, a preliminary experiment in which northern Blotting was carried out with an LMP1-negative type III cell line revealed *bfl-1* mRNA levels were still elevated relative to a type I EBV positive cell line (MutuI) (Figure 3 1) As EBNA2 is the second major effector of phenotypic change in EBV infected cells, EBNA2 was a likely candidate for regulating *bfl-1* expression

This thesis presents the novel findings that EBNA2 up-regulates expression of the cellular *bfl-1* gene by increasing transcription from the *bfl-1* promoter in a B cell specific manner and that this up-regulation is likely to involve a mechanism requiring the cellular transcription factor CBF1 and possibly members of the Ets transcription factor family Despite the fact that EBNA2 and activated Notch/Notch-IC overlap in the subset of genes which they regulate through their shared ability to interact with CBF1, the evidence presented here also suggests that, like CD23, *bfl-1* may be responsive to EBNA2 but not Notch-IC

In order to assess the role of EBNA2 in regulating the *bfl-1* gene and other *bcl2* family members, it was essential to have a system in which EBNA2 could be expressed as the sole EBV protein independently of other EBV latent proteins For this reason two different systems were employed, where (i) EBNA2 expression was induced in the absence of tetracycline and (ii) EBNA2 was activated by addition of estrogen

Although previous studies (Floettmann *et al*, 1996) using the tet-regulatable system mainly focused on the effects of LMP1 induction on expression of cell surface receptors

involved in mediating cell signaling responses affecting cellular activation and proliferation, these studies also demonstrated that this system was suitable for expressing EBNA2 in a tightly controlled fashion in BL cells and was thus suitable for studying the phenotypic changes induced by EBNA2 expression in a BL cell environment

Initial experiments in this study, using the DG72-tTA-EBNA2 cell line, revealed the novel finding that when present as sole EBV protein, EBNA2 expression led to an increase in *bfl-1* mRNA and protein levels and these results were verified by both RPA and Northern blot analyses (Figures 3 3 and 3 5) The increase in the level of *bfl-1* mRNA could have been attributable either to an increased rate of transcription from the promoter of the *bfl-1* gene or a stabilizing effect of EBNA2 on *bfl-1* mRNA Although LMP1 expression has been shown to stabilize the *bfl-1* transcript (D'Souza *et al*, 2000), the possibility that EBNA2 expression was in some way stabilizing *bfl-1*mRNA was unlikely for a number of reasons, (i) all evidence suggests that the primary function of EBNA2 is as a *trans*-activator of viral and cellular genes (ii), the fold-activation of the *bfl-1* promoter in transfection experiments is similar (6-8 fold) to the increase m *bfl-1* mRNA induction in response to EBNA2 expression seen in Northern Blot and RPA analyses and (iii) this induction occurs immediately upon activation of EBNA2 in BL-derived cell lines (after 3hours) (Figures 3 11 and 3 12) and when EBNA2 is detectable m the Dg75-tTA-EBNA2 cell line after 12 hours (RPA Figure 3 3 lane not shown) Thus *bfl-1* induction appears to be an early and direct effect of EBNA2 expression These results comply with several lines of evidence, which indicate that *bfl-1* and its mouse homologue A1 are early response genes (Lin *et al*, 1993, Hu *et al*, 1998) Also the significant induction of *bfl-1* mRNA levels in response to EBNA2 expression were observed at a time-point when physiologically relevant levels of EBNA2 are observed, since *bfl-1* mRNA up-regulation is seen where EBNA2 levels are comparable to endogenous levels in a typical LCL (X50-7)

A 5 fold induction of *bfl-1* mRNA in response to EBNA2 expression was recorded in conjunction with EBNA2 expression after 24 hours, Bfl-1 protein was detected at low levels prior to EBNA2 induction but levels were immediately up-regulated in response to

EBNA2 activation with a 5 fold induction detected after 24 hours (Figure 3 3) Although multiprobe RPA analysis provided a quantitative way of measuring *bfl-1* mRNA induction, northern blotting was carried out to confirm the increase in the level of *bfl-1*mRNA and to determine the size the transcript from the *bfl-1* gene (Figure 3 5) One *bfl-1* mRNA of ~0 8Kb was detected in all cases, in agreement with the previously reported size of the transcript from this gene using Northern blotting (Kenny *et al*, 1997) However recent studies employing reverse–transcriptase polymerase chain reaction (RT–PCR) and primers specific for *bfl-1* cDNA revealed two *bfl-1* transcripts The original *bfl-1* transcript, and a second transcript that encodes a shorter protein *bfl-1S* (Ko *et al*, 2003) Using gene mapping and splicing site prediction databases, Ko *et al*, 2003 suggest a 3-exon structure for the *bfl-1* gene The production of transcriptional variants of *bfl-1* was found to result from the alternative inclusion or exclusion of the 56bp exon II The shorter transcript encodes a 175 amino acid protein while the longer transcript codes for a 163 amino acid protein The longer transcript encodes a shorter protein since the inclusion of exon II results in early translational termination by an upstream stop codon (Ko *et al*, 2003) The suggested three exon structure of *bfl-1* and splicing involved make it similar to that of ICH1 (Caspase 2) which similarly consists of three exons and produces two splice variants, Ich-1 a pro-apoptotic protein and Ich-1S an anti-apoptotic protein produced by exclusion of a 61bp exon (Ko *et al*, 2003) Both *bfl-1* proteins however have anti-apoptotic properties and have been shown to protect against apoptosis in a variety of cell types (D'Sa-Eipper and Chinnadurai 1996, Karsan *et al*, 1996, Zong *et al*, 1999, Wang *et al*, 1999, Ko *et al*, 2003) However the two variants may do this by different mechanisms since the Bfl-1S protein is directed to the nucleus and lacks the trans-membrane domain common to anti-apoptotic members of the Bcl2 family including *bfl-1* (Ko *et al*, 2003) It is important to note that the Northern blotting and RPA experiments presented in this thesis would not have distinguished between the two *bfl-1* transcripts as (i) they are almost identical in length and (ii) the RPA probe used is from the 5' end of the *bfl-1* mRNA where no sequence differences exist between the two variants Hence the degree to which either splice variant is induced by EBNA2 yet remains to be determined.

A second system was also used, in which the expression of functional EBNA2 was controlled by the presence of estrogen Using the BL41-K3 and BL41/P3HR1-9A cell lines, it has been shown that EBNA2 fusion protein is virtually inactive in the absence of estrogen (Kempkes *et al*, 1995a) However in the presence of hormone, the EBNA2 fusion protein is activated to its functional form and functions as wild type EBNA2 in that it up-regulates the expression of the cell surface markers CD21 and CD23 and trans-activates known EBNA2 responsive promoters including LMP1, TP1, TP2, BamH1 C and W promoters (Kempkes *et al*, 1995a) The interaction of EBNA2 with CBF1 is also rendered dependent on the presence of estrogen in these cell lines (Kempkes *et al*, 1995, Strobl *et al*, 2000) Activation of the EBNA2 fusion protein in these cell lines was verified by probing for the induction of CD21 (a known target gene of EBNA2) by Northern blotting, which showed up-regulation after only 3 hours following EBNA2 activation (Figures 3 7 and 3 8) The activated EBNA2 fusion protein also up-regulated *bfl-1*mRNA and protein levels in both cell lines and again the effect was almost immediate to EBNA2 activation suggesting the effect on *bfl-1* is an early and direct effect of EBNA2 activation (Figure 3 9) These results confirm that *bfl-1* is an EBNA2 responsive gene, this time using a second well-worked EBV-negative B-cell line (BL41)

Since LMP1 is a target gene of EBNA2 (Abbot *et al*, 1990, Fahraeus *et al*, 1990, Wang *et al*, 1990, Zimber-Strobl *et al*, 1991) and EBNA2 trans-activates the LMP1 promoter, it had to be verified that the effect of EBNA2 activation on *bfl-1* mRNA in the BL41/P3HR1 9A cell line was indeed due to activation of EBNA2 and not EBNA2-induced LMP1 Thus Western blotting was carried out on extracts from the BL41/P3HR1 9A cell line (Figure 3 10) It can be seen however that LMP1 is not detected in this cell line prior to or post-activation of EBNA2, thus implying that LMP1 is not involved in the up-regulation of *bfl-1*mRNA in this cell line The fact that (i) both native EBNA2 (expressed in the Dg75tTA EBNA2 cell line) and the ER-EBNA2 (in the BL41 K3 cell line) both up-regulated the *bfl-1* gene in the absence of other EBV latent proteins and (ii) the observation of elevated *bfl-1*mRNA in an EBV positive but LMP1 negative cell line (BL41P3HR1 9A), provided compelling evidence that EBNA2 trans-activates this gene in an LMP1 independent manner Although LMP1 has been shown to independently up-

regulate a number of the same genes as EBNA2 including CD21, CD23 and more recently *bfl-1* (Wang *et al*, 1990, Peng *et al*, 1992, Kieff E 1996, D'Souza *et al*, 2000), the ensemble of results presented so far, also adds the *bfl-1* gene to the list of cellular genes which can be independently activated by expression of either LMP1 or EBNA2 Other genes however such as the cellular BATF gene are EBNA2 but not LMP1 responsive (Johansen *et al*, 2003)

Although EBNA2 expression generally results in the transcriptional up-regulation of LMP1 this is not the case for the BL41P3HR1-9A cell line, and the molecular basis for this has yet to be investigated It is possible that the LMP1 gene has been inactivated by mutation or deletion, in any case the absence of detectable levels of LMP1 even after activation of a functional EBNA2 capable of up-regulating both *bfl-1* and CD21 independently, provides further evidence that *bfl-1* is directly responsive to EBNA2 These results are further confirmed by the RPA analyses in Figures 3 11 and 3 12

Cyclohexamide (an inhibitor of translation) was added to the E2/ER and ER-mNotch-IC cell lines, in the presence or absence of estrogen, and six hours later, samples were taken for mRNA analysis (in accordance with Strobl *et al*, 2000) Although very faint bands were visible in the Northern blot experiments, analysis of the *bfl-1*mRNA transcripts in the RPA analyses showed significant induction of *bfl-1* upon activation of EBNA2 and mNotch-IC in the presence of cyclohexamide in the estrogen responsible cell lines This suggests that upon activation of these trans-activators, an up-regulatory effect on *bfl-1* occurs in the absence of new protein synthesis (Figures 3 11 and 3 12) Since activation of mNotch-IC did not appear to up-regulate *bfl-1*mRNA, the effect of cyclohexamide is more difficult to interpret though possibly, cyclohexamide is indirectly stabilising *bfl-1* mRNA molecules by preventing the re-synthesis of short-half life proteins that otherwise promote its degradation (Figure3 55)

