

Epstein-Barr Virus Latent Proteins Regulate Expression of the Anti- Apoptotic Cellular *bfl-1* Gene

A dissertation submitted for the degree of Ph D

By

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2005

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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 Doctor of Philosophy 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.'

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Date: Friday, 29th January, 2005.

Acknowledgements

Firstly I would like to acknowledge my supervisor Dr Dermot Walls for giving me the opportunity to work as part of his research group. Thanks for all the support and guidance over the last few years, and especially for the time and effort put into the preparation of this thesis. Many thanks to Brendan and Pam for teaching me the tricks of the trade, and to Sinead and Eva for all their help over the past couple of years. I couldn't have found a nicer bunch of people to work with and will always remember the laughs we've had in X270, not to mention the various nights out!

Thanks also to the other post-grads, post-docs and technical staff in DCU for always being willing to lend a helping hand and making the School of Biotechnology a great place to work. A special thanks to Caroline for being a great friend and for the various nights in the Slipper-we never thought we'd see the day we finished!

Thanks to the girls Claire, Dee, Cat, Michelle, Sarah and Sara-Jane for always showing an interest in my work, and understanding when I was so busy that I neglected you! Thanks to Seonadh and Hill - who would have thought we'd end up with 3 PhDs between us!

A very special thanks to my parents for endless financial support, and to Dad for the taxi service back and forth over the M50. Thanks to Fergal and Eugene for their support and to Phil for being the best personal assistant anyone could ask for!

Endless thanks to Dave for being such a great listener and wonderful source of knowledge and advice when I was writing up. For your love, support, friendship and all the laughs-thank you.

ABSTRACT

The ubiquitous and oncogenic human herpes-virus 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, including apoptosis, proliferation and differentiation. These host-virus interactions are likely to play a crucial role in the development of EBV-associated malignancies such, as Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma and tumours in immunosuppressed individuals. It has previously been shown in our laboratory that two EBV latent proteins, latent membrane protein 1 (LMP1) and EBV nuclear antigen 2 (EBNA2), which are major effectors of cellular phenotypic change, can independently regulate expression of the cellular *bfl-1* gene. 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. In this study, it is reported that LMP1 and EBNA2 regulate *bfl-1* activity through interactions with components of the NF- κ B and Notch signalling pathways respectively. NF- κ B composed of p65 sub-units trans-activated the *bfl-1* promoter in the EBV-negative cell line DG75, and an NF- κ B-like binding site at position -52 to -43 relative to the transcription start site was essential for this effect. An RBP-J κ /CBF1 mutant blocked EBNA2-mediated trans-activation of *bfl-1* in DG75 cells, indicating an important role for this DNA-binding protein in *bfl-1* trans-activation by EBNA2. Although RBP-J κ /CBF1 is also essential for signalling by the cellular equivalent of EBNA2, intra-cellular Notch (Notch1C), this protein was not found to trans-activate the *bfl-1* promoter. Both EBNA2 and LMP1 are expressed in EBV-infected cell lines, and EBNA2 is responsible for induction of LMP1. Blocking of either EBNA2- or LMP1-mediated signalling in EBV-infected cell lines did not dramatically affect the level of *bfl-1* promoter activity. However, when both EBNA2 and LMP1 signalling were blocked simultaneously, a significant decrease in the level of *bfl-1* activity was observed. These data indicate a role for both EBNA2 and LMP1 in the regulation of the promoter for the *bfl-1* gene in the context of the EBV-infected cell. These findings are relevant to our understanding of EBV persistence in the infected host, and its role in malignant disease.

Abbreviations

A	Absorbance
aa	Amino acid
AIDS	Acquired immuno-deficiency syndrome
AP	Alkaline phosphatase
AP1	Activator protein 1
APS	Ammonium persulphate
ATP	Adenosine tri-phosphate
BART	<i>Bam</i> HI A rightward transcript
BATF	B cell specific transcription factor
BCA	Bicinchomnic acid
BCIP-NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BCR	B cell receptor
BH	Bcl-2 homology
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
BTM	Basal transcription machinery
CAT	Chloroamphenicol acetyl transferase
cdk	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
cDNA	Complementary DNA
chx	Cyclohexamide
c-IAP	Cellular inhibitor of apoptosis
CIP	Calf intestinal phosphatase
CMV	Cytomegalovirus
Cp	C promoter
CR	Conserved region
CST	Complementary-strand transcript
CTAR	C-terminal activating region
C-terminal	Carboxy terminal
Cyt c	Cytochrome c
DEAE	Diethyl aminoethyl
DEPC	Diethylpyro-carbonate

dH ₂ O	Distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide
DS	Dyad symmetry
DS-DNA	Double stranded DNA
EA	Early antigen
EA-D	Diffuse early antigen
EA-R	Restricted early antigen
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBNA-LP	Epstein-Barr virus nuclear antigen-leader protein
EBV	Epstein-Barr virus
EC	Extracellular
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ERE	EBNA2-responsive element
ERK	Extracellular signal-regulated kinase
est	Estrogen
FBS	Foetal bovine serum
FR	Family of repeats
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp	Glycoprotein
HA	Hemagglutinin
HAT	Histone acetyltransferase
HD	Hodgkin's disease
HDAC	Histone deacetylase
HRT	Hairy-related transcription factor
HSV	Herpes simplex virus

HTLV	Human T-cell leukaemia virus type
IC	Intracellular
ICAM	Intercellular cell adhesion molecule
Ig	Immunoglobulin
IKK	I κ B kinase complex
IL	Interleukin
IM	Infectious mononucleosis
IP-CNS	Immunoblastic primary central nervous system
IR	Internal repeat
ITAM	Immunoreceptor tyrosine-based activation motif
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	c-Jun NH ₂ terminal kinase
LB	Luria-Bartani broth
LCL	Lymphoblastoid cell line
LFA	Lymphocyte-function-associated antigen
LMP	Latent membrane protein
LPS	Lipopolysaccharide
Luc	Luciferase
MA	Membrane antigen
MHC	Major histocompatibility complex
M-MLV RT	Moloney Murine leukemia virus reverse transcriptase
MMP	Matrix metalloproteinase
MRNA	messenger RNA
Mut	Mutant
NF- κ B	Nuclear factor κ B
NIK	NF- κ B inducing kinase
NK	Natural killer cell
EREB	Estrogen-responsive Epstein-Barr virus nuclear antigen
NLS	Nuclear localisation signal
NPC	Nasopharyngeal carcinoma
N-terminal	Amino terminal
O D	Optical density

ONPG	<i>o</i> -nitrophenyl- β -D-galactopuranoside
ORF	Open reading frame
ori	Origin of replication
p53	Protein 53
p38/MAPK	p38/mitogen activated protein kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEST	Proline-, glutamate, serine-, threonine-rich
PI3K	Phosphatidylinositol 3-kinase
PMA	Phorbol-12-myristate 13-acetate
pRb	Retinoblastoma protein
PTK	Protein tyrosine kinase
PTLD	Post transplant lymphoproliferative disorder
Qp	Q promoter
RBP-J κ	J κ -recombinant-binding protein
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
Rnase	Ribonuclease
RPA	Ribonuclease protection assay
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
S	Subunit
SDS	Sodium dodecyl sulphate
sIgG	Surface immunoglobulin G
SKIP	Ski-interacting protein
SuH	Suppressor of hairless
SV	Simian virus
TACE	Tumour necrosis factor α -converting enzyme
TAD	Trans-activation domain
TAE	Tris acetate ethylenediamine tetraacetic acid
tBid	Truncated Bid
TBP	TATA box binding protein
TBS	Tris buffered saline

TBS-T	Tris buffered saline + Tween 20
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TES	Transformation effector site
Tet	Tetracycline
TFB	Transformation buffer
T _m	Melting temperature
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TR	Terminal repeat
TRADD	TNFR-associated death domain
TRAF	TNFR-associated factors
UL	Unique long
upH ₂ O	Ultra pure water
US	Unique short
UV	Ultra violet
v/v	Volume per volume
VCA	Viral capsid antigen
w/v	Weight per volume
Wp	W promoter
wt	Wild type

UNITS

%	Percentage
°C	Degrees Celsius
bp	Base pairs
cm	Centimetre
cpm	Counts per million
g	Grams
Kb	Kilobase pairs
kDa	Kilo Dalton
Kg	Kilogram
L	Litres
lb/sq	Pounds per square inch
M	Molar
mA	Milliamperes
mg	Milligrams
ml	Millilitres
mM	Millimolar
ng	Nanograms
nm	Nanometres
pmole	Picomoles
U	Enzyme units
V	Volts
x g	G force
μF	Micro Faraday
μg	Micrograms
μl	Microlitre
μM	Micromolar

Publications

Pegman, P M , D'Souza, B N , **Smith, S.M** , Edelstein, L C , Rowe, M , Kempkes, B and Walls, D (submitted December, 2004) CBF1-dependent activation of the anti-apoptotic *bfl-1* gene by the Epstein-Barr Virus nuclear antigen 2

D'Souza, B N , Edelstein, L C , Pegman, P M , **Smith, S.M** , Loughran, S T , Clarke, A, Mehl, A , Rowe, M , Gelinas, C and Walls, D (2004) Nuclear factor- κ B-dependent activation of the anti-apoptotic *bfl-1* gene by Epstein-Barr virus latent membrane protein 1 and activated CD40 receptor *J Virol* **78**, 1800-1816

Poster presentations

Pegman, P M , **Smith, S.M** , D'Souza, B N , Loughran, S L , Rowe, M , Kempkes, B , Gelinas, C and Walls, D The Epstein-Barr virus nuclear antigen 2 transcriptionally activates the cellular anti-apoptotic *bfl-1* gene by an RBP-J κ /CBF1 dependent pathway International Symposium of Epstein-Barr virus and associated diseases Regensburg, Germany, 2004

D'Souza, B N , Pegman, P M , **Smith S.M** , Loughran, S T , Clarke, A , Mehl, A , Floettmann, E , Edelstein, L , Gelinas, C , Rowe, M and Walls, D Transcriptional activation of the cellular anti-apoptotic *bfl-1/A1* gene by the Epstein-Barr virus latent membrane protein 1 Society for General Microbiology- Irish Branch Meeting Microbial Diseases in the Immunocompromised patient Department of Biology, National University of Ireland, Maynooth, 2003

Pegman, P M , D'Souza, B N , **Smith, S.M** , Floettmann, E , Edelstein, L , Gelmas, C , Kempkes, B , Rowe, M and Walls, D Transcriptional activation of the anti-apoptotic *bfl-1/A1* gene is regulated by Epstein-Barr virus nuclear antigen 2 Society for General Microbiology- Irish Branch Meeting Microbial Diseases in the Immunocompromised patient Department of Biology, National University of Ireland, Maynooth, 2003

D'Souza, B N , Pegman, P M , **Smith S.M** , Loughran, S T , Clarke, A., Mehl, A , Floettmann, E , Edelstein, L , Gelinas, C , Rowe, M and Walls, D
Transcriptional activation of the *bfl-1/A1* gene by the EBV latent membrane protein 1 Irish Association for Cancer Research Meeting, Kilkenny, 2003

Pegman, P M , D'Souza, B N , **Smith, S.M** , Floettmann, E , Edelstein, L , Gelinas, C , Kempkes, B , Rowe, M and Walls, D Expression of the anti-apoptotic *bfl-1/A1* gene is regulated by Epstein-Barr virus nuclear antigen 2 Irish Association for Cancer Research Meeting, Kilkenny, 2003

Oral presentations

“Epstein-Barr virus latent genes regulate expression of the anti-apoptotic cellular *bfl-1* gene ” Biological Seminar Series, DCU, Glasnevin, Dublin 9 April 20th, 2004

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CHAPTER 1

An Introduction to Epstein-Barr Virus Biology

1.1 The discovery of Epstein-Barr virus (EBV)

Cancer is one of the most common diseases known to man, with more than 10 million people diagnosed with the disease every year throughout the world (World Health Organisation, 2004). The disease is complex and can have both genetic and environmental causes. Viruses have also been found to be involved in the development of some cancers. It is estimated that virus infections account for approximately 15 % of the worldwide cancer incidence (zur Hausen, 1991), demonstrating the urgent need to identify the exact role for viruses in cancer. Viruses may contribute to the development of human tumours by different mechanisms, indirectly by inducing immunosuppression or by modifying the host cell genome without persistence of viral DNA, or directly by expressing oncoproteins or by altering the expression of host cell proteins. The oncogenic viruses that are implicated in human disease are diverse and vary greatly in the complexity of their genomes, their target tissues and their requirements for additional co-factors during tumourigenesis. Some members of the human herpes viruses, most notably Epstein-Barr virus (EBV), have been linked to a number of cancers of both lymphoid and epithelial origin.

The discovery of EBV was a direct result of the association of the virus with Burkitt's lymphoma (BL). Denis Burkitt demonstrated in 1958 that the distribution of the endemic form of a tumour that mainly arises at the jaw of children was dependent on geographical and climatic factors (Burkitt, 1958). This led to speculations that a biological agent might be involved in the aetiology of the disease (Burkitt, 1962). After meeting Epstein in the early 1960s, Burkitt provided him with several lymphoma biopsies. However, Epstein failed to identify the pathogen associated with the tumours from fresh biopsies, and only after tumour cells were successfully grown in culture were Epstein, Achong and Barr able to identify replicating virus particles by electron microscopy in cell lines derived from African Burkitt's lymphoma (Epstein *et al.*, 1964). The association of EBV with BL was subsequently proven through the use of serological techniques to detect viral genes expressed in tumours. Although it was the association with BL that led to the discovery of the virus, EBV is found to be widespread in all human populations, with a prevalence of over 90 % in adults. Primary infection usually occurs during early childhood and is generally asymptomatic. However, primary infection that is delayed until adolescence or early adulthood may cause a self-limiting lymphoproliferative disease, infectious mononucleosis (IM).

Infection results in the establishment of a life-long carrier state characterised by the persistence of antibodies to several viral gene products and the secretion of infectious virus in saliva (IARC Monographs, 1997).

1.2 Classification of EBV

Humans are the exclusive natural host for EBV, which is classed as a herpes virus based on its structural properties. Taxonomists have renamed EBV as human herpes virus 4 (HHV4), but EBV is still its commonly used name (IARC Monographs, 1997). Herpes viruses are biologically classified into three subfamilies: alpha, beta and gamma, with EBV belonging to the gamma-herpes virus subfamily and the genus *Lymphocryptovirus*. The gamma-herpes viruses are classified on the basis of their biological properties rather than their genomic organization (Kieff, 1996). Viruses of this subfamily are characterised by their tropism for lymphoid cells and their capacity to induce cell proliferation *in vivo* and cell immortalisation *in vitro*. 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 virus subfamily.

1.3 Structure of EBV

Like other herpes viruses, EBV consists of a toroid protein core that is wrapped with double-stranded DNA (Figure 1.1). The DNA wrapped protein core is contained within an icosahedral nucleocapsid with 162 capsomeres approximately 100 nm in diameter. The major EBV capsid proteins are 28, 47 and 160 kDa in size. A protein tegument surrounds the nucleocapsid and is enclosed by an outer envelope with external glycoprotein spikes. The most abundant EBV proteins within the envelope and tegument are the glycoproteins gp350/220 and gp152 respectively (Dolyniuk *et al.*, 1976; Epstein and Achong, 1973).

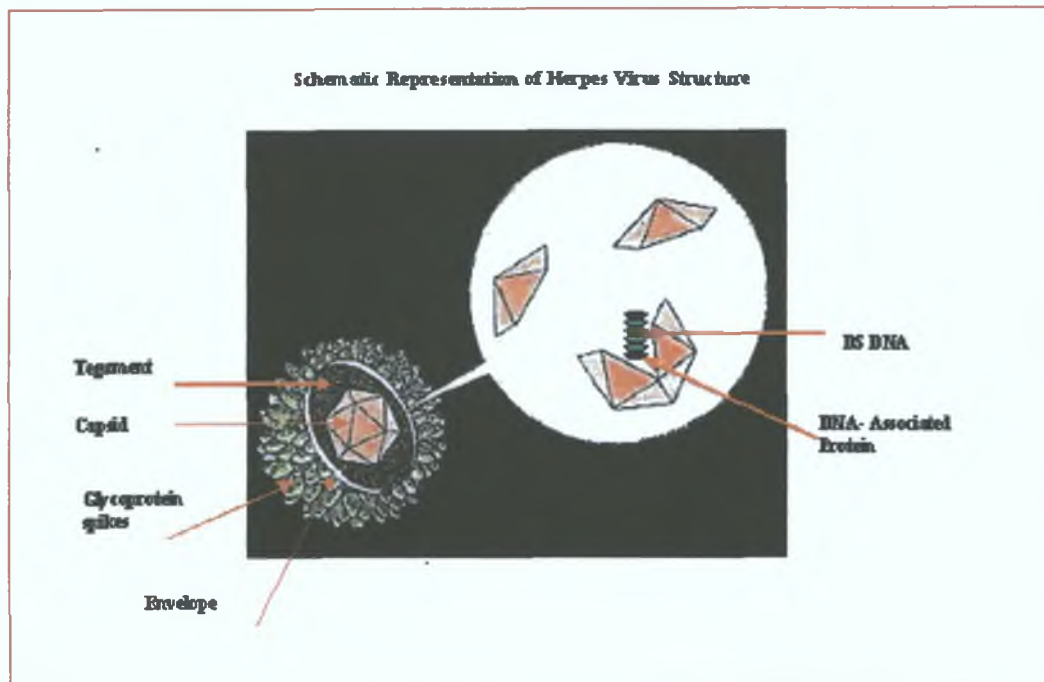


Figure 1.1: A Schematic representation of the herpes virus structure. EBV consists of a toroid protein core (illustrated in black) that is wrapped with double-stranded DNA (DS DNA, green). The DNA wrapped protein core is contained within a nucleocapsid (red). A protein tegument surrounds the nucleocapsid and is enclosed by an outer envelope with external glycoprotein spikes

1.4 EBV genome

The EBV genome is a linear, double-stranded 172 kbp DNA molecule 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 DNA of the B95.8 strain of EBV has been cloned as a set of overlapping *EcoRI* and *BamHI* restriction endonuclease fragments (Arrand *et al.*, 1981), which was an important prerequisite that enabled EBV to be the first herpes virus for which the complete DNA sequence was obtained (Baer *et al.*, 1984). As a consequence of Arrand's findings, regions of the EBV genome have become known by their position on the *BamHI* restriction endonuclease map (Figure 1.2B). Open reading frames (ORFs) are named using a four-digit number and acronym, for example BKRF1 refers to the first rightward ORF of the *BamHI* K region of the genome. Features of the EBV genome (Figure 1.2A), which are also found in other gamma-herpes viruses, include randomly reiterated 3 kb internal direct repeats (IR1) that separate the genome into short (US) and long

(UL) unique domains (Hayward *et al.*, 1980). Interspersed within the UL regions are short repeated sequences (IR2-IR4). At each end of the DNA molecule are 4-12 copies of a 500 bp terminal repeat (TR) (Kintner and Sugden, 1979). The TRs at each end of the linear genome join together to form a closed circular episome in the nucleus of latently infected cells.

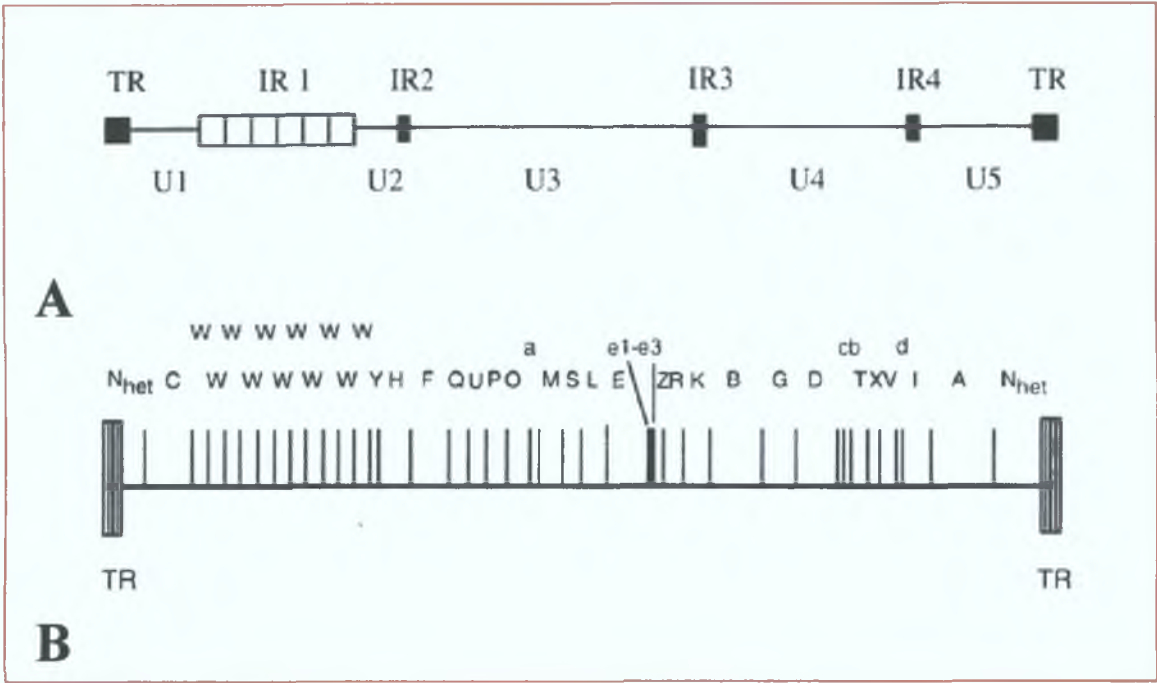


Figure 1.2: Features of the EBV genome. (A) The linear EBV genome comprises a short unique region (U1) separated from a long unique region (U2-U5) by a large repeating element, the internal repeat, IR1. The long unique region is interspersed with several minor internal repeat elements IR2-IR4. In latently infected cells, the genome fuses at the terminal repeats (TR) to form a circular episome. N_{het} denotes the heterogeneity in this region due to the variable number of TRs in different virus isolates and in different clones of EBV-infected cells. (B) The *Bam*HI restriction map of the B95.8 genome. Fragments are named according to size, with the *Bam*HI A fragment being the largest.

Two subtypes of EBV are known to infect humans, originally referred to as A and B and now called types 1 and 2 (Kieff, 1996). Type 1 and type 2 EBV appear to be identical over the bulk of the EBV genome, but show allelic polymorphism (with 50-80 % sequence homology depending on the locus) in a subset of latent genes, namely the Epstein-Barr virus nuclear antigen (EBNA)-LP, EBNA2, EBNA3A, EBNA3B and EBNA3C (Sample *et al.*, 1990). Type 1 EBV is predominant in many Western countries, whereas both types are widespread in equatorial Africa, New Guinea and perhaps certain other regions (Young *et al.*, 1987). Studies suggest that type 1 isolates are more potent than type 2 in achieving B cell transformation *in vitro*. In addition to

this broad distinction between EBV types 1 and 2, there is also minor heterogeneity within each virus type, which is most easily detected as a variation in the size of the EBNA proteins (Murray and Young, 2001). These differences have been used to trace virus transmission within families and from transplant donors to recipients. The balance of evidence to date suggests that most healthy individuals are infected with only one virus type, although a small number of healthy virus carriers do harbour multiple, perhaps sequentially acquired EBV strains (Brooks *et al.*, 2000). By contrast, most immunologically compromised patients are infected with multiple EBV strains (Murray and Young, 2001).

1.5 EBV infection *in vivo*

It is widely accepted that EBV infects more than 90 % of the world's adult population and the virus is transmitted from host to host via saliva (Thompson and Kurzrock, 2004). Primary infections occurring during early childhood are normally silent or asymptomatic. However, infection that is delayed until late adolescence or early adulthood is associated with the debilitating non-malignant lymphoproliferative syndrome infectious mononucleosis (IM), which is also known as glandular fever (IARC Monographs, 1997). IM is characterised by a variety of symptoms, including fever, malaise, lymphadenopathy (marked swelling of peripheral lymphoid tissues) and the presence of atypical dividing lymphocytes in the peripheral blood (Eliopoulos and Young, 2001). Incoming virus establishes a primary focus of lytic replication in the stratified squamous epithelium of the oropharynx (Murray and Young, 2002; Sixbey *et al.*, 1984). Lytic replication describes the full cycle of virus infection, leading to the production of new infectious virus progeny, and eventually lysis of the infected cell. Oropharyngeal infection is followed by a latent infection (type III) of B cells as they traffic in close proximity to the infected epithelial cells (Figure 1.3A), resulting in B cell proliferation and viral spread throughout the B cell compartment. Many of the proliferating cells are removed by the emerging latent-antigen-specific primary T cell responses, but some escape by down-regulating antigen expression and establishing a stable reservoir of resting EBV-positive memory B cells, in which viral antigen expression is mostly suppressed (Young and Rickinson, 2004).

1.6 EBV persistence *in vivo*

After initial infection, EBV persists in the host for life, in a circulating subset of resting memory B cells. These cells carry the virus in a latent form, and there is a low level continuous or intermittent production of infectious virus into saliva (Yao *et al.*, 1985). Several lines of evidence support a role for the B lymphocyte as the site of EBV persistence *in vivo* (Murray and Young, 2001). Firstly, therapy aimed at eliminating virus replication using long-term acyclovir treatment eliminates virus excretion from the oropharynx, but does not affect the level of latent infection in B cells (Ernberg and Andersson, 1986). As soon as the treatment is halted, virus can be detected in the oropharyngeal secretions at pre-treatment levels (Yao *et al.*, 1989). In addition, studies of EBV strains in donor-recipient pairs, before and after bone marrow transplantation, have shown that the recipient's strain disappeared from the oropharynx and was replaced by the donor's strain, indicating that the bone marrow B cells harbour EBV (Gratama *et al.*, 1990). Furthermore, patients with X-linked agammaglobulinaemia, who are deficient in mature B cells, are found to be free of EBV infection, suggesting they are not able to maintain a persistent infection (Young, 1999).

EBV is thought to exist in the peripheral blood within the IgD⁻ memory B cell pool, with EBV gene expression limited to latent membrane protein 2A (LMP2A) and possibly EBNA1 (Babcock *et al.*, 1998). The exact route of entry to the memory B cell pool is still a matter of much debate. *In vitro*, both naïve and memory B lymphocytes seem equally susceptible to EBV infection (Young and Rickinson, 2004). One view is that the naïve B cell compartment is the main target of new EBV infections *in vivo*. Viral transformation drives naïve B lymphocytes into memory by mimicking the physiological process of antigen-driven memory B cell development in lymphoid tissue, involving somatic immunoglobulin (Ig) gene hypermutation during transit through a germinal centre (Babcock *et al.*, 1998, Babcock *et al.*, 2000). However, this view is difficult to reconcile with the finding that EBV-infected cells in the tonsils from patients with IM localise to extra-follicular areas and not germinal centres. Although some of the infected cells display a naïve phenotype, the expanding clones preferentially involve cells with a mutated phenotype that are typical of antigen selected memory cells (Kurth *et al.*, 2000). An alternative view, therefore, envisages infection of pre-existing memory cells as a direct route to memory. This is consistent with the finding of EBV-

infected B cells in tonsils but does not explain the apparent disappearance of the naïve B cell population (Young and Rickinson, 2004).

The reservoir of EBV-infected memory B cells is stably maintained and becomes subject to the same physiological controls governing memory B cell migration and differentiation as a whole (Laichalk *et al.*, 2002). Such a strategy brings with it the possibility of antigen-driven recruitment of infected cells into germinal centres, entailing the activation of their latency programmes, leading to progeny that either re-enter the circulating memory pool or differentiate to become plasma cells that might migrate to mucosal sites in the oropharynx (Young and Rickinson, 2004). Differentiated plasma cells that migrate to the oropharynx undergo lytic cycle replication, providing a source of low-level shedding of infectious virus into the oropharynx, where the virus can spread to other individuals via saliva. Lytic replication in the oropharynx might also initiate new growth-transforming latency type III infections of naïve and/or memory B cells; these new infections might possibly replenish the B cell reservoir, but are more likely to be efficiently removed by the memory T cell response (Young and Rickinson, 2004). The interactions between EBV and host cells are summarised in Figure 1.3.

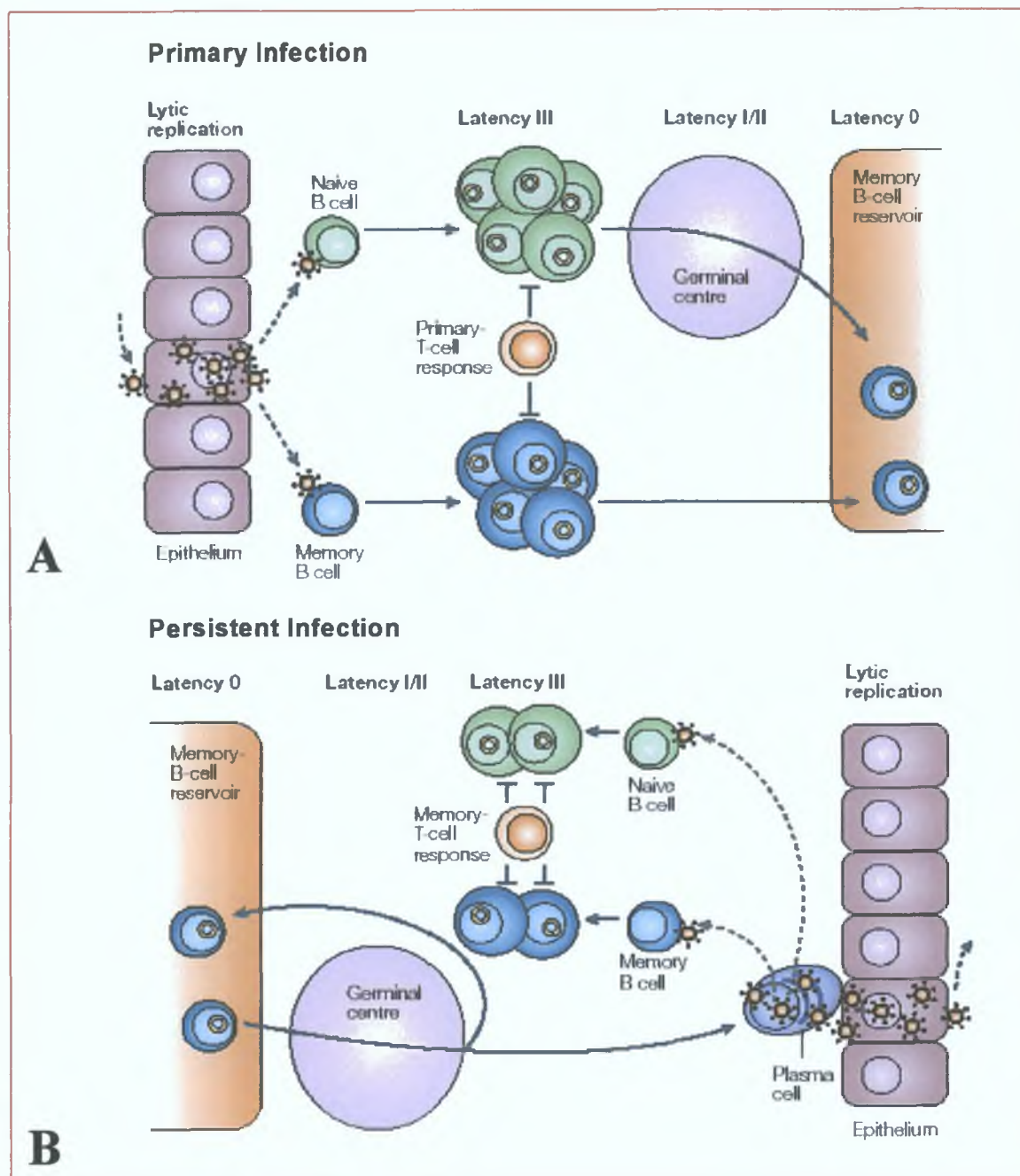


Figure1.3: Putative *in vivo* interactions between Epstein-Barr virus and host cells. (A) Primary infection. Incoming virus establishes a primary focus of lytic replication in the oropharynx (possibly in the mucosal epithelium), after which the virus spreads throughout the lymphoid tissues as a latent (type III latency) growth-transforming infection of B cells. Many of these proliferating cells are removed by the emerging latent-antigen-specific primary-T-cell response, but some escape by down-regulating antigen expression and establishing a stable reservoir of resting EBV-positive memory B cells, in which viral antigen expression is mostly suppressed (latency type 0). Different views on the sequence of events are shown. (B) Persistent infection. The reservoir of EBV-infected memory B cells becomes subject to the physiological controls governing memory B cell migration and differentiation as a whole. Occasionally, these EBV-infected cells might be recruited into germinal-centre reactions, entailing the activation of different latency programmes, after which they might either re-enter the reservoir as memory cells or commit to plasma-cell differentiation, possibly moving to mucosal sites in the oropharynx and, in the process, activating the viral lytic cycle (adapted from Young and Rickinson, 2004).