Apart from up-regulation of *bfl-1*mRNA, the mRNA species from another Bcl2 family member bik, is down regulated in response to expression or activation of EBNA2 (Figures 3 3, 3 11 and 3 12) Bik is a 185 amino acid pro-apoptotic protein member of the Bcl2 family It is thought of as the minimal death module since it only contains the BH3

homology domain prevalent in pro-apoptotic members of this family (Boyd *et al*, 1995, Elangovan *et al*, 1997) As with other pro-apototic members of the Bcl2 family including Bak and Bax, (Oltvai *et al*, 1993, Chittenden *et al*, 1995), Bik heterodimerizes with anti-apoptotic proteins Bcl2 and Bcl-x_l via its BH3 domain (Boyd *et al*, 1995, Tong *et al*, 2001) and their pro-apoptotic activities are restrained by post-translational modifications that cause their sequestration from pro-apoptotic proteins such as Bcl2 (reviewed by (Puthalakath *et al*, 2002) Expression of *bik* triggers apoptosis in a range of cell lines and is up-regulated by expression of p53 and a number of tumour suppressing therapeutic agents including doxorubicin in lymphoma cell lines (Mathai *et al*, 2002, Panaretakis *et al*, 2002) Bik expression has also been shown to induce apoptosis in a range of epithelial chemoresistant human tumor cell lines (Tong *et al*, 2001, Radetzki *et al*, 2002) In the case of the experiments presented here, the combination of an up-regulatory affect on the anti-apoptotic *bfl-1* gene and a down-regulation of the pro-apoptotic gene *bik* is not contradictory, and fits well with the concept that the apoptotic threshold of cells is controlled by the ratio of pro- and anti-apoptotic proteins expressed therein (reviewed by Korsmeyer 1995) In any case the combination of up-regulation of *bfl-1* and down regulation of *bik* would suggest a cellular environment with increased resistance to apoptosis

Bfl-1 has been shown to interact with a number of pro-apoptotic proteins including Bax and Bad (Bae *et al*, 2001) Another pro-apoptotic protein Bid has been shown to interact with Bfl-1 in vitro thus preventing it interacting with other pro-apoptotic proteins Bak and Bax (Werner *et al*, 2002) The regulation of the *bik* gene by EBNA2 expression is a subject that merits further investigation which is ongoing in the laboratory, but for the purpose of this study was not pursued any further It can be concluded that, at the mRNA level, none of the other Bcl2 family members were affected by EBNA2 induction in the Dg75tTaEBNA2, BL41K3, or BL41P3HR19A cell lines (Figures 3 3, 3 11, 3 12)

EBNA2 trans-activates a number of viral and cellular genes including the EBNAs themselves (EBV BamH1C and W promoters), LMP1, LMP2A, and the cellular CD21, CD23, c-fgr, c-myc and EBII/BLR2 genes (Woisetschlaeger *et al*, 1990, Sung *et al*,

1991, Abbot *et al*, 1990, Fahraeus *et al*, 1990, Wang *et al*, 1990, Zimber-Strobl *et al*, 1991, Laux *et al*, 1994, Calender *et al*, 1987, Wang *et al*, 1987, Cordier *et al*, 1990, Knutson *et al*, 1990, Patel *et al*, 1990, Burgstahler *et al*, 1995, Kaiser *et al*, 1999) More recently another cellular gene called BATF, has been identified as a trans-activational target of EBNA2 (Johansen *et al*, 2003) The ensemble of the results presented in this thesis adds *bfl-1* to the list of cellular genes trans-activated by EBNA2

The EBNA2 responsive elements for the Cp, LMP1, LMP2 CD21 and CD23 promoters have all been characterized (Woisetschlaeger *et al*, 1990, Sung *et al*, 1991, Abbot *et al*, 1990, Fahraeus *et al*, 1990, Wang *et al*, 1991) and are known to contain at least one CBF1 binding site (Zimber-Strobl *et al*, 2001) CBF1 binds to the DNA sequence 5'GTGGGAA3' (Ling *et al*, 1994, Tun *et al*, 1994), and this sequence was identified in each of the promoters above The relevance and importance of CBF1 binding is that EBNA2 does not bind directly to DNA but is targeted to responsive promoters via this cellular protein CBF1/RBP-jk (Ling *et al*, 1993, Waltzer *et al*, 1994)

The contribution of CBF1, in mediating the EBNA2 responsivity of the *bfl-1* promoter was assessed in two ways, (i) by assessing the requirement of the EBNA2 CBF1 binding site in region CR5 of the EBNA2 protein (Figure 3 16) (Ling *et al*, 1993) and (ii) by assessing the requirement of any putative CBF1 binding sites on the *bfl-1* promoter itself Transient transfections were performed using a pair of EBNA2 expression plasmids, pSG5EBNA2 (PDL151) and pSG5EBNA2WW323SR (PDL152) (Ling et al, 1993) The latter expresses an EBNA2 mutant that can no longer bind to CBF1 The data from these and further transfections using a series of *bfl-1* promoter reporter constructs, (Figures 3 16-3 21) led to the delineation of a region between -367 and –129 (containing a potential CBF1 binding site at position –243/–249 on the *bfl-1* promoter (Figure 3 22) In order to assess the contribution of this motif to EBNA2 responsiveness of the *bfl-1* promoter, site directed mutagenesis of this site was performed and transient transfections were also undertaken with these paired promoter reporter constructs (Figures 3 26-3 30) The results presented m this study show that both the EBNA2 CBF1 binding site (region

CR5) and the novel CBF1-binding motif on the promoter were essential for mediating EBNA2 trans-activation of the *bfl-1* promoter, thereby implying a critical role for CBF1 in this process

Limited studies with the *bfl-1* promoter have been published One has shown that NF-kB is required for mediating TNF-α induced up-regulation of the *bfl-1* promoter (Zong *et al*, 1999) The same study also showed that expression of the NF-kB subunit c-rel also led to the induction of *bfl-1* promoter activity and that this activity was mediated by binding to an NF-kB site at position –833 of the promoter (Zong *et al*, 1999) Numerous other groups have also identified *bfl-1* as an NF-kB responsive gene (Grumont *et al*, 1999, Wang *et al*, 1999, D'Souza *et al*, 2000, Cheng *et al*, 2000) More recent studies however have also shown that regulation of the *bfl-1* promoter activity may be more complex than originally anticipated with an NF-kB dependent enhanseosome-like complex binding to the 5' regulatory region of the *bfl-1* promoter, and that this enhanseosome complex includes the transcription factors NF-kB, AP1, C/EBPβ, HMGI-C and p300 (Edelstein *et al*, 2003) To date, no CBF1 dependent mechanisms of regulating the *bfl-1* promoter have been reported however, and this study provides the first evidence that CBF1 is required to mediate EBNA2 associated up-regulation of the *bfl-1* promoter, at least in a B cell context

In this study, transient transfections using a luciferase *bfl-1*-promoter reporter construct showed a dose dependent increase in *bfl-1* promoter activity in response to EBNA2 expression (Figure 3 16) However expression of the mutant EBNA2 which no longer binds CBF1 (Ling *et al*, 1993, Ling *et al*, 1995) only just trans-activated the promoter above basal levels over a range of quantities of vector tested (Figure 3 16) EBNA2 trans-activated the *bfl-1* promoter in two other well-worked EBV-negative cell lines (Bjab and BL41), while the inability of the CBF1 binding mutant of EBNA2 to substantitively trans-activate the *bfl-1* promoter was also repeated in these cell lines (Figure 3 17) These results led to the conclusion that *bfl-1*mRNA up-regulation seen in the RPA and Northern blot analyses (Figures 3 3, 3 5, 3 9, 3 11 and 3 12) was occurring

by trans-activation of the *bfl-1* promoter and that this process required EBNA2-CBF1 binding

Because of the B cell tropism of EBV, (reviewed by Faulkner 2000), many of the studies involving EBNA2 are undertaken in a B cell derived background, however EBNA2 can trans-activate genes in a cellular environment of non B cell lineage thus B cell specific transcription factors are not essential in mediating EBNA2 effects on certain genes, such as LMP1 and CD21 in the T-cell lines Jurkat and 293T (T cells) (Larcher *et al*, 1995, Peng *et al*, 1993, Wang *et al*, 2000) Nonetheless, EBNA2 also up-regulates certain genes in a B cell specific manner, including BATF (Ko *et al*, 2003) In order to investigate if the effect of EBNA2 on the *bfl-1* promoter was a B cell specific effect, similar transient transfections were undertaken in a limited range of cell lines including T cell, epithelial and smooth muscle cell lines The results imply that trans-activation of the *bfl-1* promoter may be a B cell specific effect with little trans-activation of the *bfl-1* promoter reporter construct observed in any of the other cell lines (Figure 3 18) The inability of EBNA2 to trans-activate the *bfl-1* promoter in non B cell background may indicate that B cell specific transcription factors may be essential in facilitating EBNA2 mediated trans-activation of the *bfl-1* promoter Interestingly, using Transcription Element Search Software (TESS), putative consensus binding sites for B cell and macrophage specific members of the Ets family of transcription factors (Klemsz *et al*, 1990) Ets1 and PU 1 were also identified at various positions on the *bfl-1* promoter (Figure 3 22), and the failure of EBNA2 to trans-activate the *bfl-1* promoter in non-B cell lines may imply a key role for these B cell specific transcription factors in conferring EBNA2 responsiveness on the *bfl-1* promoter

Site directed mutagenesis of the putative CBF1 binding site was undertaken and a pair of *bfl-1* promoter luciferase reporter constructs were generated, (-1374/+81 wt*bfl-1* Luc and –1374/+81 mCBF1*bfl-1* Luc), identical except for the abolition of the CBF1 binding site in one reporter construct (-1374/+81mCBF1*bfl-1* Luc) (Figures 3 23-3 25) Transient transfection studies with the paired promoter reporter constructs demonstrated the requirement of the putative CBF1 site (at position –243 to –249) on the *bfl-1* promoter

was essential for conferring EBNA2 responsiveness on the *bfl-1* promoter (Figures 3 26A and 3 27) Co-transfection with the vector expressing wild-type EBNA2 resulted in a consistent up-regulation of the promoter however the CBF1 mutated promoter was only very weakly trans-activated (Figure 3 27) These results combined with the fact that the non-CBF1 binding EBNA2 mutant expression plasmid (pSG5EBNA2WW323SR), does not trans-activate the wild type *bfl-1* promoter, all imply a crucial role for CBF1 in mediating the EBNA2 induced activity of the *bfl-1* promoter This requirement was also demonstrated in other BL cell lines BL41 and Bjab and the same trend was apparent in the estrogen responsive cell lines where activated EBNA2 consistently trans-activated (albeit to a lesser extent), the wild type but not the CBF1 mutated *bfl-1* promoter reporter construct (Figures 3 28-3 30) Although neither EBNA2 nor the CBF1 mutated EBNA2 trans-activate the mutant *bfl-1* promoter, co-transfection of the mutated *bfl-1* promoter with an LMP1 expression plasmid (pSG5LMP1) resulted in average of 4 5 fold trans-actiavtion Thus, although essential to EBNA2 trans-activation, the putative CBF1 site at position –243 to –249 is not an essential basal element required for of *bfl-1* promoter activity (Figure 3 37B)

In the absence of EBNA2/Notch-IC, CBF1 acts as a repressor of transcription (Hsieh *et al*, 1995, Hsieh *et al*, 1996) It does so in part by tethering a histone deacetylase co-repressor complex to the promoter (Dou *et al*, 1994), (Hsieh *et al*, 1995, Waltzer *et al*, 1995, Kao *et al*, 1998, Zhao *et al*, 2000) A number of proteins have been identified as members of the CBF1 repression complex, these include, SMRT and HDAC1 (Kao *et al*, 1998), and CIR, Sap30, and HDAC2 (Hsieh *et al*, 1999) as well as SKIP (Zhou *et al*, 2000, Zhou *et al*, 2000) (Figure 1 6) Some of the members of the repression complex including Sin3A and SMRT were identified in the CBF1 HDAC repression complex due to their association with other HDAC-associated repression complexes (Hassig *et al*, 1997, Heinzel *et al*, 1997, Laherty *et al*, 1997, Nagy *et al*, 1997) CBF1-HDAC repression is thought to be attributable to histone deacetylation which leads to chromatin remodelling and destabilization of transcription factor TFIID-TFIIA interactions (Olave *et al*, 1998) EBNA2 masks CBF1 repression by binding to the repression domain of CBF1 and then introducing its trans-activation domain which induces transcriptional

activation of target promoters (Hsieh *et al*, 1995, Hsieh *et al*, 1996) (Figure 1 6) EBNA2 also interacts with the SNF-SWI complex which is also involved in chromatin remodelling and this, in conjunction with dislocation of the HDAC repression complex may assist in making target promoters more accessible to the EBNA2 trans-activation domain (Wu *et al*, 1996)