1.7 EBV-associated malignancies

Long-term EBV co-exists with most human hosts without overt serious consequences. However, in a small percentage of individuals, the virus may be implicated in the development of malignancy. Experimental and clinical evidence have linked EBV to various tumours of lymphoid origin: Burkitt's lymphoma (BL), Hodgkins' disease (HD), lymphomas in immuno-compromised individuals and certain T cell lymphomas. In addition the virus has been linked to epithelial cell malignancies, including nasopharyngeal carcinoma (NPC) and gastric carcinomas. EBV-associated malignancies are outlined in Table 1.1 (adapted from Thompson and Kurzrock, 2004).

Table 1.1: EBV-associated malignancies

Disease	Subtype	% EBV positivity	Description
Burkitt's lymphoma (BL)	Endemic	> 95 %	An aggressive lymphoma occurring endemically in equatorial Africa and Papua New Guinea and non-endemically sporadically throughout the Western world. Both disorders are characterised by deregulation of <i>c-myc</i> expression, resulting from translocation of the <i>c-myc</i> gene to a location close to the enhancers of the antibody genes.
	Nonendemic	15-30 %	
Hodgkin's disease (HD)	Mixed cellularity	70 %	HD is characterised by an expansion of Reed-Sternberg cells, which are postulated to be of B cell lineage.
	lymphocyte depleted	> 95 %	
	nodular sclerosing	10-40 %	
	lymphocyte predominant	< 5 %	
Non-Hodgkin's lymphoma	Nasal T/NK	> 90 %	A non-B cell non-Hodgkin's lymphoma occurring in T cells and natural killer (NK) cells.
	Angioimmunoblastic	unknown	
	lymphadenopathy		

Post-transplant lymphoproliferative disorders (PTLD)		> 90 %	B lymphomas occurring in T-cell immuno-compromised individuals following transplantation.
AIDS-associated lymphoma	IP-CNS other	> 95 % 30-50 %	B lymphomas that occur in the immunoblastic primary central nervous system (IP-CNS) of T-cell immuno-compromised AIDS patients.
Leiomyosarcomas in immuno-suppressed individuals		frequent	Smooth muscle tumours in immuno-compromised patients.
Nasopharyngeal carcinoma (NPC)	anaplastic	> 95 %	An undifferentiated carcinoma of the nasopharynx in the Chinese province of Canton, Hong Kong, Taiwan and some parts of Alaska and Greenland.
Gastric carcinoma	Lymphoepithelioma-like Adenocarcinoma	> 90 % 5-25 %	Carcinomas of the stomach resembling NPC.
Breast carcinoma	Medullary carcinoma Adenocarcinoma	0-51 %	Carcinomas of the breast

1.8 EBV latent genes and transformation

EBV preferentially infects B lymphocytes and possesses the unique ability to transform resting B cells into permanent, latently infected lymphoblastoid cell lines (LCLs) *in vitro* (Young and Rickinson, 2004). This system has provided an invaluable tool for studying the transforming potential of the virus. Infection of other cells, mainly epithelial cells, is less efficient and not as extensively studied. B-cell transformation by EBV, resulting in the establishment of LCLs, therefore remains the dominant *in vitro* model of infection (Young and Rickinson, 2004).

In EBV-transformed LCLs, every cell carries multiple extra-chromosomal copies of the viral episome and constitutively expresses a limited set of viral gene products, the so-called latent products, which comprise six EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and EBNA-LP) and three latent membrane proteins (LMPs 1, 2A and 2B) (Kieff and Rickinson, 2001). A diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome is shown in Figure 1.4.

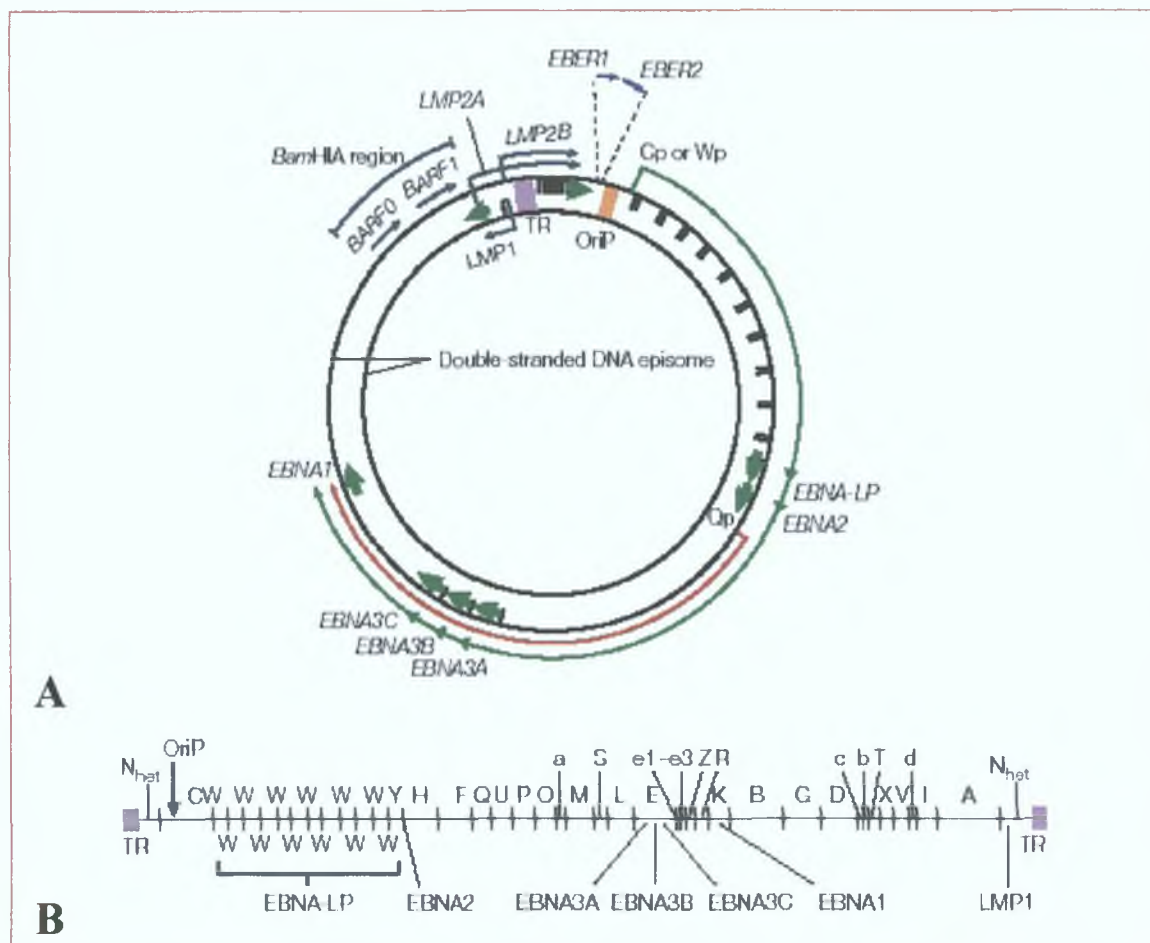


Figure 1.4: EBV latent genes. (A) A schematic representation showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The origin of replication (OriP) is shown in orange. The large green solid arrows represent exons encoding each of the latent proteins, and the arrows indicate the direction in which the genes encoding these proteins are transcribed. The latent proteins include six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and EBNA-LP) and the three latent membranes (LMPs 1, 2A and 2B). *EBNA-LP* is transcribed from a variable number of repetitive exons. *LMP2A* and *LMP2B* are composed of multiple exons, which are located on either side of the terminal repeat (TR) region, which is formed during the circularisation of the linear DNA to produce the viral episome. The blue arrows at the top represent the highly transcribed non-polyadenylated RNAs *EBER1* and *EBER2*; their transcription is a consistent feature of latent EBV infection. The long outer green arrow represents EBV transcription during a form of latency known as type III latency, in which all the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs that are generated by differential splicing of the same long primary transcript. The inner, shorter red arrow represents the *EBNA1* transcript, which originates from the Qp promoter during latency type I and latency type II. Transcripts from the *BamHI A* region can be detected during latent infection but no protein arising from this region has been definitively identified. The locations of the *BARF0* and *BARF1* coding regions are shown here. (B) Locations of the open reading frames for the EBV latent proteins on the *BamHI* restriction endonuclease map of the prototype B95-8 genome (adapted from Young and Rickinson, 2004).

Transcripts from the *Bam*HI A region of the viral genome (BART transcripts) are also detected in LCLs. In addition to the latent proteins and BARTs, LCLs display abundant expression of the small, non-polyadenylated (and therefore non-coding) RNAs, *EBER1* and *EBER2*. The function of these transcripts is not clear but they are consistently expressed in all forms of latent EBV infection (Young and Rickinson, 2004) and have served as excellent targets to detect EBV in tumours (Murray and Young, 2001). This pattern of latent EBV gene expression, which appears to be activated only in B-cell infections, is referred to as type III latency, and is a characteristic of most lymphomas in immuno-compromised individuals (Murray and Young, 2001). At least 2 other forms of EBV latency are observed. In latency type I, which is a characteristic of BL, only EBNA1, the EBERs and the BARTs are regularly detected. In latency type II, which is observed in EBV-associated HD and NPC, the EBERs, EBNA1 are expressed together with LMP1 and LMP2. The types of EBV latency are summarised in Table 1.2.

Table 1.2: EBV latency pattern and associated malignancies

Latency type	Viral genes expressed	Associated malignancies	References
Type I	EBNA1 EBERs BARTs	Burkitt's lymphoma	Sbihi-Lammali <i>et al.</i> , 1996. Rowe <i>et al.</i> , 1987.
Type II	EBNA1 EBERs LMP1 LMP2 BARTs	Hodgkin's disease Nasopharyngeal carcinoma Peripheral T/NK lymphoma	Liebowitz and Kieff, 1993.
Type III	All EBNAs EBERs LMP1 LMP2 BARTs	AIDS-associated lymphomas PTLD	Liebowitz and Kieff, 1993. Niedobitek <i>et al.</i> , 1997.
Other	EBERs EBNA1 EBNA2	Smooth muscle tumours	Lee <i>et al.</i> , 1995

In vitro studies using LCLs have provided insight into the sequence of events occurring during EBV infection of B lymphocytes. EBV infection begins with the attachment of the major viral envelope glycoprotein gp350/220 to the CD21 (also known as CR2) receptor on the surface of B cells (Nemerow *et al.*, 1987; Tanner *et al.*, 1987). CD21 is a member of the Ig super-family and functions as the receptor for the C3d component of complement. The EBV envelope glycoproteins gp350 and gp220 are translated from abundant late replication cycle EBV mRNAs, which are transcribed from the same gene. The mRNA for gp350 is not spliced, whereas the mRNA for gp220 is spliced in frame. CD21 is the only known B-lymphocyte protein that binds gp350/220 (Kieff, 1996). The penetration of B cells by EBV also involves the viral glycoproteins gp25 and gp42/38 in a complex with gp85. This complex mediates an interaction between EBV and the major histocompatibility complex (MHC) class II molecules, which serve as a co-receptor for virus entry into B cells (Knox and Young, 1995).

Post-attachment events are complex. CD21 becomes cross-linked, which triggers an initial activating signal that is thought to prepare the cell for EBV infection. EBV binding to CD21 immediately activates the tyrosine kinase *lyk* and mobilises calcium (Cheung and Dosch, 1991; Gordon *et al.*, 1986). This is followed by an increase in mRNA synthesis, blast transformation, homotypic cell adhesion, surface CD23 expression and interleukin (IL)-6 production. The viral genome is then uncoated and delivered to the nucleus where it immediately circularises (Thompson and Kurzrock, 2004). Circularisation of the viral genome and transcription from the W promoter begin a cascade of events leading to expression of all latent genes. EBV does not encode an RNA polymerase, and uses host cell RNA polymerase II for transcription of viral RNAs (Kieff, 1996). The EBV nuclear antigen leader protein (EBNA-LP) and EBNA2 are the first proteins to be detected upon EBV infection (Thompson and Kurzrock, 2004). At 24 - 48 hours post-infection, a promoter shift occurs where the C promoter (Cp) is used instead of the initial Wp promoter. Activation of the Cp promoter leads to a higher level of transcription of the EBNA mRNAs. Many transcripts now pass the polyadenylation site downstream of EBNA2 and extend to EBNA1, 3A, 3B and 3C (Kieff, 1996). The different EBNA1s are encoded by individual mRNAs generated by differential splicing of the same long "rightward" primary transcript expressed from one of two promoters (Cp or Wp) located close together in the *Bam*HI C and W regions of the genome (Murray and Young, 2001) (Figure 1.5). The processing of the transcripts is then determined by their polyadenylation sites (Kieff, 1996).

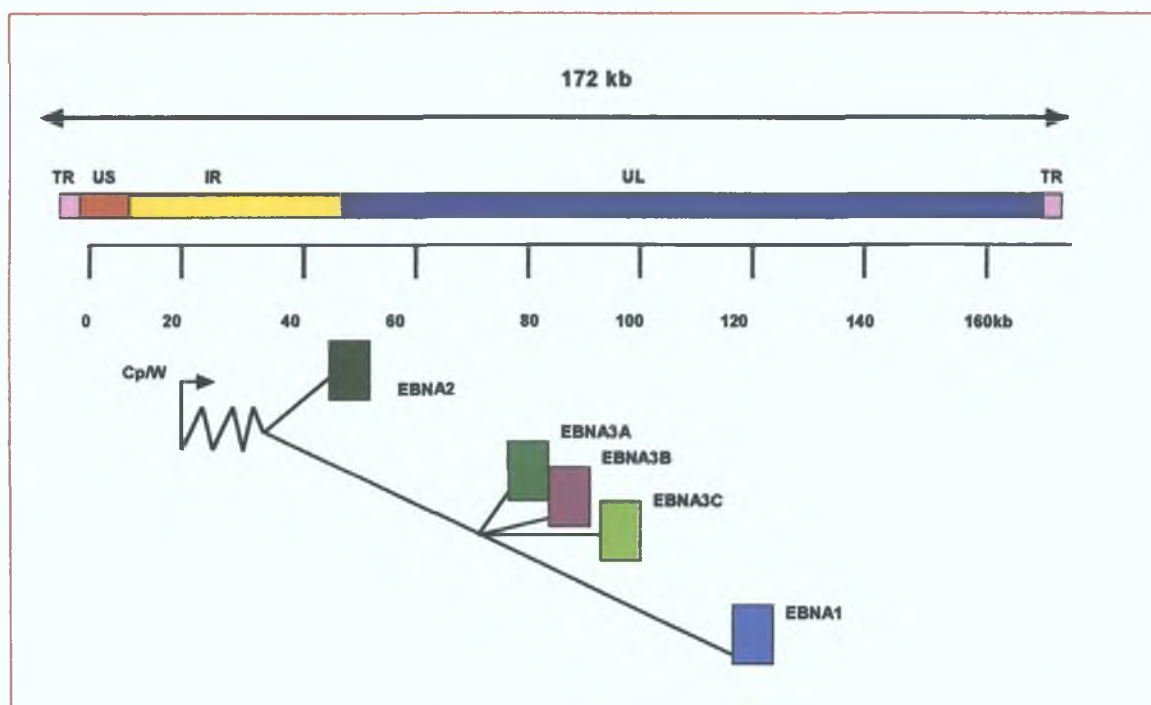


Figure 1.5: A simplified outline of the splicing of the EBV nuclear antigen coding mRNAs. All the EBNAs are transcribed from either the Cp or Wp promoter; the different EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcript.

By 32 hours post-infection, all EBNAs and latent membrane proteins (LMPs) are expressed. The LMP transcripts are expressed from separate promoters in the *Bam*HI N region of the EBV genome, with the leftward LMP1 and rightward LMP2B mRNAs apparently controlled by the same bi-directional promoter (Murray and Young, 2001). EBER expression lags behind by approximately 24 hours and does not reach substantial levels until approximately 70 hours post-infection. The EBV genome is extensively transcribed during latent infection and only a highly restricted complexity of RNA is processed into cytoplasmic polyadenylated polyribosomal mRNA. Polyadenylation may determine splicing and thereby regulate expression of 6 EBNAs from the same promoter (Kieff, 1996). However, *cis* or *trans* factors may also play a role in splice choice, although none have been identified. It is possible that the EBERs or EBNA (particularly LP) act as these factors (Kieff, 1996).