EBNA2 has also been shown to interact with SKIP in vitro and competes with SMRT for contacts on both CBF1 and SKIP, since different elements within EBNA2 confer the binding ability of EBNA2 to CBF1 and SKIP respectively it is likely that contacts on both SKIP and CBF1 are required for effective EBNA2 targeting to DNA (Zhou *et al*, 2000, Ling *et al*, 1993) SKIP was also demonstrated to interact with other members of the CBF1 repression complex including CIR, HDAC2, such that a new model for the CBF1 repression complex was constructed (See Figure 1 6 adapted from Zhou *et al*, 2000) Ultimately the ability of EBNA2 to bind CBF1 and SKIP and thereby displace the CBF1-associated repression complex from target promoters is a model which, due to the apparent importance of CBF1, may apply to EBNA2-mediated trans-activation of the *bfl-1* promoter

Optimal activation of promoters by EBNA2 often requires cooperation by other transcription factors in addition to CBF1 (Meitinger *et al*, 1994) In the LMP1 promoter, multiple transcription factor binding sites have been identified One transcription factor, PU 1/Spi-B, which also interacts directly with EBNA2, has been found to be essential in mediating EBNA2 activation of LMP1 (Johannsen *et al*, 1995, Laux *et al*, 1994) It has also been shown that a POU domain protein is involved in the EBNA2 mediated trans-activation of LMP1 (Sjoblom *et al*, 1995) In the Cp promoter, the EBNA2 responsive element also contains a CBF2 binding site adjacent to the CBF1 binding site and this also contributes to EBNA2 responsiveness (Ling et al, 1993, Fuentes-Panana et al, 1998) Also, in the LMP2A promoter, beside CBF1, two other as yet unidentified proteins bind to the EBNA2 responsive element to contribute to EBNA2 responsiveness (Hofelmayr et al, 1999) These data may be significant in the case of the *bfl-1* promoter in that sequence analysis of the promoter sequence, identified three putative Ets1 and one

possible PU 1 binding site(s) in close proximity to the crucial putative CBF1 consensus site The arrangement and proximity of multiple transcription factor binding sites in the EBNA2 responsive element of the *bfl-1* promoter is reminiscent of EBNA2 responsive elements in other EBNA2 target promoters including the Cp, CD23, LMP1 and Tp1 promoters (Table 3 1 - adapted from Le Roux *et al*, 1993) Sequence alignment of the EBNA2 responsive elements in each of these promoters and the *bfl-1* promoter reveals the presence of 2 regions of homology, firstly the core GTGGGAA sequence which recognizes/binds CBF1 (Zimber-Strobl *et al*, 1993) which was identified (in the reverse orientation) on the *bfl-1* promoter This motif has been identified in each of the other EBNA2 responsive promoters shown in Table 3 1 A possible PU 1 consensus binding site was also identified on the *bfl-1* promoter and sequence analysis shows this site as similar to the PU 1 site found in the EBNA2 responsive LMP1 promoter The LMP1 PU 1 site is also highlighted Table 3 1 (Ling *et al*, 1993) Thus the EBNA2 responsive element in the *bfl-1* promoter has a similar arrangement to other known E2REs in well-studied EBNA2 target promoters including the Cp, LMP1 and CD23 promoters

The trans-activational effect of EBNA2 is generally due to its binding of CBF1 which subsequently binds to cognate DNA sequences thereby making the EBNA2 trans-activation domain available to target promoters, (Grossman *et al*, 1994, Henkel *et al*, 1994) EBNA3 inhibition of EBNA2-CBF1 mediated trans-activation of target promoters is based on the interaction of the EBNA3 proteins with CBF1 and members of the CBF1 repression complex, (Zhao *et al*, 1996, Robertson *et al*, 1995, Dalbies-Tran *et al*, 2001, Knight *et al*, 2003) therefore any inhibitory affect of the EBNA3 proteins on EBNA2 mediated trans-activation of the *bfl-1* promoter, would further imply a key role for CBF1 in facilitating EBNA2 trans-activation of *bfl-1* To investigate this, DG75 cells were transfected with the EBNA2 expression plasmid pSG5EBNA2 and the *bfl-1* promoter reporter constructs (-1374/+81 wt*bfl-1* Luc and -1374/+81 mCBF1*bfl-1* Luc) Increasing quantities of the EBNA3 expression plasmids were also added A dose dependent decrease in EBNA2 mediated trans-activation of the *bfl-1* promoter was recorded upon addition of increasing quantities of each of the EBNA3A, 3B and 3C expression plasmids (Figures 3 31-3 33) These results again indicate a key role for CBF1 in

mediating the EBNA2 trans-activational effect on the *bfl-1* promoter These results comply with the findings of others, which show the EBNA3 proteins repress EBNA2-CBF1 mediated trans-activation of target genes including LMP1 TP2 Cp (Robertson *et al*, 1995, Zhao *et al*, 1996, Johannsen *et al*, 1995, Le Roux *et al*, 1994) and recently c-myc (Cooper *et al*, 2003)

Several lines of evidence are consistent with the possibility that EBNA3A, 3B and 3C regulate the transcription of genes with CBF1 binding sites EBNA3A, 3B and 3C can bind to CBF1 in vitro and in yeast cells (Marshall *et al*, 1995, Robertson *et al*, 1995) can associate with CBF1 in human lymphoblasts (Robertson *et al*, 1995, Robertson *et al*, 1996) and can specifically block EBNA2 trans-activation of the LMP1, LMP2 Cp, CD21 and CD23 promoters in transient transfection assays (Le Roux *et al*, 1994, Marshall *et al*, 1995, Robertson *et al*, 1995, Lin *et al*, 2002, Zhao *et al*, 1996, Dalbies-Tran *et al*, 2001) It has also been shown that EBNA3C interacts with HDAC1 and 2 and another co-repressing member of the CBF1 repression complex Sm3A (Radkov *et al*, 1999, Knight *et al*, 2003) This interaction suggests that the association of EBNA3C with CBF1 may also include complexing with HDACs and another molecule (ProTα) containing deacetylase activities in EBV-transformed cells (Knight *et al*, 2003) Thus, apart from binding to this complex and preventing CBF1 from recognizing cognate DNA sequences, EBNA3A may also actually stabilize the CBF1 repression complex similarly to RPMS1 (See figure 1 14 chapter 1) In any case the negative effects of EBNA3A or 3C on EBNA2 mediated trans-activation of the *bfl-1* promoter provides strong evidence that CBF1 and the CBF1 repression complex are involved

The EBNA3 proteins have also been shown to have trans-activational properties EBNA3C can trans-activate some EBNA2-responsive genes including LMP1 in G1 arrested cells and CD21 in EBV negative BL cells (Allday 1996, Wang *et al*, 1990) Also, similarly to EBNALP, EBNA3C has been shown to cooperate with EBNA2 in trans-activating the LMP1 promoter in EBV negative cells (Lin et al, 2002) EBNA3B can up-regulate CD40 and vimentin expression m non EBV infected BL cells (Silins and Sculley 1994) and stable co-expression of the three EBNA3s in EBV negative DG75

cells up-regulates pleckstrin (Kienzle *et al*, 1999) Also EBNA3C has a glutamine-proline rich domain that can functionally substitute for the EBNA2 acidic domain transcriptional activation (Cohen *et al*, 1991, Marshall *et al*, 1995) EBNA3A and 3B are distantly homologous to 3C however these also have similar glutamine-proline rich regions and therefore may also exert their trans-activational functions in the same manner (for sequence comparison see Sample *et al*, 1990) As a result of their trans-activational ability and in order to assess any effect of the EBNA3 proteins on the *bfl-1* promoter, each of the EBNA3 expression plasmids were individually co-transfected with the wild type and CBF1 mutated *bfl-1* promoters in the absence of EBNA2 expression vector Despite their trans-activating abilities, it can be seen in Figure 3 34 that EBNA3A EBNA3B and EBNA3C did not trans-activate the *bfl-1* promoter (-1374/+81 wt*bfl-1* Luc) or its CBF1 mutated counterpart (-1374/+81 mCBF1*bfl-1* Luc)

Epstein-Barr virus (EBV) has been found to express various spliced RNAs transcribed rightward from the region spanning nucleotide positions 150,000 to 161,000 on the EBV B95-8 genome These have been referred to as complementary strand transcripts (CSTs), *Bam*HI A rightward transcripts (BARTs), or the BARF RNAs (Chen *et al*, 1999) The function of the BARTs is unknown Deletion of the *Bam*HI-A region of the genome does not affect EBV immortalization of B cells in vitro indicating that the BARTs are unlikely to contribute directly to immortalization On the other hand, the consistent expression of these transcripts in EBV-associated epithelial and B-cell tumors suggests that they are making some contribution in vivo One such putative protein, RPMS, is nuclear in transfected cells (Chen *et al*, 1992, Smith *et al*, 2000) and was shown to bind to CBF1 in glutathione-S-transferase (GST) affinity assays and to interfere with EBNA2 and Notch-IC activation of reporters containing CBF1 binding sites in transient-expression assays (Smith *et al*, 2000, Zhang *et al*, 2001)

Since RPMS1 is known to interfere with EBNA2 trans-activation of target promoters which require CBF1, an expression plasmid containing RPMS1 was transfected with EBNA2 expression vector and a *bfl-1* promoter reporter construct (-1374/+81 wt*bfl-1* Luc) (Figure 3 35) It can be seen that RPMS1 potently down-regulated EBNA2-

associated trans-activation of the *bfl-1* promoter in a dose dependent manner These findings corroborate the involvement of a CBF1-associated complex in EBNA2 mediated trans-activation of the *bfl-1* promoter since the ability of RPMS1 to interfere with EBNA2 trans-activation is due to its involvement with CBF1 and Cir as well as other members of the co-repressor complex and subsequent stabilization of this repressor complex (Zhang *et al*, 2001) These results again comply with previous results obtained with the EBNA3 expression plasmids, which also interact with CBF1 and members of the repressor complex