EBV-infected LCLs show high levels of expression of the B cell activation markers CD23, CD30, CD39 and CD70, and of the cell adhesion molecules lymphocyte-function-associated antigen 1 (LFA1; also known as CD11a/18), LFA3 (also known as CD58) and intercellular cell adhesion molecule 1 (ICAM1; also known as CD54) (Kieff and Rickinson, 2001; Rowe *et al.*, 1987). These markers are usually absent or

expressed at low levels on resting B cells, but are transiently induced to high levels when these cells are activated into short term growth by antigenic or mitogenic stimulation, indicating that EBV-induced immortalisation can be elicited through the constitutive activation of the same cellular pathways that drive physiological B cell proliferation (Young and Rickinson, 2004). The ability of EBNA2, EBNA3C and LMP1 to induce LCL-like phenotypic changes when expressed individually in human B cell lines indicates that these viral proteins are key effectors of the immortalisation process (Wang *et al.*, 1990). The role of EBV latent genes in the *in vitro* transformation of B cells has been confirmed more recently by the generation of recombinant forms of EBV that lack individual latent genes. Studies using such viruses have confirmed the absolute requirement for EBNA2 and LMP1 in the transformation process, and have also highlighted a crucial role for EBNA1, EBNA-LP, EBNA3A and EBNA3C (Kieff and Rickinson, 2001).

Epithelial cells generally do not express CD21, suggesting that EBV enters these tissues by other, as yet unidentified, cellular receptors. Various human epithelial cells can be infected *in vitro* either by direct contact with high-titre virus supernatant or by co-cultivation with EBV-producing B cells, such as Akata (Imai *et al.*, 1998). This suggests an *in vivo* model of EBV infection whereby epithelial tissues might be infected by virtue of their close proximity to (i) infectious virus present in saliva or (ii) lytically infected B cells resident near or within epithelial tissues, for example adjacent to the sub-epithelial sinus in tonsil or within nasopharyngeal mucosa (Murray and Young, 2001).

1.9 Structure and functions of the EBV latent genes

The transformation of B cells by EBV involves the co-ordinated action of several latent gene products. EBV uses its viral proteins, the actions of which mimic several growth factors, transcription factors and apoptotic factors, to usurp control of cellular pathways that regulate diverse homeostatic cellular functions, allowing both cellular transformation and the establishment of a latent infection in the memory B cell compartment. The structural and functional properties of the EBV latent genes are addressed below.

1.9.1 Epstein-Barr virus nuclear antigen 1 (EBNA1)

EBNA1 is a 73 kDa sequence-specific DNA-binding protein which is expressed in all EBV-associated tumours and EBV-proliferating cells in healthy EBV carriers. The protein consists of a short amino terminal, a 20-40 kDa glycine-alanine repetitive sequence flanked by arginine rich regions, and a highly charged acidic carboxy terminal sequence (Hennessy and Kieff, 1983). The glycine-alanine repeat is thought to act as an inhibitor of MHC class I-restricted presentation, and appears to function in inhibiting antigen processing via the ubiquitin-proteasome pathway (Levitskaya *et al.*, 1995). Failure to present EBNA1-derived peptides results in ineffective CD8⁺ T cell responses to EBNA1 when expressed in target cells. A nuclear localisation sequence, DNA binding domain and a dimerisation domain have also been mapped on the EBNA1 protein (Figure 1.6).

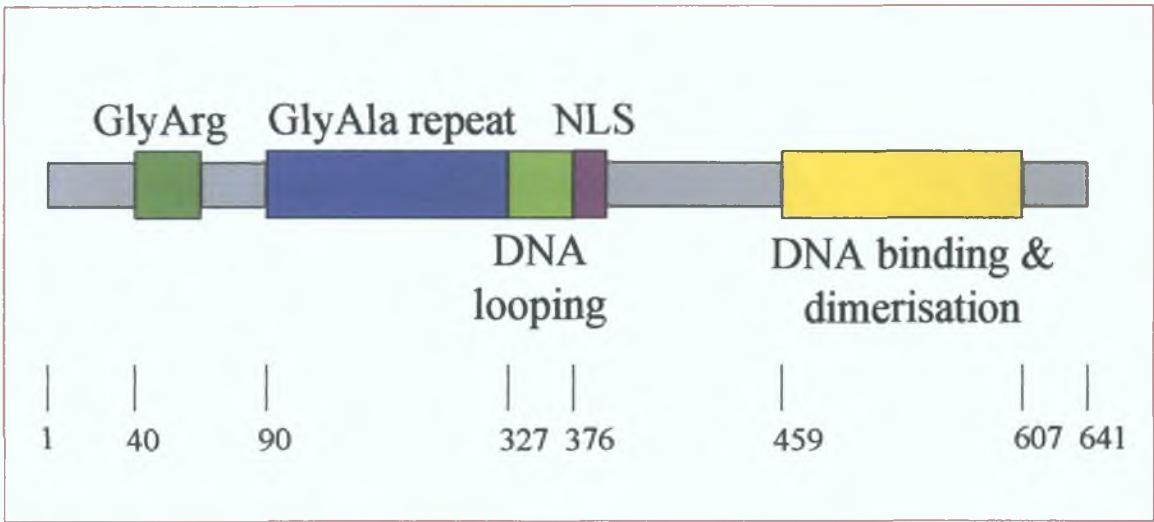


Figure 1.6: Functional domains of Epstein-Barr virus nuclear antigen 1. EBNA1 is a multifunctional 73 kDa protein of 641 amino acids. The protein consists of a short amino terminal, a 20-40 kDa glycine-alanine repetitive sequence (GlyAla) that varies in length between viral strains. The glycine-alanine sequence is flanked with arginine rich regions (GlyArg). A nuclear localisation sequence (NLS), DNA binding domain and a protein dimerisation domain have also been mapped on the highly charged acid carboxy terminus of the EBNA2 protein (adapted from Avolio-Hunter and Frappier, 1998).

EBNA1 plays a number of important roles during latent EBV infection of human host cells. Importantly, EBNA1 activates replication of the viral genome once every cellular S phase (Adams, 1987; Yates and Guan, 1991). This is important because EBV DNA rarely integrates into the host cell genome and is usually carried as circular DNA

episomes in latently infected cells (Humme *et al.*, 2003). Therefore, EBV requires a mechanism for replicating viral DNA before mitosis and distributing episomes into progeny cells during cell division. EBNA1 fulfils these tasks by both initiating virus replication and by segregating the viral episomes during cell division to ensure the EBV genome is stably maintained (Kieff and Rickinson, 2001). EBNA1 is known to associate with cellular metaphase chromosomes through chromosome binding-domains within its N-terminus, an association that is required for both the partitioning of oriP plasmids and for their replication (Sears *et al.*, 2004). Furthermore, EBNA1 activates transcription of other EBV latent gene products.

All of these functions require direct binding of EBNA1 in a sequence specific manner to oriP, the origin of viral replication, which is composed of two distinct EBNA1 binding elements (Ambinder *et al.*, 1990; Jones *et al.*, 1989; Rawlins *et al.*, 1985). These two binding elements contain various copies of the EBNA1 recognition sequence TAGGATAGCATATGCTACCCAGATCCAG (Kieff, 1996). One of these two regions, the dyad symmetry (DS) element, which contains four EBNA1 binding sites, is the site of initiation of episomal replication (Wysokenski and Yates, 1989). The other region, the family of repeats (FR) consists of 20 EBNA1 binding sites and functions as an EBNA1-dependent replication enhancer (Wysokenski and Yates, 1989). It has been established that EBNA1 binds to DNA as a dimer and that dimerisation is essential for DNA binding (Chen *et al.*, 1993; Jones *et al.*, 1989; Shah and Ambinder, 1992). Upon binding of EBNA1 to the plasmid origin of replication, EBV uses host enzymes to mediate all remaining steps in replication (Thompson and Kurzrock, 2004).

As well as supporting DNA replication, EBNA1 binding to FR trans-activates the Cp promoter, located about 3 kb away on the EBV genome, and other heterologous promoters with copies of the FR upstream (Middleton and Sugden, 1994; Reisman and Sugden, 1986; Sugden and Warren, 1989), resulting in the trans-activation of other EBV latent genes. EBNA1 binding sites are also present at +10 to +34 nucleotides just downstream of the Qp promoter (Sample *et al.*, 1992). It is thought that promoter Qp operates in response to many transcription factors to ensure and maintain EBNA1 levels but is subject to feedback regulation by excess EBNA1 (Nonkwelo *et al.*, 1997).

In addition to its involvement in DNA replication and trans-activation, the presence of EBNA1 has been suggested to also contribute a selective advantage to tumour cells.

Evidence for this is based on a transgenic mouse system where EBNA1 expression resulted in the development of B cell lymphomas (Wilson *et al.*, 1996). In addition, there is some evidence that EBNA1 may mediate some effects on immunoglobulin enhancer elements, which in turn regulate *c-myc* expression of the translocated *c-myc* locus in BL cells (Magrath *et al.*, 1993; Shiramizu *et al.*, 1991). The recent isolation of EBV-negative Akata clones that show reduced tumourigenicity in nude mice, provides further evidence that EBNA1 may be involved in tumourigenicity, since the only viral protein known to be transcribed in the EBV-positive parental cell line is EBNA1 (Shimizu *et al.* 1994).

1.9.2 Epstein-Barr virus nuclear antigen 2 (EBNA2)

EBNA2 (together with EBNA-LP) is the first latent protein detected after EBV infection (Kieff, 1996). EBNA2 is a transcriptional trans-activator that is essential for EBV driven immortalisation. The inability of one EBV strain, P3HR1, which carries a deletion of the gene that encodes EBNA2 and the last two exons of EBNA-LP, to transform B cells *in vitro* was the first indication of the crucial role of EBNA2 in the transformation process (Kieff and Rickinson, 2001). Restoration of the EBNA2 gene in P3HR1 has unequivocally confirmed the importance of EBNA2 in B cell transformation and has allowed the functionally relevant domains of EBNA2 to be identified (Hammerschmidt and Sugden, 1989; Cohen *et al.*, 1989). Additionally, EBNA2 is 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.*, 1995b).

The EBNA2 gene encodes an 83 kDa 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 than type 2 (Rickinson *et al.*, 1987). The EBNA2 proteins identified in type 1 and type 2 EBV are called EBNA2A and 2B respectively, and only share about 50 % sequence homology (Addinger *et al.*, 1985). Characteristic structures of the EBNA2 protein (Figure 1.7) are: (i) a negatively charged

region at the amino-terminus, thought to play a role in homo-dimerization, (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-J κ /CBF1, (v) an arginine-glycine rich stretch of around 18 amino acids, (vi) a negatively charged region, which harbors a trans-activation domain, and (vii) a nuclear localization signal at the carboxy-terminus (Zimber-Strobl and Strobl 2001).

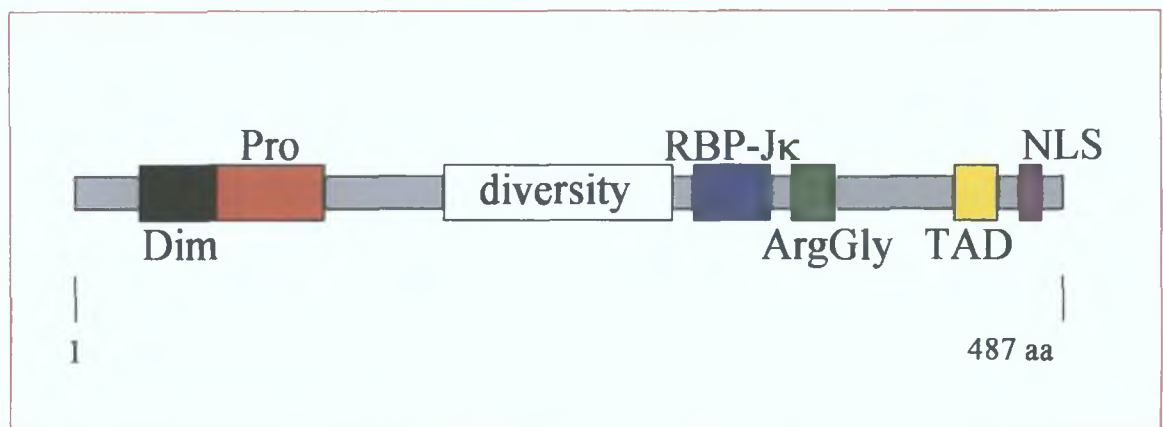


Figure 1.7: Structural domains of Epstein-Barr virus nuclear antigen 2. EBNA2 consists of a negatively charged region at the amino-terminus, which is likely to play a role in homo-dimerization (Dim), a polyproline region (Pro) consisting of 10-40 consecutive prolines depending on the virus strain, a diversity region, a domain responsible for interaction with RBP-J κ /CBF1 (RBP-J κ), an arginine-glycine rich stretch (ArgGly) and a negatively charged region carboxy terminus, which harbors a trans-activation domain (TAD) and nuclear localization signal (NLS) (adapted from Zimber-Strobl and Strobl, 2001).

EBNA2 functions as a specific trans-activator of both viral genes and a number of cellular genes that in turn are involved in the immortalisation process. EBNA2 activates the transcription of all other viral proteins expressed in LCLs by trans-activating (i) the *Bam*HI C promoter Cp (Woisetschlaeger *et al.*, 1990; Sung *et al.*, 1991), from which transcription of all EBNA genes is controlled 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.*, 1994a). In addition, EBNA2 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 FGR tyrosine kinase (Knutson, 1990; Patel *et al.*, 1990), BATF which induces expression of a B cell specific transcription factor of the same name (Johansen *et al.*, 2003), the key proliferative

transcription factor Myc (Jayachandra *et al.*, 1999; Kaiser *et al.*, 1999) as well as the chemokine receptor BLR2/EBII (Burgstahler *et al.*, 1995).

1.9.2.1 EBNA2 interacts with the DNA binding protein RBP-J κ /CBF1

EBNA2 does not bind DNA directly but interacts with a sequence-specific DNA-binding protein, J κ -recombinant-binding protein (RBP-J κ , also known as CBF1), and this is responsible for directing EBNA2 to promoters that contain RBP-J κ /CBF1 binding sites (Grossman *et al.*, 1994). The cognate DNA sequence element to which RBP-J κ /CBF1 binds is 5'-GTGGGAA-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 RBP-J κ /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). RBP-J κ /CBF1 is ubiquitously expressed and highly conserved during evolution. In *Drosophila*, this protein is known as suppressor of hairless (SuH) and is involved in signal transduction from the Notch receptor, a pathway that is important in cell fate determination (Artavanis-Tsakonas *et al.*, 1999).

Transient transfection assays using Gal4-CBF1 constructs revealed that RBP-J κ /CBF1 functions as a transcriptional repressor. RBP-J κ /CBF1 mediates repression (i) through direct contacts with the basal transcriptional machinery (BTM) (Olave *et al.*, 1998), disturbing the TFIIA-TFIID interaction, which is essential for the initiation of transcription and (ii) as a result of histone deacetylation, which leads to chromatin remodelling and a loss of transcription factor access to the nucleosome-associated promoter sequences. The RBP-J κ /CBF1 repressor complex (Figure 1.8) includes the proteins SMRT/NcoR, HDAC1, HDAC2, SAP30, CIR and SKIP (Kao *et al.*, 1998; Zhou *et al.*, 2000a; Zhou *et al.*, 2000b; Zhou and Hayward, 2001; Hsieh *et al.*, 1999). In the co-repressor complex, SKIP binds to the carboxy terminal RID-2 domain of SMRT and along with SMRT is important for nuclear entry of RBP-J κ /CBF1 (Zhou and Hayward, 2001). SKIP also interacts with Sin3A (Zhou *et al.*, 2000a) and this, along with the fact that SAP30 is part of the Sin3 complex, implies that Sin3 is also present in the complex.

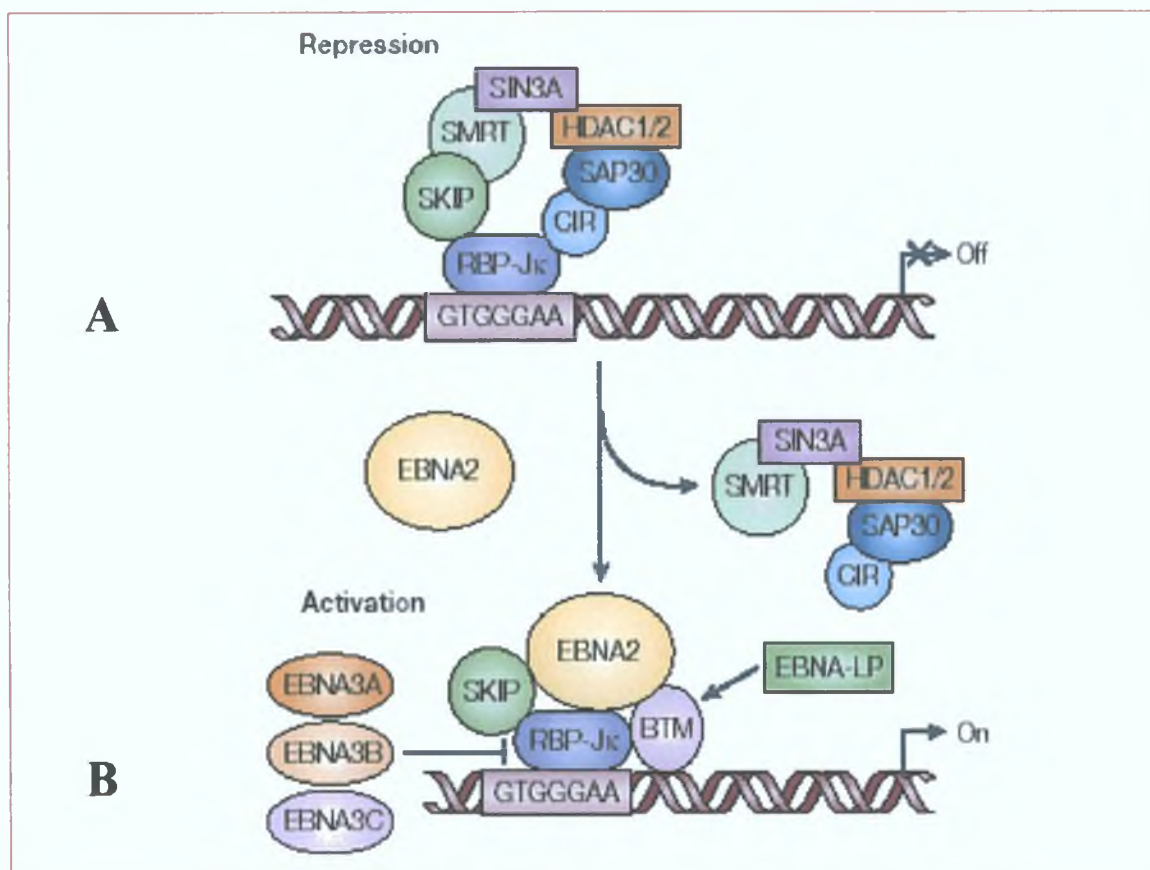


Figure 1.8: EBNA2-mediated promoter activation. (A) EBNA2 functions as a transcriptional activator by interacting with the DNA-binding κ -recombination-binding protein (RBP- κ /CBF1) and relieving transcriptional repression that is mediated by a large multi-protein complex consisting of SMRT, SIN3A, histone deacetylase 1 (HDAC1) and HDAC2. SKIP (Ski interacting protein) is another RBP- κ -interacting protein that also interacts with the SMRT-HDAC co-repressor complex. EBNA2 abolishes RBP- κ -mediated repression by competing with the SMRT-HDAC co-repressor for binding to both RBP- κ and SKIP. (B) The acidic domain of EBNA2 then recruits the basal transcription machinery (TFIIB, TFIIF and p300, not shown) to activate transcription. EBNA-LP cooperates with EBNA2 in RBP- κ -mediated transcriptional activation by interacting with the acidic activation domain of EBNA2. The EBNA3 family of proteins modulate EBNA2-mediated RBP- κ activation by interacting with RBP- κ and competing for binding and activation by EBNA2 (adapted from Young and Rickinson, 2004).

EBNA2 trans-activates promoters by (i) binding to the repression domain of RBP- κ /CBF1 to relieve repression and (ii) by bringing a strong transcriptional activation domain to the promoter (Hsieh and Hayward, 1995). These properties were demonstrated in studies where an EBNA2 mutant capable of binding to RBP- κ /CBF1, but lacking the trans-activation domain, was able to relieve repression by displacing the RBP- κ /CBF1 co-repressor complex. Transcriptional up-regulation depended on the presence of the EBNA2 trans-activation domain, which recruits co-activator proteins (Hsieh and Hayward, 1995; Wang *et al.*, 2000). During EBNA2 displacement of the RBP- κ /CBF1 co-repressor complex, adjacent domains make contact with RBP-

J κ /CBF1 and SKIP to displace SMRT (Figure 1.8). Direct competition for binding to SKIP has been demonstrated for SMRT and EBNA2 (Zhou *et al.*, 2000a). Conserved region 5 (CR5) of EBNA2 binds SKIP and conserved region 6 (CR6) binds to RBP-J κ /CBF1. The locations of CR5 and CR6 on the EBNA2 protein may be seen in Figure 1.9 below. A mutation within CR6 that abolishes EBNA2 binding to RBP-J κ /CBF1 (WW323SR) completely abolishes EBNA2-mediated trans-activation (Ling and Hayward, 1995; Ling *et al.*, 1993), and when incorporated into the virus, this mutation results in a non-immortalising EBV (Yalamanchili *et al.*, 1994). Mutations within CR5, such as the II307 mutant, which are impaired for SKIP interaction are equally impaired for the trans-activating function of EBNA2 (Zhou *et al.*, 2000a). Introduction of deletions that span CR5 into the EBV genome led to a mutant virus that either failed to immortalise B cells *in vitro* or resulted in B cell colonies that grew less than the wild type virus-immortalised controls (Harada *et al.*, 1998). Taken together, these data indicate that EBNA2 requires contact with both SKIP and RBP-J κ /CBF1 for effective activation of promoters containing RBP-J κ /CBF1 binding sites.

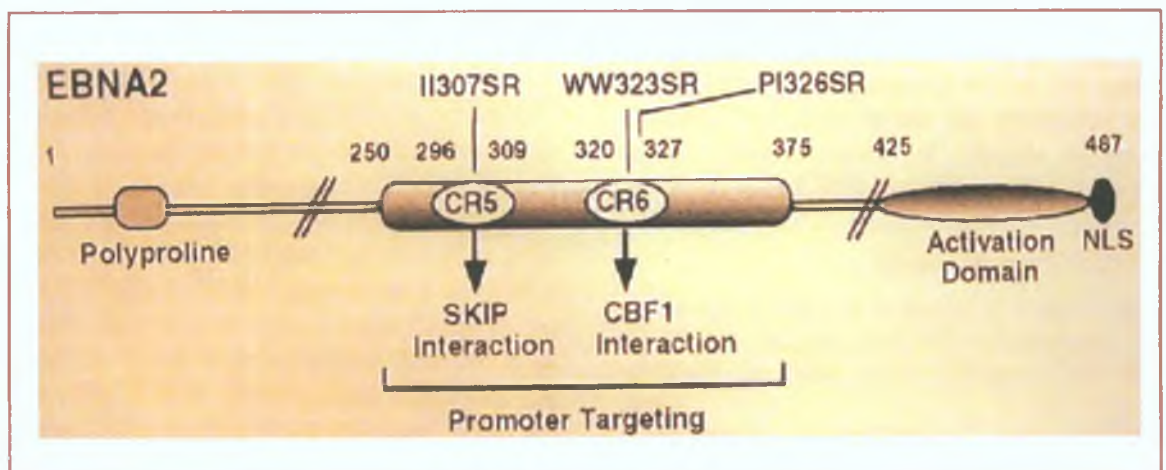


Figure 1.9: The locations of the SKIP and RBP-J κ /CBF1 interaction regions on the EBNA2 protein. A schematic representation of the EBNA2 protein illustrating the relative locations of characterized functional domains and the position of the WW323SR mutation. The amino acid numbers are indicated. CR5, CR6 and a nuclear localization signal (NLS) are also indicated.

1.9.2.2 Co-Activating Proteins Interact with EBNA2

EBNA2 interacts with other proteins to facilitate trans-activation of EBNA2-responsive genes. The acidic domain of EBNA2 interacts with factors of the basal transcription machinery to assemble the pre-initiation complex. These factors include TFIID, TATA

box binding protein (TBP), associated binding factor TAF40, TFIIB, and the co-activator p100 that interacts with TFIIE (Tong *et al.*, 1995a; Tong *et al.*, 1995b; Tong *et al.*, 1995c). The trans-activation domain also interacts with the histone acetyltransferases (HATs) p300/CBP and PCAF (Wang *et al.*, 2000). HATs facilitate chromatin opening, which is necessary for initiation of transcription. The viral protein EBNA-LP also acts as a co-activator of EBNA2 (Nitsche *et al.*, 1997; Harada *et al.*, 1997). Outside the activation domain, EBNA2 interacts with the chromatin remodelling complex hSW1-SNF, which converts the chromatin structure to facilitate transcription (Wu *et al.*, 2000a). 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).

1.9.2.3 EBNA2-responsive elements (EREs)

The promoter elements responsible for EBNA2-mediated trans-activation have been mapped for the viral Cp, LMP1 and LMP2 promoters (Woisetschlaeger *et al.*, 1990; Sung *et al.*, 1991; Fahraeus *et al.*, 1990; Wang *et al.*, 1990; Zimmer-Strobl *et al.*, 1991; Laux *et al.*, 1994a) and for the promoter of the cellular gene CD23 (Wang *et al.*, 1991). These EBNA2-responsive elements (EREs) are all relatively large, are orientation independent and confer EBNA2-responsiveness to heterologous promoters. Furthermore, they all contain at least one RBP-J κ /CBF1 binding site (Zimmer-Strobl and Strobl, 2001). Although the interaction between EBNA2 and RBP-J κ /CBF1 is necessary for induction of gene expression, additional factors bind within the enhancer elements to confer EBNA2-responsiveness (Meitinger *et al.*, 1994). In the LMP1 promoter, binding of PU.1/Spi-B, which is also thought to interact with EBNA2, is absolutely necessary for EBNA2-mediated trans-activation (Johannsen *et al.*, 1995; Laux *et al.* 1994b; Sjoblom *et al.*, 1995). 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 LMP2A promoter, beside RBP-J κ /CBF1 two further proteins bind within the EBNA2-responsive region and contribute to EBNA2-mediated trans-activation. These proteins, however, have not been identified to date (Hoefelmayr *et al.*, 1999). In the Cp promoter, binding of the hnRNP protein AUF1 appears to cooperate with RBP-J κ /CBF1 in EBNA2-responsiveness (Fuentes-Panana *et al.*, 2000).

1.9.2.4 Viral and cellular proteins counter-regulate EBNA2 activity

EBNA2 mediated trans-activation through RBP-J κ /CBF1 can be disrupted by a number of viral and cellular proteins. The EBV nuclear antigens EBNA3A, EBNA3B and EBNA3C compete with EBNA2 for binding to RBP-J κ /CBF1 (Johannsen *et al.*, 1996; Robertson *et al.*, 1995; Le Roux *et al.*, 1994). The RBP-J κ /CBF1-EBNA3C complex does not bind to DNA and so transcriptional activation is blocked by loss of access to RBP-J κ /CBF1 and loss of RBP-J κ /CBF1 promoter targeting. The positive regulation of EBNA2 by EBNA-LP binding and the balancing negative regulation by the EBNA3 family places EBNA2 activity largely under viral control and the level of the RBP-J κ /CBF1 complex (Hayward, 2004). 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), which interacts with RBP-J κ /CBF1 and the RBP-J κ /CBF1-associated co-repressor CIR, to stabilize the RBP-J κ /CBF1 co-repressor complex, thereby negatively regulating the trans-activational effect of EBNA2 (Zhang *et al.*, 2001). Furthermore, a cellular LIM protein, KyoT2, has also been identified, which dislocates RBP-J κ /CBF1 from DNA by competing with EBNA2 for binding to RBP-J κ /CBF1 (Taniguchi *et al.*, 1998).

1.9.2.5 RBP-J κ /CBF1 links EBNA2 to the cellular Notch signalling pathway

RBP-J κ /CBF1 also plays an important role in the Notch signalling pathway, which regulates cell fate decisions in many different organisms from worm to humans (Artavanis-Tsakonas *et al.*, 1999). The Notch signalling pathway was originally discovered through genetic studies in the fruit fly *Drosophila melanogaster*, and the name derives from the effect of some Notch alleles to create notched wings (Hansson *et al.*, 2004). Cloning of the Notch gene (Wharton *et al.*, 1985; Kidd *et al.*, 1986) showed that it encodes a single trans-membrane receptor (Figure 1.10). The amino-terminus contains a signal peptide followed by multiple epidermal growth factor (EGF)-like repeats, which function as a ligand binding domain, and cystine-rich Lin12/Notch repeats. The intracellular part contains the RAM domain and ankyrin repeats which are both important for RBP-J κ /CBF1 interaction. Also, in the intracellular region lie three nuclear localization signals (NLS), and a C terminal PEST (proline-, glutamate, serine-, threonine-rich) region, which regulates protein stability (Weinmaster, 1997). A possible

trans-activation domain has also been identified at the carboxy-terminus (Kurooka *et al.*, 1998; Dumont *et al.*, 2000). Four Notch genes have been isolated and identified in vertebrates (Notch1, Notch2, Notch3 and Notch4) and the structures of the proteins encoded by these genes are strikingly similar to *Drosophila* Notch (Artavanis-Tsakonas *et al.*, 1995; Greenwald, 1994; Lardelli *et al.*, 1995; Uyttendaele *et al.*, 1996). Like *Drosophila* Notch, the Notch1 and Notch2 proteins contain 36 tandemly arranged EGF-like repeats, while Notch3 and Notch4 have 34 and 29 repeats respectively (Weinmaster, 1997).

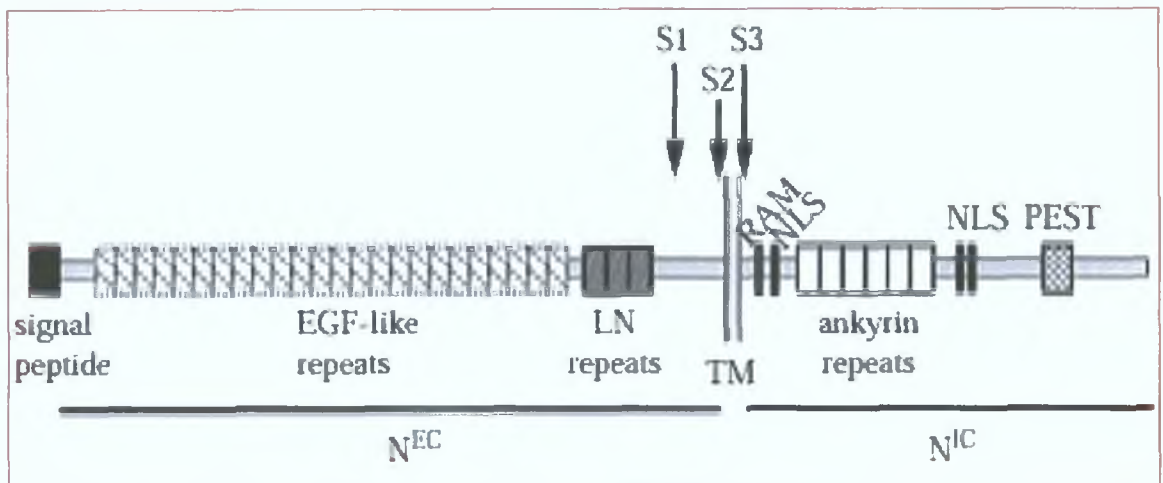


Figure 1.10: Structure of the Notch receptor. The N-terminal of the Notch protein contains a signal peptide followed by multiple epidermal growth factor (EGF)-like repeats, that function as a ligand binding domain, and cysteine rich Lin12/Notch repeats (LN). These regions comprise the extracellular domain of the Notch protein (N^{EC}). The intracellular part (N^{IC}) harbours the RAM domain and a series of ankyrin repeats, which both interact with RBP-J κ /CBF1, 3 nuclear localisation signals (NLS), and a C-terminal PEST region. The arrows (S1 to S3) indicate the sites of proteolytical cleavage during protein maturation and following binding of ligand (adapted from Zimmer-Strobl and Strobl, 2001).