RPMS1 binds to CBF1 and co-localizes in the nucleus and further evidence for the direct interaction of CBF1 and RPMS1 in vivo comes from the ability of RPMS1 to relocate a mutant CBF1 from the cytoplasm to the nucleus of transfected lymphocytes (Zhou *et al*, 2001) In binding to CBF1, RPMS joins the EBNA2 and EBNA3A, -3B, and -3C proteins as an EBV-encoded protein that interacts with CBF1 and consequently has the potential to modify the Notch signaling pathway The ability of RPMS1 to counteract the trans-activational effect of EBNA2 and Notch-IC mediated trans-activation is due to the fact that RPMS1 competes with SKIP for binding to CIR (Zhang *et al*, 2001) SKIP is a component of the CBF1-corepressor complex and is an important cofactor for both EBNA2 and Notch-IC activation of CBF1-repressed promoters since interaction between SKIP and SMRT is important in tethering the HDAC co-repressor complex to CBF1 (Zhou *et al*, 2000, Zhou *et al*, 2001) One interpretation of these experiments is that changing the SKIP-CIR interaction in some way disadvantages the EBNA2-SKIP or Notch-IC-SKIP interaction and prevents a stable association of EBNA2 or Notch-IC with CBF1 (Fig 1 8) In the case of the data in this study, the negative effect of RPMS1 on EBNA2 mediated trans-activation of the *bfl-1* promoter, provides further substantiation to the theory that CBF1 and the CBF1 repression complex are involved in regulating EBNA2 associated stimulation of *bfl-1*

Although both EBNA2 and LMP1 can independently trans-activate the *bfl-1* promoter, and LMP1 can trans-activate the CBF1 mutated *bfl-1* promoter, EBNA2 and LMP1 do not cooperate to trans-activate the *bfl-1* promoter (Figure 3 38) EBNA2 and LMP1 have been shown to cooperate in the regulation of the cellular CD21 and CD23 promoters

[Wang, 1990], however, this was not found to be the case with the *bfl-1* promoter, in fact addition of increasing quantities of an LMP1 expression plasmid resulted in a dose dependent decrease in EBNA2-associated trans-activation of the *bfl-1* promoter (Figure 3 38) Although LMP1 has been shown to have a cytotoxic effect on cell growth in certain transfection assays, (Martin *et al*, 1993), and even a cytostatic effect on cell growth under stable expression conditions (Floettmann *et al*, 1996) it is not likely that this is the case here as similar increasing quantities of LMP1 (in the absence of EBNA2) did not result in a significant reduction in *bfl-1* promoter activation and all transfection results were normalised by correcting for β-galactosidase activity (Figure 3 37A) These results with the *bfl-1* promoter may suggest that some common element is required by both EBNA2 and LMP1 to trans-activate the *bfl-1* promoter, thus competition between EBNA2 and a downstream signaling target of LMP1 for binding of this common element may inhibit promoter trans-activation Alternatively, binding of either EBNA2 or an LMP1 responsive protein may modify the transcription factors involved rendering them inaccessible to the other Since LMP1 can trans-activate the CBF1 mutated *bfl-1* promoter, that particular CBF1 binding site is not a candidate for the common element used by both EBNA2 and LMP1, however other transcription factors which both EBNA2 and LMP1 use to trans-activate other promoters may be candidates here, including AP1 and members of the Ets/PU 1 family LMP1 activation of the p38 mitogen activated protein kinase (MAPK) pathway targets transcription factors such as ATF2 (reviewed in Nebreda *et al*, 2000) and AP1, which are required to facilitate LMP1 activation of IL6 and IL 8 promoters (Eliopoulos *et al*, 1997) Recent studies which show that EBNA2 trans-activates the BATF gene in B cells (Johansen *et al*, 2003) may suggest a possible role for AP1 as an import factor in mediating LMP1 activation of the *bfl-1* promoter (which appears to be a B cell specific effect) since EBNA2 up-regulation of BATF (a member of the AP1 family) inhibits AP1 target gene expression by heterodimerizing with jun and competing with Fos Jun complexes for binding to AP1 consensus sequences Thus LMP1 may require AP1 sites in the *bfl-1* promoter to exert its trans-activational effect and in the presence of EBNA2 activation of BATF1 this may not be possible Interestingly putative AP1 sites have also been identified at the 5' end of the *bfl1*-promoter (Edelstein *et al*, 2003), and the same study also showed that both Ap1 and

CEBPβ were important members of the NFk-B dependent enhanseosome complex on the *bfl-1* promoter again indicating the possibly complex transcriptional requirements of the *bfl-1* promoter Although an AP1 site has been identified at the 5' end of the promoter at position –864, (Edelstein *et al*, 2003) it is possible that more proximal as yet unidentified transcription factor binding sites may also be involved in heterodimerizing with CBF1 or Nf-kB in conferring EBNA2 or LMP1 responsivity on the *bfl-1* promoter In agreement with this, other recent experiments in our laboratory have shown that the elimination by mutation of an additional novel AP1-like binding site in the *bfl-1* promoter leads to a significant loss of LMP-1-associated trans-activation, indicating a role for this transcription factor in this context

Another family of transcription factors with which EBNA2 associates to direct it to target promoters is the Ets family of transcription factors whose members include Spi-1/PU 1, Ets-1, and Spi-B Indeed EBNA2 activation of the LMP1/TP2 promoter is mediated by CBF1, PU 1 and Spi-B (Johannsen *et al*, 1995, Laux *et al*, 1994) This family of transcription factors has also been shown to interact with AP1 and NF-kB, both downstream targets of LMP1 signaling, suggesting a possible role for Ets family members (Bassuk *et al*, 1995, Thomas *et al*, 1997) in mediating LMP1 effects on the *bfl-1* promoter If the putative Ets-1/PU 1 sites identified on the *bfl-1* promoter are targeted by both EBNA2 and downstream signaling elements of LMP1, binding of one or other (i e EBNA2 or a downstream signaling element of LMP1) may be inhibiting binding of the other or causing transcription factor modification which inhibit use for the other protein In any case the possibility of a common portal required by both EBNA2 and LMP1 in addition to CBF1 and NF-kB binding is a likely scenario and one which is supported by the requirement of both EBNA2 and LMP1 for numerous transcription factors in the trans-activation of target promoters

EBNA-LP has been found to cooperate with EBNA2 in the trans-activation of the LMP1/LMP2 and EBV BamH1 C latency promoters, (Harada and Kieff 1997 and Nitsche *et al*, 1997) The molecular basis for EBNA-LP mediated enhancement of EBNA2 trans-activation of promoter activity is still not fully understood although the 66

amino acid repeat domains within EBNA-LP are required for this augmenting effect (Harada and Kieff 1997, Nitsche *et al*, 1997) and the same studies also map the critical EBNA2 domain for interaction with EBNA-LP to the EBNA2 activation domain between amino acids 425 and 487 (Figure 1 7) EBNA-LP has also been shown to cooperate with EBNA2 to stimulate cyclin D2 expression and induce transition from the G^0 to G^1 phases of the cell cycle during the immortalisation of resting B-lymphocytes by EBV (Sinclair *et al*, 1994).

In order to assess any cooperative function of EBNA2 and EBNA-LP in the trans-activation of the *bfl-1* promoter, transient transfection assays were performed in which EBNA2 and EBNA-LP expression plasmids were co-transfected into DG75 cells (Figure 3 39A) This study showed that both EBNA-LP expression plasmids, pSG5LP and pJT125 (containing four and two W repeats respectively) significantly enhanced EBNA2 activation of the *bfl-1* promoter in a dose dependent manner in DG75 and Bjab BL cell lines (Figures 3 39A, 3 39B, 3 40) Two EBNA-LP expression plasmids were used, one of which expressed an EBNA-LP with two W repeats, pJT125 (Peng *et al*, 2000) and the other which expressed an EBNA-LP containing four W repeats pSG5LP (Harada and Kieff 1997) The isoform containing four W repeats is generally accepted as the standard EBNA-LP expression plasmid (Nitsche *et al*, 1997), while the isoform containing two W repeats has been found to contain the minimum number of W repeats required for trans-activation of the LMP1 and Cp promoters Thus EBNA-LP expression plasmids expressing this two W repeat isoform (pJT125) and one expressing the four W repeat isoform (pSG5LP) were employed (Harada and Kieff 1997, Nitsche *et al*, 1997, Peng *et al*, 2000)

Transient transfection data from this study revealed that although both EBNA-LP isoform co-operated with EBNA2 to trans-activate the *bfl-1* promoter, (Figures 3 39A-C) the shorter EBNA-LP isoform with only two W2 domains consistently resulted in higher levels of trans-activation of the *bfl-1* promoter than its longer (4xW repeat) counterpart, this may be due to higher levels of EBNA-LP expression by the shorter protein (which could be determined by Western blotting), or it may be that the smaller protein is more potent and binds more efficiently Nonetheless these data correlate with the findings of others who also found higher activity with the shorter isoform (Nitsche *et al*, 1997, Peng

et al, 2000) With regard to which isoform is the more relevant in vivo, this has yet to be identified To date EBNA-LP has not been found to have any independent trans-activational effect on viral or cellular promoters however in this study, EBNA-LP was found to trans-activate the *bfl-1* promoter 4 to 6 fold in a dose dependent manner in the absence of EBNA2 Further to this, the trans-activational effect of EBNA-LP on the *bfl-1* promoter was found to depend upon the CBF1 consensus sequence on the *bfl-1* promoter as EBNA-LP trans-activation of the CBF1 mutant *bfl-1* promoter (-1374/+81 wt*bfl-1* Luc) was less than 2 fold It can be seen from the results that this was the case for both isoforms of EBNA-LP assayed (Figure 3 39A, B and C)

It can be seen from Figure 3 39C that deletion of the Y2 domain abolished the co-activating function of EBNALP in the trans-activation of the *bfl-1* promoter These results are reminiscent of the findings of others, which showed deletion of the EBNALP Y2 domain (in the context of the $(W_1W_2)_2$ Y1Y2 isoform) abolished its co-activating functions in augmenting EBNA2 mediated trans-activation of the Cp and LMP1 promoters (Nitsche *et al*, 1997, McCann *et al*, 2001) Thus the Y2 domain also appears critical in mediating the EBNA2 cooperation activity in trans-activating the *bfl-1* promoter in the context of the $(W_1W_2)_4$ Y_1Y_2 isoform Other studies involving mutational analysis of EBNA-LP revealed that EBNA-LP expression plasmids expressing an EBNA-LP protein lacking the entire 45-amino-acid Y1 Y2 unique domain still cooperated with EBNA2 in trans-activating the LMP1 promoter but that the co-operative function of EBNA-LP was reduced with a mutant EBNA-LP expression plasmid expressing an EBNALP lacking the Y2 domain (Nitsche *et al*, 1997) More recent studies using EBNALP constructs lacking the Y2 domain demonstrated that deletion of the Y2 domain either alone or together with Y1 led to a complete loss of co-operative function, despite nuclear localization as identified using immunofluorescence (McCann *et al*, 2001)

Another mutant EBNALP expression plasmid in which conserved serine residues in each of the two W2 repeats were substituted for alanines (residues 49 50 and 51 in the first W2 repeat and residues 115, 116 and 117 in the second W2 repeat), was also used (Figure 3 38) This construct, pRSP83 (Peng *et al*, 2000) failed to cooperate with EBNA2 in

trans-activating the *bfl-1* promoter These serine residues at positions 49 50 and 51 in the W2 repeat domains have been shown to be important in mediating the EBNA2-cooperative function to EBNALP, on other EBNA2 responsive promoters (Peng *et al*, 2000), similarly mutation of these residues abolishes EBNA-LP EBNA2 co-operativity for the trans-activation of the *bfl-1* promoter These results comply with other studies, (Peng *et al*, 2000, McCann *et al*, 2001), which also show this mutagenised EBNA-LP had no EBNA2 co-operativity due to the fact that the mutant proteins no longer localized exclusively to the nucleus

The importance of serine residues in conferring EBNA2 co-operativity on EBNA-LP suggests that phosphorylation may be an important feature of the relationship between EBNA-LP and EBNA2 and on the basis of the data in this study, (Figures 3 40) using the pRSP83 construct, phosphorylation may also be important in facilitating the cooperative function between EBNA-LP and EBNA2 in the trans-activation of *bfl-1*