Notch receptors are activated by interaction with membrane bound ligands. The ligands that bind and activate Notch receptor belong to the DSL family, defined by the invertebrate ligands Delta, Serrate and LAG-2 (Henderson *et al.*, 1994; Mello *et al.*, 1994; Tax *et al.*, 1994). Vertebrate genes encoding both Delta-like (Delta 1 and Delta 2) and Serrate-like (Jagged 1 and Jagged 2) subtypes have been isolated from humans, rats, mice, chickens and frogs (Weinmaster, 1997). The basic picture emerging from many different studies has the extracellular domain of the ligands, expressed on the surface of one cell, interacting with the extracellular domain of the Notch receptor on an adjacent cell.

Data from several groups have led to a model of Notch activation involving proteolytic cleavages at three sites (S1 to S3) (Baron, 2003). The location of these sites on the Notch protein is indicated in Figure 1.10. After its synthesis in the endoplasmic reticulum (ER), the 300 kDa Notch receptor is transported through the secretory pathway to the trans-Golgi network, where it is constitutively cleaved by a furin-like convertase at S1, generating an N-terminal fragment of 180 kDa that contains the Notch extracellular subunit (NotchEC) and a C-terminal fragment of about 120 kDa containing a short extracellular part, the trans-membrane domain and the intracellular part (NotchIC). The two fragments are reassembled in the Golgi apparatus to a heterodimer, which can then be expressed on the cell surface (Blaumueller *et al.*, 1997; Logeat *et al.*, 1998). Proteolytic processing of the 300-kDa precursor is an essential step in the formation of the biologically active receptor, as only the cleaved fragments are present at the cell surface (Logeat *et al.*, 1998). Ligand-receptor interactions between cells in physical contact, result in two further cleavages. At first the disintegrin-metalloprotease tumour necrosis factor α -converting enzyme (TACE) catalyses the cleavage at S2, which removes most of the extracellular part (Mumm *et al.*, 2000; Brou *et al.*, 2000). S2-processed Notch acts as a substrate for a third proteolytic cleavage at S3 (Schroeter *et al.*, 1998), which releases the intracellular region of the Notch receptor (NotchIC). S3 cleavage is mediated by the γ -secretase complex, an enzyme complex that constitutively cleaves certain trans-membrane domains with short extracellular stubs (De Strooper *et al.*, 1999). A functional γ -secretase complex is composed of four proteins: presenilin, nicastrin, Pen-2 and Aph-I (Edbauer *et al.*, 2003), and presenilin is thought to contain the protease activity (Selkoe and Kopan, 2003). As a result of S3 cleavage, NotchIC is translocated to the nucleus, where it interacts via the RAM domain and ankyrin repeats with RBP-J κ /CBF1 (Jarriault *et al.*, 1995). In the absence of a Notch signal, RBP-J κ /CBF1 can repress transcription through recruitment of the HDAC-containing co-repressor complex. However, similarly to EBNA2, binding of NotchIC displaces the repressor complex, resulting in the activation of transcription. Several target genes have been described, which are induced through the interaction of NotchIC with RBP-J κ /CBF, for example the hairy enhancer of split complex [E(spl)] related genes Hes1 and Hes5, Hey1, Hey2 and HeyL (Jarriault *et al.*, 1995; Nishimura *et al.*, 1998; Maier and Gessler, 2000), ERBB-2 (Chen *et al.*, 1997), NF- κ B2 (Oswald *et al.*, 1998) and hairy-related transcription factor (HRT) (Nakagawa *et al.*, 2000). Figure 1.11 summarises the activation of NotchIC as a direct result of ligand binding.

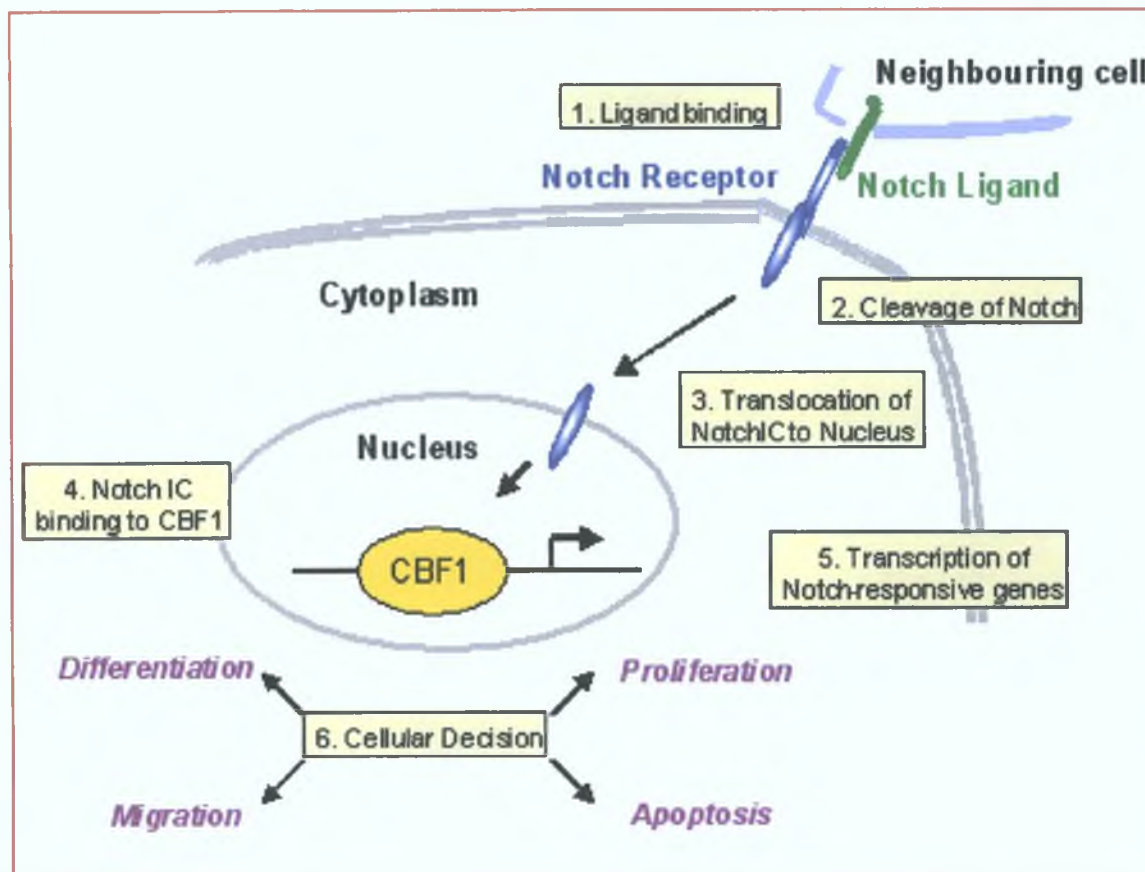


Figure 1.11: The Notch signalling pathway. (1) Ligand (Delta 1, Delta 2, Serrate 1, Serrate 2) on a neighbouring cell binds to the Notch receptor. (2) The ligand-receptor interaction results in a series of cleavages of the Notch receptor: At first the disintegrin-metalloprotease tumour necrosis factor α -converting enzyme (TACE) catalyses cleavage at S2, which removes most of the extracellular part of the Notch protein. S2-processed Notch acts as a substrate for a proteolytic cleavage at S3, which releases the intracellular region of the Notch receptor (NotchIC). S3 cleavage is mediated by the γ -secretase complex. (3) As a result of S3 cleavage, NotchIC is translocated to the nucleus, where it interacts via the RAM domain and ankyrin repeats with RBP-J κ /CBF1. (5) Binding of NotchIC to RBP-J κ /CBF1 displaces the co-repressor complex, allowing transcriptional activation of Notch responsive genes. (6) Expression of Notch responsive genes determines the fate of the cell.

1.9.2.6 EBNA2 and Notch-IC overlap in their functions and in their target genes

Both EBNA2 and NotchIC trans-activate genes by interacting with RBP-J κ /CBF1. Although there is no obvious sequence homology between these two proteins, they interact with similar regions of RBP-J κ /CBF1 and replace repressor proteins with their trans-activation domains. Furthermore, there are parallels between EBNA2 and NotchIC in the ways in which transcriptional activation through RBP-J κ /CBF1 is modified. As was the case for EBNA2, co-activating proteins interact with NotchIC.

These co-activating proteins include the HATs PCAF, GCN5 (Kurooka and Honjo, 2000) and p300/CBP (Oswald *et al.*, 2001; Wallberg *et al.*, 2002), which play a key role in chromatin opening and initiation of transcription. In addition, full transcriptional activation by NotchIC requires the human homologue of *Drosophila* Mastermind, MAML1, which binds to the ankyrin repeat domain of Notch and also recruits p300/CBP (Wu *et al.*, 2000b; Kitagawa *et al.*, 2001; Fryer *et al.*, 2002). As for EBNA2, NotchIC activity is also regulated in part by loss of RBP-J κ /CBF1 binding. The LIM protein KyoT2 was identified in a yeast two-hybrid screen as an RBP-J κ /CBF1-interacting protein and shown to prevent DNA binding by RBP-J κ /CBF1 (Taniguchi *et al.*, 1998). MINT/SHARP antagonizes NotchIC binding to RBP-J κ /CBF1 (Oswald *et al.*, 2002; Kuroda *et al.*, 2003). Overall, however, NotchIC function may be regulated less at the point of the RBP-J κ /CBF1 complex and more through processes such as differential expression of Notch ligands, receptor recycling and protein turnover (Tanigaki *et al.*, 2003; Nickoloff *et al.*, 2003).

Since both EBNA2 and Notch-IC proteins share a common mechanism for regulating target gene expression, it not surprising that they also overlap in the range of functional activities and in the genes that they regulate. This is reflected in the ability of both proteins to activate (i) the same RBP-J κ /CBF1-regulated genes in reporter assays (Hofelmayr *et al.*, 1999), (ii) EBV latency promoters (with the exception of LMP1) (Strobl *et al.*, 2000) and (iii) effect expression of the same cellular genes. Examples of the latter are up-regulation of BATF (Johansen *et al.*, 2003) and CD21 (Strobl *et al.*, 2000) and down-regulation of the Ig enhancer (Strobl *et al.*, 2000; Morimura *et al.*, 2001). It has also been demonstrated that EBNA2 can activate the NotchIC-responsive Hes1 promoter. Most likely by induction of Hes1, EBNA2 has the ability to suppress differentiation of the myogenic cell line C2C12, similar to activated Notch (Sakai *et al.*, 1998; Kuroda *et al.*, 1999). Additionally EBNA2 and Notch-IC have been shown to protect cells from apoptosis 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). The set of promoters, which is regulated by EBNA2 and NotchIC, is overlapping but not identical. Only EBNA2, but not NotchIC, can induce LMP1, CD23 and *c-myc* expression and maintains proliferation of immortalized B cells (reviewed in Zimmer-Strobl and Strobl, 2001). In order to understand the extent to which EBNA2 is a functional equivalent to Notch, it will be important in the future to

identify further target genes of both these proteins and compare their roles in cell fate decisions, such as cell proliferation and differentiation.

EBNA2 immortalises B lymphocytes in culture to generate continually proliferating LCLs, demonstrating that EBNA2 stimulates B cell survival and growth proliferation in the setting of EBV infection. Since EBNA2 associates with the RBP-J κ /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). It has not been clear how to integrate these facts with work demonstrating that a dominant function of Notch1IC in hematopoiesis is to induce common lymphoid progenitors to adopt a T cell fate at the expense of B cell development (Izon *et al.*, 2002; Han *et al.*, 2002; Radtke *et al.*, 2002). Although the role of Notch in the B cell compartment remains incompletely understood, recent findings allow speculation on the ways in which the EBNA2 usurping of Notch function may sustain a lifelong EBV infection in the host (Hayward, 2004). Firstly, there is evidence that Notch signalling increases the formation of hematopoietic stem cells. Jagged1 is expressed on bone marrow stroma and on stromal cell lines and co-culture of murine marrow precursors with Jagged1 increased the formation of precursor cell populations (Varnum-Finney *et al.*, 1998; Varnum-Finney *et al.*, 2003). Osteoblastic cells also express Jagged1 and increased exposure to osteoblasts augments primitive haematopoietic cell growth in a Notch-dependent manner (Calvi *et al.*, 2003). EBNA2-induced B lymphocyte proliferation may therefore be recapitulating a precursor stem cell response. This proliferation is thought to be important in expanding the infected cell population after primary EBV infection to permit establishment of a life-long latency in the memory B cell compartment (Hayward, 2004).

1.9.3 The Epstein-Barr virus nuclear antigen 3 family (EBNA3A, EBNA3B and EBNA3C)

The EBNA3 family members are encoded by alternatively spliced transcripts initiated at the Cp promoter (Kieff, 1996). Conserved sequences are confined to the N-terminal third of the EBNA3 proteins (Rowe, 1999). Studies with EBV recombinants have shown that EBNA3A and EBNA3C are essential for B cell transformation *in vitro*, whereas EBNA3B is dispensable (Roberston, 1997). EBNA3C can induce the up-

regulation of both cellular (CD21) and viral (LMP1) gene expression (Allday and Farrell, 1994), and repress the Cp promoter (Radkov *et al.*, 1997). EBNA3C may also interact with the retinoblastoma protein, pRb, to promote transformation (Parker *et al.*, 1996). Although not essential for transformation, EBNA3B has been shown to induce expression of vimentin and CD40 (Silins and Sculley, 1994). The EBNA3 family of proteins act as transcriptional regulators by associating with the RBP-J κ /CBF1 transcription factor and disrupting its binding to the cognate J κ sequence and to EBNA2. This results in repression of EBNA2-mediated trans-activation (Robertson, 1997). Thus, EBNA2 and the EBNA3 proteins work together to precisely control RBP-J κ /CBF1 activity, thereby regulating the expression of cellular and viral promoters containing RBP-J κ /CBF1 binding sites. More recently, EBNA3C has been shown to interact with the human histone deacetylase 1 (HDAC1), which in turn contributes to the transcriptional repression of Cp by RBP-J κ /CBF1 (Radkov *et al.*, 1999).

1.9.4 Epstein-Barr virus nuclear antigen leader protein (EBNA-LP)

EBNA-LP, also known as EBNA5, is one of the first viral proteins produced during EBV infection of B lymphocytes (Kieff, 1996). EBNA-LP is encoded by the first ORF (leader sequence) of the extensively spliced primary transcript, originating from either the Wp or Cp promoter, and encodes a protein of variable size (20 -130 kDa) depending on the number of *Bam*HI W repeats contained by a particular EBV isolate. EBNA-LP, although apparently not essential for transformation, greatly enhances the efficiency of the process (Hammerschmidt and Sugden, 1989; Mannick *et al.*, 1991). EBNA-LP interacts with EBNA2 and is required for the efficient outgrowth of virus transformed B cells *in vitro* (Mannick *et al.*, 1991; Sinclair *et al.*, 1994). Expression of EBNA-LP together with EBNA2 in resting B lymphocytes results in the activation of cyclin D2, which is necessary to drive cells into the G1 phase of the cell cycle (Sinclair *et al.*, 1994). EBNA-LP can also cooperate with EBNA2 to up-regulate expression of the major EBV effector protein of B cell transformation, LMP1 (Nitsche *et al.*, 1997). Furthermore, EBNA-LP can bind and inactivate the cellular p53 and pRb protein tumour suppressor gene products (Szekely *et al.*, 1993), also resulting in progression into the cell cycle.

1.9.5 Latent membrane protein 1 (LMP1)

The frequent detection of LMP1 in many EBV-associated malignancies led to the suggestion that this protein contributes to tumourigenesis. Several studies have shown that LMP1 possesses oncogenic properties. The expression of LMP1 leads to transformation of rodent fibroblast cell lines and renders them tumourigenic in nude mice (Wang *et al.*, 1985). In BL-derived cell lines, LMP1 induces many of the phenotypic changes observed in EBV infection, including the up-regulation of B cell activation markers and cell adhesion molecules (Kieff and Rickinson, 2001). LMP1 can also suppress cell death induced by a variety of stimuli through the up-regulation of anti-apoptotic proteins, such as Bcl-2, Mcl-1, Bfl-1, A20 and c-IAPs (Henderson *et al.*, 1991; Fries *et al.*, 1996; Wang *et al.*, 1996; D'Souza *et al.*, 2000; Laherty *et al.*, 1992; Hong *et al.*, 2000). In addition, LMP1 expression blocks the normal process of differentiation in epithelial cells, reminiscent of the undifferentiated phenotype frequently observed in NPC (Dawson *et al.*, 1990). Furthermore, LMP1 expression in carcinoma cell lines promotes IL-8 production (Eliopoulos *et al.*, 1999b) and up-regulation of matrix metalloproteinases (MMPs) (Yoshizaki *et al.*, 1998), suggesting that in addition to its transforming potential, LMP1 may influence angiogenesis and metastasis in EBV-associated tumours. 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 hyper-proliferation and lymphomagenesis, respectively (Kulwichit *et al.*, 1998; Wilson *et al.*, 1990).

LMP1 is a 63 kDa integral membrane phosphoprotein belonging to the tumour necrosis factor receptor (TNFR)/CD40 super-family and functions as a constitutively active receptor (Gires *et al.*, 1997), which mimics the cellular growth signal that normally results from the binding of CD40 ligand (Thompson and Kurzrock, 2004). The LMP1 protein can be divided into three domains (Figure 1.12). Firstly, an amino-terminal cytoplasmic tail (amino acids 1-23), which tethers LMP1 to the plasma membrane and orientates the protein. Secondly, a trans-membrane region, consisting of six hydrophobic trans-membrane loops, which are involved in self-aggregation and oligomerisation (amino acids 24-186). Third, a long carboxy-terminal cytoplasmic region (amino acids 187-386), which possesses most of the signalling activity of the molecule (Young and Rickinson, 2004). LMP1 signals mainly from the intracellular compartments (Lam and Sugden, 2003). Both oligomerisation and localisation within

glycosphingolipid-rich membrane rafts are essential for the initiation of signalling (Clausse *et al.*, 1997; Eliopoulos and Young, 2001; Higuchi *et al.*, 2001) resulting in the activation of several signalling pathways in a ligand-independent manner.

At least four signalling pathways have been implicated in the function of LMP1, namely nuclear factor κ B (NF- κ B), c-Jun NH₂ terminal kinase (JNK), p38/mitogen activated protein kinase (p38/MAPK) and janus kinase/signal transducers and activators of transcription (JAK/STAT) (Murray and Young, 2001). NF- κ B is a key transcription factor involved in regulation of cell growth and apoptosis. NF- κ B controls the expression of numerous cytokines, including lymphotoxin, which is an autocrine growth factor for EBV-transformed cells (Thompson and Kurzrock, 2004). p38/MAPK is also a central signalling pathway that regulates the activation of the transcription factor ATF2 (Eliopoulos *et al.*, 1999b). LMP1 induces activation of another member of the MAPK family, namely JNK, resulting in the activation of the pleiotropic transcription factor AP-1 (Eliopoulos *et al.*, 1999a). The JAK/STAT pathway is activated by a number of growth factors and cytokines that regulate transcription and control diverse cellular functions, such as proliferation, apoptosis and cell surface marker expression. LMP1 binds JAK3 leading activation of the transcription factor STAT1 (Gires *et al.*, 1999). Taken together, the activating cascades associated with LMP1 lead to enhanced expression of B cell surface adhesion molecules (LFA1, CD54 and CD58), B cell activation markers (CD23, CD39, CD40, CD44 and MHC class II), and morphological changes such as cellular clumping (Thompson and Kurzrock, 2004). As LMP1 activates transcription factors that are key modulators of cell growth, they might also be relevant to the role of EBV in some cancers.

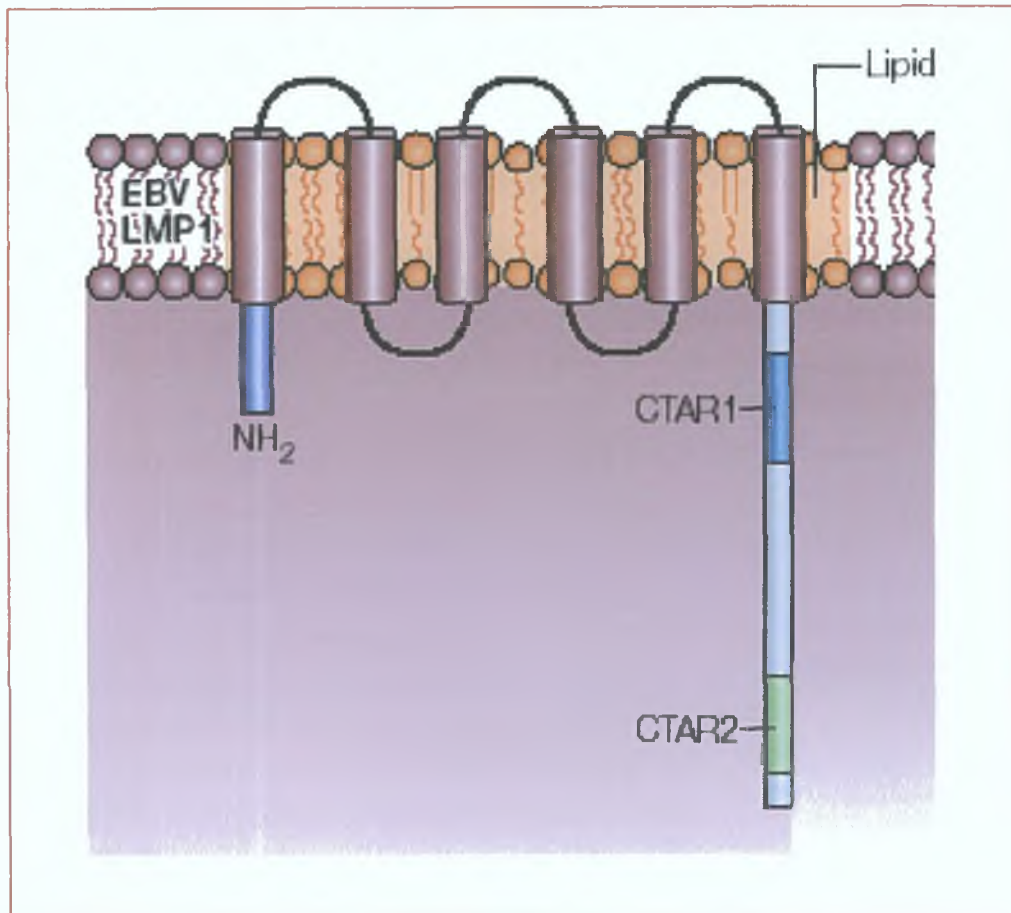


Figure 1.12: Structure of LMP1. The EBV LMP1 is an integral membrane protein of 63 kDa and can be subdivided into three domains. Firstly, an amino-terminal cytoplasmic tail, which tethers LMP1 to the plasma membrane and orientates the protein. Secondly, a trans-membrane region, consisting of six hydrophobic trans-membrane loops, which are involved in self aggregation and oligomerisation. Third, a long carboxy-terminal cytoplasmic region, which possesses most of the signalling activity of the molecule. Two distinct functional domains referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2) have been identified on the basis of their ability to activate the NF- κ B signalling pathway (Huen *et al.*, 1995) (adapted from Young and Rickinson, 2004).

It is the cytoplasmic C-terminus of LMP1 that is responsible for the transduction of signalling cascades that result in primary B cell transformation and phenotypic changes. Within the C-terminus of LMP1 there are at least two major activating domains (Figures 1.12 and 1.13), C-terminal activating region 1 (CTAR1) and CTAR2. CTAR1 (also known as transformation effector site 1, TES1) is located proximal to the membrane (amino acids 186-231), binds TNFR-associated factors (TRAFs) (Devergne *et al.*, 1996; Huen *et al.*, 1995), and is essential for EBV-mediated B-cell immortalisation (Izumi *et al.*, 1997; Kaye *et al.*, 1995; Kaye *et al.*, 1999). CTAR2/TES2, which is located near the C-terminus (amino acids 351-386), supports the long-term growth of immortalised B cells (Izumi and Kieff, 1997) and recruits the TNFR-associated death domain

(TRADD) protein and receptor-interacting protein (RIP) (Eliopoulos *et al.*, 1999a; Huen *et al.*, 1995; Izumi *et al.*, 1999). The molecular interactions and signalling pathways engaged by LMP1 are summarised in Figure 1.13). The activities of CTAR1 and CTAR2 affect diverse signalling cascades and provide the basis for the molecular explanation of the transforming properties of LMP1.

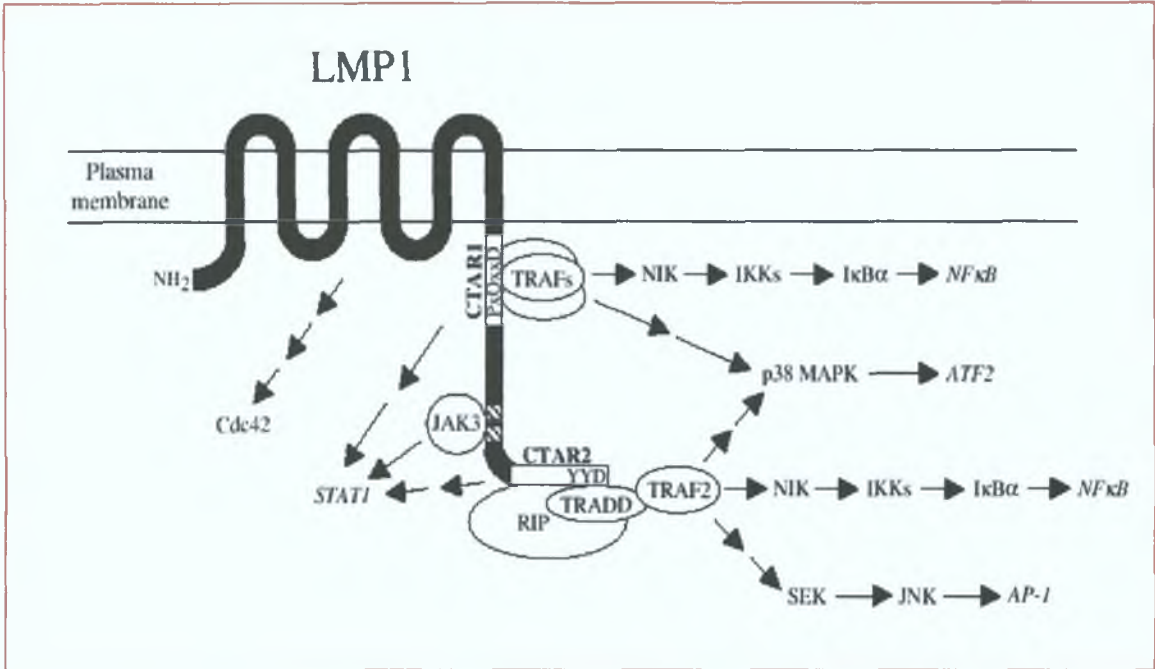


Figure 1.13: Schematic representation of the molecular interactions and signalling pathways engaged by LMP1. The C-terminus of LMP1 contains at least two activating regions, referred to as C-terminus activation regions 1 and 2 (CTAR1 and CTAR2). CTAR1, which is essential for EBV-mediated B cell immortalisation, binds TRAF1, TRAF2, TRAF3 and TRAF5 and activates the NF-κB and p38 signalling pathways. CTAR2 supports the long-term growth of immortalised B cells and recruits TRADD to activated downstream signals, such as NF-κB, JNK, and p38. Both LMP1 C-terminal domains also mediate the activation of the JAK/STAT pathway, although an intermediated region has also been shown to bind JAK3 and induce STAT binding activity independently of CTAR1 and CTAR2. The transmembrane domains of LMP1 are responsible for the activation of the small GTPase Cdc42 leading to cytoskeletal changes (adapted from Eliopoulos and Young, 2001).

1.9.5.1 LMP1-mediated activation of the NF-κB signalling pathway

NF-κB signalling has been extensively studied and it has been shown to play a role in proliferation, differentiation, apoptosis, oncogenesis and inflammation. The list of potential activators of NF-κB is diverse and includes pro-inflammatory stimuli such as

TNF, cytokines such as CD40 ligand and IL-1, bacterial products such as lipopolysaccharide (LPS), as well as stress signals. Additionally, many viruses, including, human T-cell leukaemia virus type I (HTLV-1), herpes simplex virus-8 (HSV-8) and EBV, utilize this pathway to promote survival and transformation of infected target cells (Eliopoulos and Young, 2001). The NF- κ B pathway provides an attractive target to viral pathogens for several reasons. Activation of NF- κ B is a rapid event that occurs within minutes after exposure to a relevant stimulus and does not require *de novo* protein synthesis. Additionally, viruses have evolved strategies that result in the modulation of the NF- κ B pathway in order to enhance host cell survival and evasion of immune responses. Furthermore, activation of NF- κ B constitutes an obvious target because many of the NF- κ B-responsive genes (growth factors, cytokines, proto-oncogenes, and anti-apoptotic proteins) profoundly influence the host cell cycle (Hiscott *et al.*, 2001). In the case of EBV, LMP1 has appropriated the NF- κ B signalling pathway into its mechanism of B cell transformation. Indeed, there is recent evidence to suggest that suppression of EBV-mediated NF- κ B activation compromises the viability of EBV-infected LCLs (Cahir-McFarland *et al.*, 2000).

The eukaryotic transcription factor NF- κ B was originally identified as a protein that bound to a specific DNA sequence within the intronic enhancer of the Ig kappa light chain in mature B- and plasma cells (Sen and Baltimore, 1986). NF- κ B has subsequently been identified in most cell types, and specific NF- κ B binding sites have also been identified in promoters and enhancers of a number of inducible genes. The transcription factor NF- κ B consists of homo- or hetero-dimers composed of different subunits. The subunits are all members of the NF- κ B/Rel family of structurally related proteins. The Rel proteins share an approximately 300-amino acid amino terminal Rel homology domain that contains sequences essential for dimerisation, DNA binding and nuclear transport (Hiscott *et al.*, 2001). Each dimer combination exhibits differences in DNA binding affinity and trans-activation potential (Delfino and Walker, 1999). Five different Rel proteins have been identified to date, namely RelA (p65), RelB, NF- κ B1 (p50/p105), NF- κ B2 (p52/p100) and c-Rel. Family members c-Rel, RelA and RelB possess C-terminal trans-activation domains, while p50/p105 and p52/p100 function as either short DNA binding domains or larger inactive proteins which contain C-terminal inhibitory domains (Hiscott *et al.*, 2001). Because of the potential for generating diverse homo- and hetero-dimers, these proteins may allow transcriptional specificity by

forming combinations that can interact specifically with structural variations of the NF- κ B DNA binding site.

In its inactive form NF- κ B is sequestered in the cytoplasm, bound by members of the I κ B family of inhibitor proteins. The various stimuli that activate NF- κ B result in the phosphorylation of I κ B by the multi-subunit I κ B kinase complex (IKK). IKK contains two catalytic subunits, IKK α and IKK β , and the regulatory subunit IKK γ [NF- κ B essential modulator (NEMO)] (Karin, 1999). Phosphorylation of I κ B by the IKK complex, targets the I κ B molecules for ubiquitination (the covalent attachment of multiple ubiquitin molecules), whereupon the I κ B inhibitor is degraded by the 26S proteasome, allowing the release of NF- κ B proteins. The phosphorylation and subsequent release of NF- κ B exposes the nuclear localisation signals (NLS) on the NF- κ B subunits, resulting in translocation of the molecule to the nucleus. In the nucleus, NF- κ B binds to promoters bearing κ B consensus sequences, leading to activation of transcription. The biochemistry of NF- κ B activation is summarised in Figure 1.14.