Although CBF1 binding has been shown to be crucial in mediating EBNA2 trans-activation of a number of target promoters including LMP1, CD21, Cp and possibly c-myc, (Woisetschlaeger *et al*, 1990, Sung *et al*, 1991, Laux *et al*, 1994) it is often insufficient on its own to confer optimal EBNA2 responsivity on target promoters (Meitinger *et al*, 1994) Apart from CBF1 a number of additional transcription factors including CBF2 and AUF1 (Fuentes-Panana *et al*, 1998, Fuentes-Panana *et al*, 2000) are critical in mediating EBNA2 responsivity on the Cp promoter and additional transcription factors are required in the EBNA2 trans-activation of the LMP2A promoter (Hofelmayr *et al*, 1999) Additionally, two members of the Ets family of transcription factors, PU 1/Spi-1 and Spi-B have been found to interact with EBNA2 in the trans-activation of the LMP1/TP2 promoters (Johannsen *et al*, 1995, Laux *et al*, 1994) In an attempt to identify a role for other possible transcription factor binding sites on the *bfl-1* promoter, sequence analysis using a number of online databases revealed the presence of putative Ets1 and PU 1 binding motifs located in close proximity to the consensus CBF1 binding site already identified (Figure 3 22) Both PU 1 and Ets-1 are members of the Ets family of transcription factors The identification of a number of putative Ets-1 and Pu 1 binding sites in close proximity to each other and proximal to the CBF1 consensus sequence on

the *bfl-1* promoter, is reminiscent of the EBNA2 responsive elements in other EBNA2 responsive promoters (Table 3 1) It is possible therefore that EBNA2 (and possibly LMP1) mediated trans-activation of the *bfl-1* promoter may require downstream targeting of the putative Ets1 and PU 1 sites identified on the *bfl-1* promoter This may explain the inhibiting property of LMP1 expression on EBNA2 activation of the *bfl-1* promoter, as both may compete through different mechanisms to target these same sites thereby competitively inhibiting the trans-activational effect of the other Site directed mutagenesis was undertaken and putative ets1 sites at nucleotide positions -213, -176 and a putative PU 1 site at position -143 were mutagenised with the core GGAA motif of these Ets family transcription factors replaced by the GCAG (Table 3 2) Transient transfections with these ets1/PU 1 mutant *bfl-1* reporter constructs (-213 mEts1*bfl-1* Luc, -176 mEts1*bfl-1* Luc and -143 mPU 1*bfl-1* Luc) and the pSG5EBNA2 expression plasmid showed that all three putative Ets-1 sites and the putative PU 1 site contributed to mediating optimal trans-activation of the *bfl-1* promoter by EBNA2 (Figure 3 44) Mutation of the 'double' Ets-1 consensus sequence at position -176 on the *bfl-1* promoter, reduced EBNA2 trans-activation of the *bfl-1* promoter from 6 5 fold to less than 2 fold, similarly mutation of the putative PU 1 consensus sequence at position -143 reduced EBNA2 trans activation of the *bfl-1* promoter from 6 5 fold to about 2 fold Mutation of the putative Ets-1 binding site at position -213 also reduced EBNA2 trans-activation of the *bfl-1* promoter, but to a lesser extent from 7 fold to about 3 5 fold (Figure 3 44) In the case of all three mutant expression plasmids, the trans-activational effect of EBNA2 was compared to the trans-activational effect of EBNA2 on the truncated -367/+81 *bfl-1* Luc reporter construct as it was in this background that the mutagenesis was performed These data may imply that similar to the LMP1 promoter, EBNA2 may trans-activate the *bfl-1* promoter via recruitment by both CBF1 and PU 1, as well as other transcription factors namely Ets-1 which may also be involved in targeting EBNA2 to the *bfl-1* promoter

Modulation of gene expression by ETS family members can involve combinatorial interactions with other transcription factors, including Sp1, AP-1, c-myb, Nf-kB and CBF1 (Bassuk *et al*, 1997, Eisenbeis *et al*, 1995, Nagulapalli *et al*, 1995, Thomas *et al*, 1997, Sementchenko *et al*, 2000) Ets proteins including PU 1 (also known as Spi-1)

and Ets-1 most often bind DNA as monomers however they interact with other cellular DNA-binding proteins to form ternary protein-protein complexes, (Fitzsimmons *et al*, 1996, Thomas *et al*, 1997) sometimes in the activation of B cell and lymphoid specific promoters (Fitzsimmons *et al*, 1996, Nagulapalli *et al*, 1995 Pongubala *et al*, 1992, Thomas *et al*, 1997) Further to this, NF-kB , AP1 and Ets1 synergistically combine to trans-activate the GM-CSF promoter (Granulocyte-Macrophage colony stimulating Factor) Since these transcription factor often act in concert with other transcription factors in trans-activating target genes it is therefore likely that in the case of both LMP1 and EBNA2 a number of these transcription factors in combination as well as CBF1 or Nf-kB are required to trans-activate the *bfl-1* promoter Similarly to the LMP1 promoter (Johannsen *et al*, 1995), EBNA2 trans-activation of the *bfl-1* promoter, may require a combination of transcription factors including PU 1 and CBF1 Interestingly, EBNA2 trans-activation of the LMP1 promoter occurred only in B cells but not in T or epithelial cell lines tested (Johannsen *et al*, 1995) Similarly EBNA2 trans-activation of the *bfl-1* promoter only occurred in B cells, not in the T or muscle cell lines tested (Figure 3 18) The fact that the trans-activational effect of EBNA2 on both *bfl-1* and LMP1 promoters, in transient transfections appears to be limited to B cell lines (Figure 3 18 and (Johannsen *et al*, 1995) and that LMP1 trans-activation of the *bfl-1* promoter is also B cell specific (D'Souza *et al*, 2000) may indicate that B cell specific transcription factors such as PU 1 and Ets1 are required to mediate the effects of EBNA2 and LMP1 on the *bfl-1* promoter The identifcation of possible binding sites for the B cell and macrophage specific Ets family of transcription factors on the *bfl-1* promoter indicates that these may be the likely B cell specific factors required

Further evidence for the requirement of Ets-1 in mediating EBNA2 trans-activation of the *bfl-1* promoter came from transient transfection data using a dominant negative Ets-1 expression plasmid (Figure 3 45) This dominant negative expression plasmid contains the cDNA sequence encoding human Ets-1, which lacks the transcription activation domain and corresponds to amino acids residues 306 ± 441, (which has been introduced into the pCEP4 expression plasmid, Invitrogen) (Nakada *et al*, 1999, Kim *et al*, 2000) Co-transfection of the dominant negative expression plasmid (up to 7ug) with the

pSG5EBNA2 expression plasmid resulted in a dose dependent reduction in EBNA2 associated trans-activation of the *bfl-1* promoter EBNA2 mediated trans-activation of the *bfl-1* promoter was reduced from over 5 fold to less than two fold with addition of increasing quantities of the dominant negative expression plasmid Unexpectedly, addition of 10ug of the dominant negative reproducibly partially restored EBNA2 trans-activation of the *bfl-1* promoter to 3 fold A similar trend was also observed with the truncated –367/+81 *bfl-1* Luc promoter A possible explanation as to this effect is that over-expression of the dominant negative leads to some indirect effect resulting in blocking expression of itself, or possibly the dominant negative molecule loses activity through dimerization when the protein is present in high concentration This phenomenon however, has not been reported in similar experiments elsewhere, in other studies expression of the dominant negative was shown to down regulate Ets-1 target genes uPA, (urokinase type plasminogen activator) and MMP family members (matrix metalloprotease) (Nakada *et al*, 1999) Nonetheless the combined effects of the Ets-1 dominant negative results, and mutation of the putative Ets-1 sites on the *bfl-1* promoter, are evidence of an important role for Ets-1 and PU 1 sites, particularly the double Ets-1 site at position –176 to –163 on the *bfl-1* promoter, in facilitating EBNA2 mediated trans-activation of this promoter

Notch-IC activates the transcription of CBF1 repressed promoters by displacing the SMRT-Histone deacetylase co-repressor complex by binding to CBF1 and SKIP via its Ram and Ankyrin repeat domains respectively (Zhou *et al*, 2000) Similarly, EBNA2 activates transcription through binding to CBF1 and SKIP, members of the CBF1 repression complex, thereby displacing the co-repressor complex to facilitate access to its trans-activation domain (Zhou *et al*, 2000) Specific regions of EBNA2 designated Cr5 and Cr6 (Figure 1 7) specifically interact with SKIP and CBF1 respectively (Hsieh *et al*, 1995, Zhou *et al*, 2000) Although there *is no* obvious sequence homology between EBNA2 and Notch, they interact with similar regions of CBF1 and replace repressor proteins by their trans-activation domains Thus the interaction of EBNA2 and CBF1 mimics Notch binding to CBF1 therefore the early steps in EBV immortalization may mimic an aspect of Notch signaling (Hsieh *et al*, 1995, Kao *et al*, 1998, Zhou *et al*,

2000) In addition both Notch-IC and EBNA2 activated transcription can be negatively regulated by EBV RPMS and KyoT2 (Zhang *et al*, 2001, Taniguchi *et al*, 1998) The set of promoters, which is regulated by Notch-IC and EBNA2, is overlapping but not identical It has been shown, that EBNA2 can activate the Notch-IC responsive Hes1 promoter Similarly both EBNA2 and activated Notch suppress differentiation of the myogenic cell line C2C12 by activation of Hes1 (Sakai *et al*, 1998, Kuroda *et al*, 1999) Since EBNA2--mediated immortalization of human B cells is dependent on its interaction with and function through CBF1, (Yalamanchili *et al*, 1994) it raises the possibility that dysregulated Notch function may also immortalize B cells In Burkitts lymphoma cell lines infected with a mutant EBV lacking EBNA2, activated Notch1 can mimic EBNA2s ability to activate CD21 expression and down regulate Igμ transcription (Strobl *et al*, 2000) also both EBNA2 and activated Notch1 induce the BATF gene in EBV negative cells (Johansen *et al*, 2003), however, in contrast to EBNA2, activated Notch1 fails to activate either CD23, c-myc or the LMP1 viral protein (Strobl *et al*, 2000) Further to this, activated Notch1 can only transiently maintain proliferation of lymphoblastoid cell lines that lack EBNA2 expression but continue to express LMP1 (Hofelmayr *et al*, 2001) Thus although both EBNA2 and activated Notch activate target genes through CBF1, not all EBNA2 responsive genes are Notch-IC responsive genes and vice versa Since CBF1 appears to be critical in mediating EBNA2 reactivity on the *bfl-1* promoter, it was plausible then, that Notch-IC might also activate *bfl-1* expression via CBF1. In order to determine if *bfl-1* was also responsive to activated Notch as well as EBNA2, a number of transient transfections were performed Up-regulation of *bfl-1* at the protein and mRNA levels, in response to Notch-IC activation, was also examined using Western blot and RPA analyses Although both the EBNA2 (pSG5EBNA2) and Notch-IC (ED1) expressed functional EBNA2 and Notch-IC proteins, as demonstrated by their ability to trans-activate the pGA981-6 reporter containing a total of 12 CBF1 sites, only pSG5EBNA2 trans-activated the *bfl-1* promoter (Figures 3 46A and 3 46B) The mouse Notch-IC expression plasmid was titrated over a range of quantities in co-transfections with the *bfl-1* promoter and failed to activate the *bfl-1* reporter construct (Figure 3 46B) The RAM domain in the Notch receptor has been shown to mediate the interaction between CBF1 and Notch-IC (Zhou *et al*, 2000) and unsurprisingly the RAM deleted

mouse Notch-IC expression plasmid ED4 also failed to trans-activate the *bfl-1* promoter (Figure 3 47) Similarly despite the functionality of EBNA2 and Notch-IC in another B cell line Bjab, again shown by their ability to trans-activate the synthetic pGA981-6 reporter construct, only EBNA2 could trans-activate the *bfl-1* promoter (Figure 3 48A) Comparable experiments were also carried out in the BL41-P3HR1-mNotch-IC (CL31) cell line, where mouse Notch-IC activation was induced by addition of estrogen The *bfl-1* promoter (-1374/+81 wt*bfl-1* Luc), its CBF1 mutated partner (-1374/+81 mCBF1*bfl-1* Luc) and control reporter constructs, pGa981-6 and pGa50-7 vectors were transfected into the estrogen responsive cell line and the levels of trans-activation of the promoters assessed m the usual way by the luciferase assay As per the other transient transfections in the Dg75 and Bjab cell lines, although Notch-IC (in this case activated by the addition of estrogen) trans-activated the pGa981-6 vector, it failed to trans-activate either *bfl-1* promoter reporter constructs (Figure 3 49B) Taken together these results suggest that *bfl-1* is an EBNA2 but not a mouse Notch1 responsive gene in B cell lines In order to extend this analysis, human Notch1-IC and human Notch2-IC expression plasmids were also obtained and co-transfected with the appropriate promoter constructs in the Dg75 cell line Results obtained here were similar to those obtained with the mouse Notch-IC expression plasmids Although both human Notch1IC and human Notch2IC trans-activated the pGa981-6 vector demonstrating their ability to function in this B cell background, neither trans-activated the *bfl-1* promoter (Figures 3 50A-C) Since both EBNA2 and Notch-IC proteins share an affinitiy for CBF1 it is possible that Notch is capable of interacting with CBF1 when the latter is bound to the *bfl-1* promoter, even though significant trans-activation is not detectable Its inability to trans-activate the promoter may indicate (i) that the Notch-IC trans-activaion domain is much weaker than that of EBNA2, or (ii) that trans-activation via the CBF1 site requires interaction with other additional factors, something achieved by EBNA2 but not Notch-IC, or (iii) Notch may not bind to or have reduced affinity for the CBF1-corepressor complex formed at the –243/-249 site on the bfl1 promoter One way to determine if a Notch-CBF1 interaction is occurring on the *bfl-1* promoter was to co-transfect EBNA2 and Notch expression plasmids to see if co-expression of Notch and EBNA2 would result m decreased EBNA2-mediated trans-activation This competition experiment was carried out for the mouse and

human Notch-IC expression plasmids (pED1, pJT111) and the human Notch2-IC expression plasmid (pJT112) Increasing quantities of pJT111/pJT112/ED1 were co-transfected with pSG5EBNA2 and the subsequent trans-activation of the *bfl-1* promoter analysed using the luciferase assay Despite sharing 70% homology and functional homology as reported to date, human Notch1-IC did not significantly repress EBNA2 mediated trans-activation of the *bfl-1* promoter suggesting it may not interact or at least does not impede EBNA2 interaction, with CBF1 Although this effect could also be due to Notch interaction with another element in the *bfl-1* promoter which may effect EBNA2 trans-activation In contrast to co-transfection with human Notch2IC (pJT112) and mouse Notch1-IC (ED1) which reduced EBNA2 mediated trans-activation of the *bfl-1* promoter by ~50% and ~70% respectively Although this result does not prove an interaction with CBF1 it showed that Notch2 and mNotch1-IC are in some way impeding EBNA2 trans-activation of the *bfl-1* promoter, which may be due to competition for binding to the CBF1 site, or other EBNA2-critical sites on the *bfl-1* promoter Northern blot and Western blot analyses using the Cl31 cell line suggest that any effect of Notch-IC activation on *bfl-1* mRNA and protein is transient and complete after 6 hours Unfortunately due to inconsistencies with the gel loading, it is difficult to see the effect of Notch-IC activation on *bfl-1* mRNA after 3 hours in Figure 3 55, however after Notch1-IC activation for 3 hours, *bfl-1* mRNA is up-regulated 4 5 fold relative to pre activation levels These results suggest that *bfl-1* mRNA is transiently up-regulated by Notch-IC activation and these results are also supported by western blotting which shows a 2 fold increase in *bfl-1* protein at six hours after Notch1-IC activation Again this up-regulation is transient with protein levels decreasing to pre-activation levels by 9 hours The discrepancy between the Western and Northern blotting results and the promoter reporter data may suggest that transfection conditions need to be optimised to see the early transient effect of mNotch-IC activation on *bfl-1* Transfections were generally harvested 48hr post transfection, by which time any transient effect of Notch-IC may no longer have been detectable

Bfl-1 is an anti-apoptotic protein which has been shown to protect cells from apoptosis induced by p53, serum starvation and TNFα-induced cytotoxicity It also exhibits

proliferative and potent cooperative transforming properties in vitro (D'Souza *et al*, 2000, D'Sa-Eipper *et al*, 1996, Karsan *et al*, 1996 and Zong *et al*, 1999) Functional dissection of the *bfl-1* protein has revealed that (D'Sa-Eipper *et al*, 1998) the BH1, 2 and 4 domains are essential in conferring the anti-apoptotic and transforming properties of this protein The fact that mutations which abolish the anti-apoptotic function also abolish the oncogene co-operating function also suggest that both of these functions are linked (D'Sa-Eipper *et al*, 1998, D'Sa-Eipper *et al*, 1996)

Elevated levels of *bfl-1* mRNA are a feature of EBV infected cell lines and the EBV latent protein LMP1 has been found to up-regulate *bfl-1* mRNA in EBV negative BL cell lines (D'Souza et al, 2000) Although LMP1 is in part responsible for the EBV induced up-regulation of *bfl-1* mRNA in EBV positive cell lines, other EBV proteins are also likely to play a role in regulating the *bfl-1* gene (D'Souza *et al*, 2000) This is apparent since steady state levels of *bfl-1* mRNA in LCL and type III BL cell lines are dramatically higher than in cell lines in which LMP1 alone is induced (D'Souza *et al*, 2000) Physiologically, the up-regulation of *bfl-1* mRNA by LMP1 in BL cell lines has been shown to protect them from apoptosis induced by growth factor withdrawal (D'Souza *et al*, 2000)

The results presented in this study indicate for the first time that expression of the anti-apoptotic *bfl-1* gene is up-regulated by the EBV latent protein EBNA2 The physiological effect of this in BL cells was then investigated Initially the susceptibility of Dg75 cells to apoptosis had to be determined Dg75 cells displayed a relatively high anti-apoptotic threshold, although apoptosis was induced in Dg75 cells using growth factor withdrawal by culturing cells in 0 5% and 0 1% serum supplemented media (Figure 3 56) Apoptosis was also induced by simultaneously serum starving cells and exposing them to ionomycin Cell viability was assessed using trypan blue exclusion and the mode of cell death was analysed using Acridine orange staining (Figure 3 57) Apoptotic cells were identified by their distinctive morphology and staining pattern, appearing condensed and fluorescing brightly Healthy cells on the other hand are larger and more diffusely stained (Gregory *et al*, 1991) (Figure 3 56) Having ascertained that DG75 cells could be triggered to apoptose by serum starvation, this cell line was stably transfected with EBNA2 expression plasmids and the protective effect afforded by EBNA2, and thus *bfl-1*

induction in this cell line in response to growth factor withdrawal, was analysed by acridine orange staining and Flow Cytometry It was observed that pSG5EBNA2 and pSG5EBNA2WW323SR expression afforded some protection of these BL cells from apoptosis (Figures 3 64 and 3 65) Percentage cell death was quantified by counting apoptotic cells after acridine orange staining and it can be seen that cell death is reduced from over 60% in cells not expressing EBNA2 to 38% in cells expressing wild type EBNA2 Expression of the EBNA2WW323SR protein, also consistently reduced cell death (49%), relative to cells not expressing EBNA2 however the protective effect was consistently lower than that seen in the EBNA2-expressing cell line DG75-pSG5EBNA2 (Figure 3 64) These results suggest that CBF1 binding, or a downstream effect of this may be involved in mediating this anti-apoptotic effect in Dg75 cells Since EBNA2 up-regulates *bfl-1* and this induction of *bfl-1* requires CBF1, *bfl-1* may have a key role in mediating this anti-apoptotic effect of EBNA2 expression in the DG75 cell line EBNA2 however has also been shown to up-regulate the expression of the proto-oncogene c-myc in lymphocytes (Kempkes *et al*, 1995) and this may also have a role in this protective effect Further to this EBNA2 up-regulation of *bfl-1* may also be involved in increasing the anti-apoptotic threshold of EBV infected cells (Gregory *et al*, 1991) The anti-death property of EBNA2 expression has been shown elsewhere, when EBNA2 expression protected transfected B cells from Nur 77 induced apoptosis (Lee *et al*, 2002)

FACS analysis is a more quantitative method for detecting apoptosis, thus, in this study the stably transfected cell lines (Dg75-pSG5, Dg75-pSG5EBNA2 and Dg75-pSG5EBNA2WW323SR) were stained with propidium iodide and using light scatter data, the percentage of apoptotic cells was calculated with CellQuest software (BectonDickinson) It can be seen in Figure 3 65 that both cell pools expressing EBNA2 (Dg75pSG5EBNA2 and Dg75pSG5EBNA2WW323SR) and both cell pools not expressing EBNA2 (Dg75 and Dg75-pSG5) apoptose under conditions of serum starvation however, the percentage of cells dying is reduced in those cell lines expressing EBNA2 In the Dg75 cell line and the Dg75-pSg5 cell line, ~77% and ~83% of cells respectively, are apoptotic after seven days in serum deprived media, however in the Dg75-pSG5-EBNA2 and Dg75-pSG5EBNA2WW323SR cell lines, the percentage of apoptotic cells respectively, is ~39% and 58% respectively Thus EBNA2 expression

provides a measure of protection from growth factor withdrawal induced apoptosis in Dg75 cells CBF1 may be also have a role in this protective effect as again, the percentage of cells apoptosing was consistently higher in the cell line in which the CBF1-binding defective EBNA2 was expressed, compared to the cell line in which wild type EBNA2 is expressed It can also be seen from Figure 3 66 that EBNA2 expression results in an accumulation of cells in the G1 phase, this effect has been noted elsewhere and may be due to the combined up-regulation of c-myc (Kempkes *et al*, 1995a) as well as *bfl-1* in this cell line Levels of c-myc and *bfl-1* protein in this cell line could be detected by Western blotting It is also possible that pro-apoptotic proteins such as bik are also being down-regulated in response to EBNA2 expression as seen in Figure 3 3 In effect the combined up-regulation of anti-apoptotic and down regulation of pro-apoptotic proteins by EBNA2 expression is likely to be responsible for the relative resistance to apoptosis, of these EBNA2 expressing cells

EBNA2 trans-activation of the EBV latency C promoter, the LMP1 promoter and the cellular CD23 promoters is dependent on an interaction with the DNA-binding protein CBF1 for promoter targeting (Ling *et al*, 1993, Ling *et al*, 1994) Since a CBF1 binding deficient EBNA2 cannot trans-activate the *bfl-1* promoter (Figure 3 16) and site directed mutagenesis of the putative CBF1 binding site on the *bfl-1* promoter at nucleotide positions –243 to –249 also abolishes EBNA2 trans-activation of the *bfl-1* promoter, (Figure 3 26A), it is likely that CBF1 binding may play a role in the EBNA2 mediated trans-activation of the *bfl-1* promoter It was therefore important to determine by EMSA/bandshift assay if CBF1 and EBNA2/CBF1 interactions were occurring at the –243/-249 CBF-1 like binding site on the bfl-1 promoter Initially as a control, the effect of EBNA2 induction on CBF1 binding activity m DG75-tTA-EBNA2 cells was assessed by EMSA using a double-stranded oligonucleotide containing the CBF1 binding site between nucleotide positions -359 to –388 on the Cp promoter (Ling *et al*, 1994) which has previously been shown to form a stable complex with EBNA2 in EMSA analyses (Ling *et al*, 1994) However this study failed to detect any direct interaction between EBNA2 and the CBF1 binding site on the Cp promoter As this interaction has been previously published it was expected that documented high molecular weight protein DNA complex would have been detected Thus conditions in the binding reaction may

need to be optimized in order to facilitate protein-DNA interaction Similarly this study failed to detect any direct interaction between EBNA2 and CBF1 at the putative CBF1 binding site on the *bfl-1* promoter This may again be due to sub-optimal binding conditions or, the bfl-1 binding site in question may have a low affinity for CBF1 Another possibility is that further transcription factors are required to mediate the EBNA2/CBF1 reaction on this promoter Since the results show that members of the Ets family may have a role in mediating EBNA2 associated activation of the *bfl-1* promoter, they may be candidates here Despite the fact that EMSA analyses failed to demonstrate a direct interaction between the putative *bfl-1* CBF1 and EBNA2, the weight of evidence from transient transfection data and mutagenesis of the promoter shows that CBF1 has a critical role in mediating EBNA2 activation of *bfl-1*

SUMMARY

It has already been shown that regulation of the cellular *bfl-1* gene (also known as *A1*) is an important EBV-host cell interaction that promotes resistance to programmed cell death (or apoptosis) in a BL-derived cell line LMP1 has been shown to regulate the expression of this gene and the signalling pathway used by the EBV Latent Membrane Protein 1 (LMP1) to regulate *bfl-1* expression has been characterized

This thesis presents a second mechanism, independent of LMP1, by which EBV regulates bfl-1 mRNA levels We show that the EBV nuclear antigen 2 (EBNA2), a second major effector of phenotypic change in the infected cell, can *trans*-activate the *bfl-1* promoter through interactions with components of the cellular Notch signalling pathway The main findings of this study include

- Induction of EBNA2 expression using a tetracycline-based regulation system, leads to increased *bfl-1* mRNA levels in an EBV-negative BL-derived cell line
- When EBNA2 is expressed as an inactive fusion with the hormone-binding domain of the estrogen receptor, the steady-state level of *bfl-1* mRNA increases upon activation of EBNA2 function by addition of oestrogen

- EBNA2 *trans*-activates the *bfl-1* promoter in BL cell lines by a mechanism that is dependant upon its ability to bind CBF1, a DNA-binding nuclear component of the cellular Notch signalling pathway
- A novel CBF-1-like binding site is present on the *bfl-1* promoter that is essential for EBNA2-mediated *trans*-activation
- EBNA2-mediated *trans*-activation is modulated by other EBV latent proteins that are known to co-operate with EBNA2 (such as EBNA-LP), or that have been shown to interact with the CBF1-co-repressor complex (EBNA3A, 3B, 3C and RPMS)
- LMP1 and EBNA2 do not cooperate to *trans*-activate the *bfl-1* promoter and may require a common factor to exert their trans-activational effect on *bfl-1*
- Activated Notch receptor (Notch-IC), of which EBNA2 is regarded as a viral functional homologue, does not *trans*-activate *bfl-1* in BL-derived cell lines
- Novel Ets1 and PU 1 like binding sites are present on the *bfl-1* promoter which have a critical role in EBNA2 *trans*-activation
- EBNA2 induced up-regulation of Bfl-1 in an EBV negative BL cell line may provide some protection from apoptosis triggered by growth factor withdrawal

CHAPTER 5

BIBLIOGRAPHY

BIBLIOGRAPHY

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APPENDIX

SOLUTIONS FOR DNA MANIPULATION

TE buffer

10 mM	Tris-Cl
1 mM	EDTA pH 8 0

Solutions for mini-preparation of plasmid DNA

Solution I

50 mM	Glucose
25 mM	Tris Hcl (pH 8 0)
10 mM	EDTA (pH 8 0)

Solution II (Prepared fresh)

0 2 M	NaOH
1 % (w/v)	SDS

Solution III

60 ml	5 M potassium acetate
11 5 ml	Glacial acetic acid
28 5 ml	Distilled water

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate

DNase-free RNase

RNase A (1 mg/ml) in sterile water

Heat to 100^{0}C for 30 min Cool slowly and store –20^{0}C

Solutions for Maxipreparations of DNA – Qiagen Buffers

Buffer P1 (Resuspension buffer)

50 mM	Tris-c1, pH 8 0

10 mM	EDTA
100 µg	RNase A

Store at 4^0C after the addition of RNase A

Buffer P2 (Lysis buffer)

200 mM	Sodium Hydroxide
1% (w/v)	SDS

Prepared fresh and stored at room temperature

Buffer P3 (Neutralization buffer)

3 0 M	Potassium acetate pH 5 5

Stored at 4^0C

Buffer QBT (Equilibration buffer)

750 mM	NAcl
50 mM	MOPS pH 7 0
15% (v/v)	Isopropanol
0 15% (v/v)	Triton X –100

Stored at room temeprature

Buffer QC (Wash buffer)

1 0 M	NAcl
50 mM	MOPS pH 7 0
15% (v/v)	Isopropanol

Stored at room temperature

50% (v/v) Glycerol

25 ml	Distilled
25 ml	Glycerol

Autoclaved and stored at room temperature

0.5 M EDTA

186 1 g	EDTA
800 ml	Distilled water
6 g	NaOH pelletes

pH to 8 0 with 5 M NaOH Volume was adjusted to 1 L with water

50X TAE

242 g	Tris
57 1 ml	Acetic acid
100 ml	0 5 M EDTA pH 8 0

Adjusted to 1L with water

10X TBE

108 g	Tris
50 g	Boric acid
40 ml	0 5 M EDTA pH 8 0

Adjusted to IL with water

Ethidium bromide

0 1 g/10 ml water (10 mg/ml)

Stored in dark at room temperature

Agarose gel loading dye

40% (w/v) sucrose

0 25% (w/v) bromophenol blue

BACTERIAL GROWTH MEDIA

LB agar

10 g	Tryptone
5 g	Yeast extract
5 g	NacL

15 g Agar technical

Autoclaved and plates stored at 4^0C

LB Agar Plus Ampicillin

Ampicillin was added to a final concentration of 100 μg/ml to LB agar (50^0C)

Plates were stored at 4^0C

Tetracycline stock

5mg /ml 50mg of tetracycline (Sigma) was added to 10ml of 80% ethanol

LB Agar Plus Tetracycline

Tetracycline was added to a final concentration of 12 5μg/ml to LB agar (50^0C)

Plates were stored at 4^0C

LB broth (per L)

10 g Bacto-tryptone

5 g Yeast extract

5 g NAcl

Autoclaved and stored at 4^0C

LB Ampicillin broth

Ampicillin was added to LB broth to a final concentration of 100 μg/ml from stock solutions (100 mg/ml in dH_2O, stored at -20^0C) Stored at 4^0C

LB Tetracycline broth

Tetracycline was added to LB broth to a final concentration of 10μg/ml from stock solution (5mg/ml m 80% ethanol)

SOB medium (per L)

20 g Trytpone

5 g	Yeast extract
0 5 g	Nacl
10 ml	KCl (250 mM)

Adjusted pH to 7 0 with 5 M NaOH

Autoclaved, cooled to –5^{0}C and added

10 ml 1 M $MgcL_2$

Stored at 4^{0}C

SOC medium (per L)

1 L	SOB
7 5 ml	50% glucose (filter sterilised)

Stored at 4^{0}C

Ampicillin stock solution (50 mg/ml)

50 mg of ampicillin per ml of sterile H_2O

Filter sterilised and stored at –20^{0}C

Buffers for Use with the Promega Altered sites II In Vitro Mutagenesis System.

2M Ammonium Acetate (pH 4 6)

15 4g of ammonium acetate dissolved in 50ml dI H_2O Brought to pH 4 6 with glacial acetic acid and then to a final volume of 100ml with dI H_2O

7 5M Ammonium Acetate (pH 7 5)

57 18g ammonium acetate dissolved in 50ml dI H_2O Brought to pH 7 5 NaOH and then to a final volume of 100ml with dI H_2O

Annealing 10X Buffer

200mM Tris-cl-(pH7 5)

100mM $MgCl_2$

500mM NaCl

Kinase 10X buffer

700mM Tris-Cl (pH 7 6)

100mM $MgCl_2$

50mM DTT

TFB1

30mM potassium Acetate

10mM $CaCl_2$

50mM $MnCl_2$

100mM RbCl

15% Glycerol

Adjust pH to 5 8 with 1M acetic acid Filter sterilize (0 45um) Astrored at room temperature

TFB2

100mM MOPS or PIPES (pH 6 5)

75mM $CaCl_2$

10mM RbCl

15% glycerol

Adjust pH to 6 5 with 1M KOH Filter Sterilize and store at room temperature

CELL CULTURE MEDIA/SOLUTIONS

Supplemented RPMI (200 ml)

176 ml	RPMI 1640
20 ml	Foetal calf Serum (Decomplemented –50^0C for 30 min)
2 ml	200 mM L-glutamine
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 µg/ml)

Supplemented DMEM High Glucose (C33A)

178 ml	DMEM high glucose with L-glutamine
20 ml	Foetal calf Serum (Decomplemented, 50^0C for 30 min)

2 ml Pemcillin/Streptomycin (1000 U/ml-1000 µg/ml)

10X Phosphate Buffered Saline (PBS)

14 24 g Na_2HPO_4 $2H_2O$ (8 mM)

2 04 g KH_2PO_4 (1 5 mM)

80 0 g NAcl (137 mM)

2 0 g KCI (2 7 mM)

pH 7 5 and make up to 1 litre

Diluted 1 in 10 in sterile distilled water and used at a 1 X working concentration

Geneticin G418 (stock solution 50 mg/ml) for tetracycline inducible cell lines

0 1 g Geneticin

2 ml RPMI 1640

Filter sterilised using a 0 2 micron filter, aliquoted and stored at -20^0C 20 µl of the stock solution was added per ml of media to give a final concentration of 1 mg/ml

Geneticin G418 for addition to Estrogen responsive cell lines.

BL41-ER/EBNA2 (K3) Kept in permanent selection by addition of G418 to a final concentration of 1 6mg/ml from stock solution

BL41P3HR1-ER/EBNA2 (9A) Kept in permanent selection by addition of G418 to a final concentrarion of 1 2mg/ml from stock solution

Hygromycin B (stock solution 50 mg/ml supplied)

Ten micro litres of the stock solution was added per ml of media to give a final concentration of 500 µg per ml Stored at 4^0C

Hygromycm for addition to the estrogen responsive cell line Cl31

BL41P3HR1-ER/mNotchIC (Cl31) Kept in permanent selection by addition of Hygromycin to a final concentration of 0 8mg/ml from stock solution

Tetracycline (stock 5 mg/ml)

5 mg	Tetracycline
1 ml	100% Ethanol

Stored at -20^{0}C, 1 µl of tetracycline was added t o5 ml of media to give a final concentration of 1 µg per ml

Ionomycin (stock solution)

2mM in DMSO

Stored m aliquots at -20°C

Estrogen. Stock solution.10mg/ml

100mg ß-estradiol (Sigma) was added to 10ml of 70% ethanol

Estrogen for activation of EBNA2 or Notch.

Estrogen was added to a final concentration of 1uM from stock solution

Cyclohexamide

Prepared as a 50mg/ml stock in dI H_2O

Cyclohexamide was added to a final concentration of 10mg/ml to stop protein synthesis

Solutions for modified DEAE-Dextran Transfection Protocol

T.E.

10 mM	Tris (pH 7 4)
1 mM	EDTA

Prepared fresh on the day of use using autoclaved of Tris and EDTA It is important to ensure that the pH of the Tris is at 7 4 room temperature prior to use

TBS

25 mM	Tris (pH 7 4)
137 mM	Nacl
5 mM	KCl

0 7 mM	$CaCl_2$
0 5 mM	$MgCl_2$
0 6 mM	Na_2HPO_4

Prepared from autoclaved stocks, aliquoted and filtered before use Again the pH of the Tris is critical

DEAE Dextran

1mg/ml in TBS, prepared fresh and filter sterilised

β-galactosidase assay

100 x Mg solution

0 1 M Mg cl_2

4 5 M 2-mercaptoethanol

Stored at -20°C

1 x ONPG substrate (o-nitrophenyl β-D-galactopyranoside)

4mg/ml in 0 1M sodium phosphate buffer pH7 5

Stored at -20°C

SOLUTIONS FOR PROTEIN ISOLATION

Suspension buffer

0 1 M	Nacl
0 01 M	Tris-Cl (pH 7 6)
0 001 M	EDTA (pH 8 0)
1 µg/ml	leupeptm
100µg/ml	PMSF

Stored at 4^0C

2X SDS gel loading buffer

100 mM	Tris-cl
10%	2-mercaptoethanol
4% (w/v)	SDS
0 2% (w/v)	Bromophenol blue
20% (v/v)	Glycerol

Two times loading buffer was prepared without 2-mercaptoethanol and stored at room temperature 2-mercaptoethanol was added just prior to use from a 14 4M (100%) stock

Protease Inhibitors (stock solutions)

2 mg/ml	Leupeptin in water
0 1 M	PMSF (phenylmethylsulfonyl fluoride) in isopropanol

Stored at -20°C (PMSF was stored away from light)

SOLUTIONS FOR SDS PAGE/WESTERN BLOTTING

1 M	Tris-Cl pH 6 8
1 5 M	Tris-Cl pH 8 8
10% (w/v)	SDS
10% (w/v)	Ammonium persulphate (APS)
Acrylagel	
Bis-acrylagel	
TEMED	

10X Tris glycine running buffer (500 ml)

15 138 g	Tris
71 125 g	Glycine
5 0 g	SDS

Made up to 500 ml with distilled water

Destain

100 ml	Acetic acid

400 ml	Methanol
500 ml	Distilled water

Coomassie blue stain

1 g	Coomassie blue R
200 ml	Destain

Transfer Buffer (10X stock solution)

30 3g	Tris
144 2g	Glycine

Adjusted to pH 8 3, made up to 1 L with distilled water, stored at room temperature

Transfer Buffer (1X working Solution)

100 ml	10X Stock Solution
200 ml	Methanol
700 ml	Distilled H_2O

Stored at 4°C

TBS (1X)

6 1 g	Tris
8 8 g	Nacl

Made up to 1 L with distilled water and adjusted to pH 7 5 with HCl

Autoclaved and stored at room temperature

TBST (0.1%, v/v)

1 L	TBS (as above)
I ml	Tween 20

Blotto

1X TBS (as above)

0 05% (v/v)Tween 20

5% (w/v) non-fat dry milk (Marvel)

0 02% NaN_3

Sodium azide (10%) (w/v)

1g	NaN_3
10ml	Distilled water

REAGENTS FOR RNA ANALYSIS

RNA sample buffer

50 % (v/v)	Deionized formamide
8 3 % (v/v)	Formaldehyde
0 027 M	MOPS pH 7 0
6 7 mM	Sodium acetate

RNA loading buffer (containing ethidium bromide)

50% (v/v)	High grade glycerol
1 mM	EDTA (pH 8 0)
0 4 % (w/v)	Bromophenol blue
0 1 µg/ml	Ethidium bromide

Aliquoted and stored at -20^0C

5 X MOPS Buffer (Formaldehyde gel running buffer)

0 1 M MOPS (pH 7 0)

40 mM Sodium Acetate

5 mM EDTA (pH 8 0)

20 6g of 3 –(*N*-morpholino*)* propanesulfonic acid (MOPS) was dissolved in 800 ml of DEPC treated 50 mM sodium acetate The pH of the solution was adjusted to 7 0 using 2 M NaOH 10 ml of DEPC-treated 0 5 M EDTA (pH 8 0) was added and the volume of the solution was adjusted to 1L using DEPC-treated H_2O The 5X solution was filter sterilised through a 0 2 micron filter prior to use

Formaldehyde gel

1 part Formaldehyde

3 5 parts agarose in DEPC H_2O

0 68 g	Agarose
35ml	DEPC H_2O

The agarose solution was boiled until fully dissolved and allowed to cool to 60^0C The following were then added

11 ml	5X MOPS buffer
10 ml	Formaldehyde
56 ml	Final volume

The gel was cast in a fume hood and allowed to set for approximately 45 mm The gel was electrophoresed in 1X formaldehyde gel running buffer

RNA loading buffer

50% (v/v)	High grade glycerol
1 mM	EDTA (pH 8 0)
0 25% (w/v)	Bromophenol blue
0 25% (w/v)	Xylene cyanol FF

DEPC-treated overnight, autoclaved and stored at room temperature

20XSSC

175 3g	Nacl
88 2g	NaCitrate

pH to 7 0 using a few drops of 10M NaOH and made up to 1 litre using up H_2O DEPC treated and autoclaved Stored at room temperature

Hybridization Buffer

50% (v/v)	Deionized formamide
6X	SSC

1% (w/v)	SDS
0 1% (v/v)	Tween 20
100μg/ml	tRNA

Prepared fresh prior to use stored at room temperature

REAGENTS FOR RIBONUCLEASE PROTECTION ASSAY

Five percent denaturing polyacrylamide gel

A five percent denaturing polyacrylamide gel was prepared for resolution of labelled riboprobes and analysis of protected RNA fragments The following formula was employed to determine the amount of acrylamide and bis-acrylamide required

Va	= volume of acrylamide
Vb	= volume of bis-acrylamide
Vt	= total volume of gel mix (150 ml)
C	= % crosslinking
A	= % gel

$$Va = \frac{Avt}{30} \qquad Vb = \frac{ACVt}{200}$$

Va = 5*150/30 = 25 ml Vb = 5*5 2*150/200 = 19 5 ml

5 % Denaturing PAGE

63 g	Urea
25 ml	Acrylamide
19 5 ml	Bisacrylamide
15 ml	10X TBE

Made up to 150 ml with up H_2O

Ninehundred μl of 10% (w/v) APS and 150 μl TEMED were added, and mixed briefly, before pouring

10X TBE (per 500 ml)

54 g	Tris base
27 5 g	Boric acid
20 ml	0 5 M EDTA (pH 8 0)

One X concentration was used for polyacrylamide gel preparation

10% (w/v) Ammonium persulphate

0 1 g APS/ml ultra pure H_2O

Elution Buffer

0 5M ammonium acetate

10 0 mM Magnesium acetate

1mM EDTA pH 8 0

0 1% SDS

made up in DEPC-treated upH_2O

Developer (5 L)

1 50 L	H_2O
1 25 L	Developer
2 25 L	H_2O

Stirred for 2 min

Fixer (5 125 L)

3 625 L	H_2O
1 250 L	A fixer
0 250 L	B fixer

Stirred for 2 min

REAGENTS FOR EMSA

Nuclear Protein Extraction buffers

Buffer A	Final Conc	Stock	for 100ml
Hepes pH 7 9 (NaOH)	10mM	1M	1ml
KCl	10mM	2M	500ul
$MgCl_2$	1 5M	1M	150ul
H_2O			98 5ml

Make aliquots of 10ml each

Before use add 50ul of DTT (1M)

50ul Pefabloc (Roche) to each aliqout

Buffer B			
Hepes pH 7 9 (NaOH)	20mM	1M	2ml
Glycerol		86-88%	25ml
NaCl		5M	8 4ml
$MgCl_2$		1M	150ul
EDTA (pH8 5)		0 5M	40ul

Make aliquots of 10ml each

Before use add 50ul of DTT

50ul Pefabloc

Poly (dI-dC).Poly (dI-dC) (stock)

Dissolve10 U in 393 5 µl ultrapure H_20

Store in aliquots at -20°C

Nuclear extracts were quick frozen and stored in liquid Nitrogen Protein content was assayed by the BCA assay

Reagents for Shift Reaction

Binding buffer (4 x)	Final Concentration	Stock	For 10ml
Hepes (pH 7 9)	40mM	1M	400ul
EDTA (pH 8)	4mM	0 5M	80ul
KCL	800mM	1M	8ml
Ficoll	16%		1 6g

1ml aliquots were then frozen

DTT was added to a final concentration of 4mM

And Pefabloc was added as before

BSA was made up to 1mg/ml in dI H_2O

Bandshift polyacrylamide gel

The formula used to calculate the volume of Accugel [40% (29 1) Acrylamide Bisacrylamide solution] used to prepare a gel of a given percentage is as follows

V_a = volume of accugel to be used (ml)

V_t = Total volume of gel casting solution required (ml)

X = % gel desired

$$V_a = \frac{(V_t)(X)}{40}$$

For a 4% or 5% gel of 50 ml volume

Solution (stock)	Volume (ml)		
	4%	5%	8%

Accugel	5 0	6 2	10 0
10xTBE	5 0	5 0	5 0
Water (ultrapure)	39 9	38 7	34 9

Then, 5µl of 1M DTT was added to the gel mixture followed by 50mg of APS and 15µl of TEMED and the gel mixture swirled briefly and poured into the gel mould The comb was then inserted and the gel allowed to set for at least 45 min

Binding Reaction. (20ul)

Binding Buffer 4X	5ul
Poly dIdC	2ul
BSA (1mg/ml)	2ul
Binding Mix)	9ul

Binding Mix	9ul
Antibody	5ul
Nuclear Extract	10ug

Incubated for 5 minutes at room temperature

^{32}P labelled oligonucleotide,	1-2ul
dI H_2O	20ul

Incubated for 30 minutes at room temperature

Loaded onto a 4-6% pAA gel in TBE one extra lane contained Bromophenol Blue to visualise the leading edge of the gel

In supershift reactions, nuclear extracts and Antibody were incubated at room temperatrure for 30 minutes prior to addition of labelled oligonucleotide