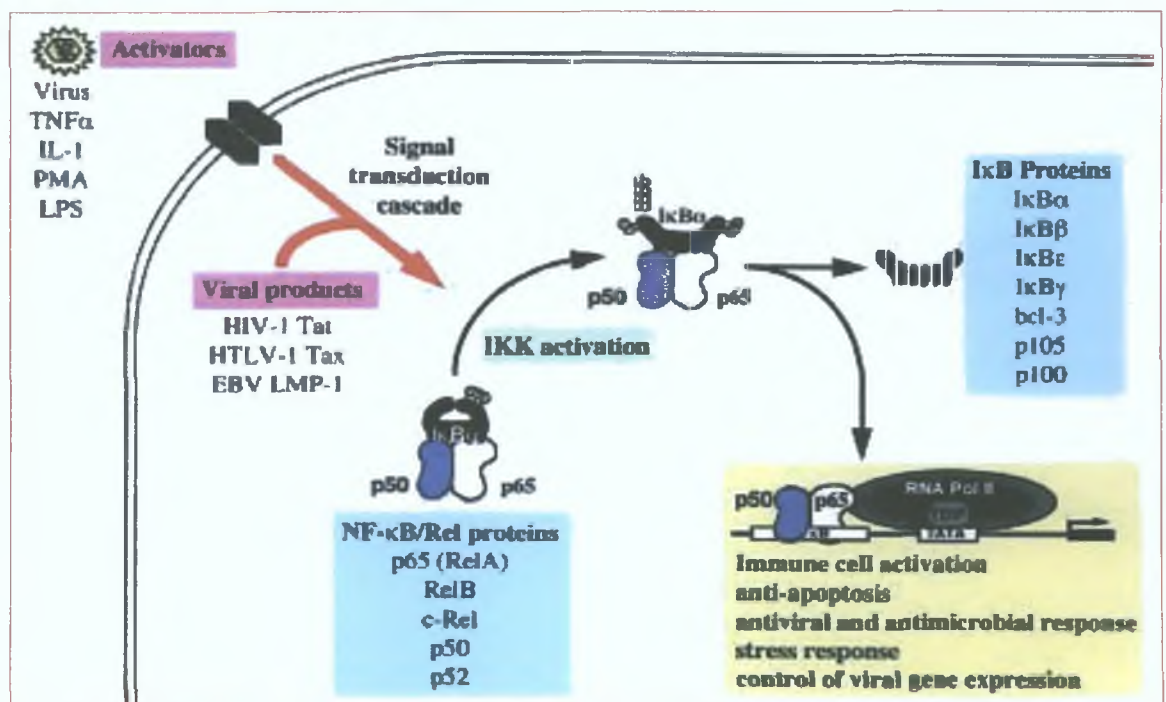


Figure 1.14: The biochemistry of NF- κ B activation. NF- κ B is sequestered in the cytoplasm by the inhibitory I κ B proteins. Stimulation by a diverse array of pathogens and other inducers including viruses, cytokines and stress-inducing agents leads to the activation of signalling cascades that culminate with the activation of the IKK complex and phosphorylation of the I κ B inhibitor. NF- κ B DNA binding subunits are released and translocate to the nucleus where they trans-activate NF- κ B-responsive genes. Target potential genes are selectively regulated by the distinct transcriptional activation potential of different NF- κ B subunit combination (adapted from Hiscott *et al.*, 2001).

NF- κ B plays a key role in most LMP1-stimulated gene expression (Devergne *et al.*, 1998; He *et al.*, 2000; Mehl *et al.*, 2001) and this activity is critical for the up-regulation of anti-apoptotic gene products, such as A20 and c-IAPs, cytokines such as IL-6 and IL-8, as well as cell surface antigens such as CD40 and CD54 (ICAM1). Moreover, inhibition of NF- κ B impairs the ability of LMP1 to promote transformation and tumourigenicity in Rat-1 fibroblasts (He *et al.*, 2000). This effect is consistent with the observation that the LMP1 domains, which are critical for immortalisation of EBV-infected B-cells, namely CTAR1 and CTAR2 are also responsible for NF- κ B signalling (Huen *et al.*, 1995; Mitchell and Sugden, 1995). Both CTAR1 and CTAR2 are able to independently activate NF- κ B (Huen *et al.*, 1995). CTAR2 accounts for the majority (70-80 %) of LMP1-mediated NF- κ B activation via its interaction with TRADD. The remaining LMP1-mediated NF- κ B activation is achieved through CTAR1 via interaction with TRAF proteins (Murray and Young, 2001).

Analysis of nuclear extracts isolated from LMP-1 expressing cells, or from cells transfected with LMP1 mutants lacking CTAR1 or CTAR2, have demonstrated the presence of a variety of NF- κ B complexes bound to NF- κ B specific sequences. LMP1-mediated NF- κ B activation has been extensively studied and shares similarities to the pathways activated by clustered TNF receptors or CD40. The interactions of TRAF2 and TRAF5 with CTAR1 appear to be critical for NF- κ B signalling, as mutations in the core of the TRAF binding motif or over-expression of dominant negative TRAF2 or TRAF5 molecules abrogate CTAR1 mediated NF- κ B activation (Eliopoulos and Young, 2001). In addition, TRAF1 or TRAF3 may influence these signals indirectly by competing or synergising with TRAF2. Therefore, whilst TRAF1 cannot induce NF- κ B *per se*, its over-expression significantly augments CTAR1-induced NF- κ B activation (Devergne *et al.*, 1996).

The ability of TRAF2 or TRAF5 to recruit NF- κ B inducing kinase (NIK) initiates an established cascade of phosphorylation events, which promote phosphorylation of I κ B proteins, resulting in the activation of NF- κ B. NIK appears to be responsible for these LMP1 signals because expression of a kinase-inactive NIK mutant functions as a dominant-negative inhibitor of CTAR1-mediated activation of NF- κ B *in vitro* (Sylla *et al.*, 1998; Eliopoulos *et al.*, 1999a). Similar experiments using dominant negative mutants of IKK α and IKK β have demonstrated their contribution to CTAR1-emanating signals (Sylla *et al.*, 1998) and the ability of LMP1 to promote phosphorylation and

degradation of I κ B α has long been recognised (Herrero *et al.*, 1995). Thus the experimental evidence suggests that the CTAR1 domain of LMP1 activates NF- κ B through a TRAF2 \rightarrow NIK \rightarrow IKK \rightarrow I κ B α pathway (Figure 1.13) (Eliopoulos and Young, 2001).

The CTAR2 region of LMP1 requires TRADD but not RIP for NF- κ B activation (Izumi *et al.*, 1999). The N-terminus of TRADD strongly binds to TRAF2 and it is possible that the interaction initiates a cascade similar to that engaged by CTAR1. Indeed, dominant-negative mutants of TRAF2, NIK and IKKs inhibit CTAR2-induced NF- κ B signalling. Thus the available evidence suggests that a TRADD \rightarrow TRAF2 \rightarrow NIK \rightarrow IKK \rightarrow I κ B α pathway is responsible for NF- κ B activation by the CTAR2 domain of LMP1 (Eliopoulos and Young, 2001). Taken together, the experimental evidence has demonstrated that NF- κ B signalling is mediated entirely through the LMP1 carboxy tail and may account for most of the pleiotropic affects of this key viral protein.

1.9.6 Latent membrane proteins 2A and 2B (LMP2A and LMP2B)

The gene encoding LMP2 yields two distinct proteins: LMP2A and LMP2B. The first exons of LMP2A and LMP2B are the only unique exons, whereas the remaining eight exons are common to both messages (Laux *et al.*, 1989; Sample *et al.*, 1989). As a result, the structures of LMP2A and LMP2B are similar (Figure 1.15); both contain 12 trans-membrane domains and a 27 amino acid cytoplasmic C-terminus. However, LMP2A has a 119 amino acid cytoplasmic N-terminus domain, encoded in exon 1, whereas exon 1 is non-coding in LMP2B (Kieff, 1996). 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. LMP2A aggregates in patches within the plasma membrane of latently infected B cells (Longnecker and Kieff, 1990). By analogy to LMP1, LMP2A probably functions as a constitutively activated receptor via cross-linking caused by aggregation of multiple LMP2A molecules through the association of their trans-membrane domains (Gires *et al.*, 1997). Although mRNAs for both LMP2A and LMP2B are expressed in immortalized B cells, genetic analyses of the LMP2 gene have shown that neither product is required for transformation of B cells by EBV (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). However, LMP2A plays an important role in modifying normal B cell development to favour maintenance of EBV latency.

In primary B lymphocytes, cross linking the B cell receptor (BCR) leads to an intricate signal cascade including the recruitment and activation of the Src family of protein tyrosine kinases (PTKs); subsequent activation and recruitment of other kinases, phosphatases or adaptor proteins; the hydrolysis of phospholipids; mobilisation of intracellular calcium; activation of protein kinase C; activation of nuclear transcription factors and transcription of BCR signal specific genes (Freuhling *et al.*, 1998). These genes include the EBV immediate early genes, resulting in the induction of viral lytic replication (Miller *et al.*, 1994). EBV-infected B cells are blocked in their ability to transduce signals through the BCR and LMP2A has been demonstrated to be responsible for this effect (Freuhling *et al.*, 1998).

The LMP2A N-terminal domain contains eight tyrosine residues, two of which form an immunoreceptor tyrosine-based activation motif (ITAM) (Freuhling and Longnecker, 1997). This is the same motif that is present on the BCR and is required for signalling by the BCR when it binds cognate antigen. Upon phosphorylation, the ITAM present on the BCR plays a central role in mediating lymphocyte proliferation and differentiation by the recruitment and activation of the Src family of PTKs, preferably with Lyn and Fyn, and the hemopoietic-cell-specific kinase Syk. LMP2A also interacts with these PTKs through its phosphorylated ITAM (Figure 1.15) and this association appears to negatively regulate PTK activity (Freuhling and Longnecker, 1997). As one of the repercussions of BCR activation is the induction of the EBV lytic cycle (Miller *et al.*, 1995; Kieff *et al.*, 2001), resulting in the induction of expression of EBV genes, many of which are highly immunogenic, LMP2A has an important function in blocking BCR-stimulated activation of the EBV lytic cycle and helping EBV to avoid detection by the immune system (Miller *et al.*, 1995). Recent studies also show that LMP2A associates with lipid rafts (Dykstra *et al.*, 2001; Higuchi *et al.*, 2001), thus LMP2A blocks BCR signalling by interfering with raft association of the BCR (Dykstra *et al.*, 2001). Expression of LMP2A in the B cells of transgenic mice alters normal B cell development, allowing BCR-negative B cells to exit the bone marrow and survive in the peripheral lymphoid organs (Caldwell *et al.*, 1998). This suggests that LMP2A can drive the proliferation and survival of B cells in the absence of signalling through the

BCR. Taken together, these data support a role for LMP2A in modifying the normal programme of B cell development to favour the maintenance of EBV latency and to prevent inappropriate activation of the EBV lytic cycle. A modulatory role for LMP2B in regulating LMP2A function has been suggested (Scholle *et al.*, 1999).

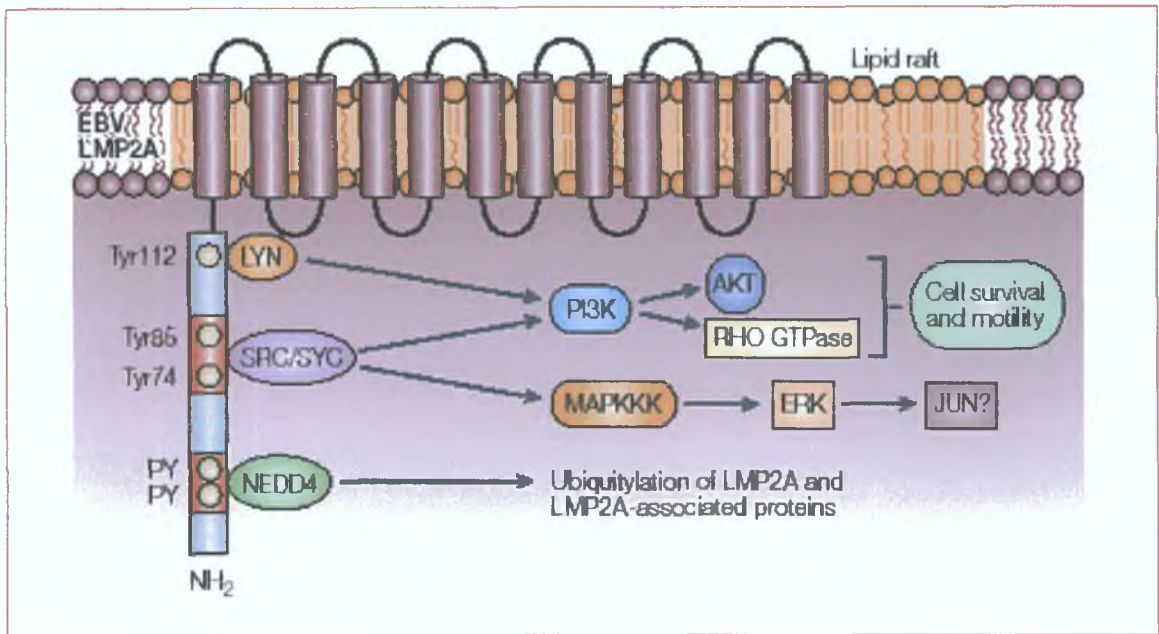


Figure 1.15: Structure and function of LMP2. The structures of EBV LMP2A and LMP2B are similar; both have 12 trans-membrane domains and a 27-amino acid cytoplasmic carboxyl terminus. In addition, LMP2A has a 119-amino acid cytoplasmic amino-terminal domain that contains eight tyrosine residues, two of which (Tyr74 and Tyr85) form an immunoreceptor tyrosine-bases activation motif (ITAM). The phosphorylated ITAM recruits members of the SRC family of protein tyrosine kinases and the SYK tyrosine kinase and negatively regulates their activities. A membrane-proximal tyrosine residue (Tyr112) binds the LYN tyrosine kinase and mediates the constitutive phosphorylation of the other tyrosine residues in LMP2A. The LMP2A ITAM blocks signalling from the B cell receptor (BCR) by sequestering these tyrosine kinases and by blocking the translocation of the BCR into lipid rafts. LMP2A also recruits NEDD4-like ubiquitin protein ligases through phosphotyrosine (PY) motifs, and these promote the degradation of LYN and LMP2A by a ubiquitin-dependent mechanism. LMP2A interacts with the extracellular signal-regulated kinase 1 (ERK1) mitogen activated protein kinase (MAPK), and this results in the phosphorylation of two serine residues (Ser15 and Ser102) in LMP2A, and might contribute to LMP2A-induced activation of JUN (adapted from Young and Rickinson, 2004).

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). Furthermore, the consistent expression of LMP2A in HD and NPC suggests an important function for this protein in oncogenesis but this remains to be shown.

LMP2A also recruits Nedd4-like ubiquitin protein ligases; this might promote Lyn and Syk ubiquitination in a fashion that contributes to a block in B cell signalling (Ikeda *et al.*, 2000). Furthermore, recent reports show that LMP2A can transform epithelial cells; this effect is mediated, at least in part, by activation of the phosphoinositide 3-kinase-Akt pathway (Scholle *et al.*, 2000). 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 co-localise with LMP1 in the plasma membrane (Longnecker and Kieff, 1990) and enhance LMP1 mediated activation of the AP-1/JNK and NF- κ B pathways (Dawson *et al.*, 2001; Kieser *et al.*, 1997). Recent studies found that LMP2A regulates c-Jun protein through extracellular signal-regulated kinase (ERK) (Chen *et al.*, 2002).

1.9.7 Epstein-Barr virus-encoded RNAs 1 and 2 (EBER1 and EBER2)

EBERs 1 and 2 are non-polyadenylated, uncapped, non-coding RNAs of 167 and 172 nucleotides respectively (Thompson and Kurzrock, 2004). They are expressed in abundance in nearly all EBV-infected cells with the exception of oral hairy leukoplakia lesions from AIDS patients and some hepatocellular carcinomas (Sugawara *et al.*, 1999). In addition to the latent proteins, EBER1 and EBER2 are expressed in all forms of latency. However, recombinant EBV with EBER genes deleted can transform lymphocytes, suggesting that the EBERs are not essential for transformation (Swaminathan *et al.*, 1991). The EBERs assemble into stable ribonucleoprotein particles with the autoantigen La and ribosomal protein L22, and bind the interferon-inducible, double-stranded-RNA-activated protein kinase PKR (Takada and Nanbo, 2001). PKR has a role in mediating the antiviral effects of the interferons, and it has been suggested that EBER-mediated inhibition of PKR function might be important for viral persistence (Nanbo *et al.*, 2002). Expression of the EBERs in BL cell lines has been found to increase tumourigenicity, promote cell survival and induce interleukin-10 (IL10) expression (Takada and Nanbo, 2001; Ruf *et al.*, 2000; Kitiwaga *et al.*, 2000). Furthermore, transfection of the EBER genes into the EBV-negative Akata cell line restored the oncogenic potential that was originally present in the EBV-positive Akata cells but was lost in the EBV-negative subclones (Komano *et al.*, 1999). Such studies indicate that EBV genes that were previously shown to be dispensable for transformation in B cell systems might make more important contributions to the

pathogenesis of some EBV-associated malignancies, and to EBV persistence, than was previously appreciated (Young and Rickinson, 2004).

1.9.8 The *Bam*HI A rightward transcripts (BARTs)

The EBV *Bam*HI A rightward transcripts were first described in nasopharyngeal tumour tissues where they are the most abundant viral transcripts (Hitt *et al.*, 1989). BARTs were subsequently detected in all EBV latently infected cells (Brooks *et al.*, 1993; Deacon *et al.*, 1993; Chen *et al.*, 1999b). These highly spliced transcripts are commonly referred to as either *Bam*HI A rightward transcripts (BARTs) or complementary-strand transcripts (CSTs) (Karran *et al.*, 1992; Smith *et al.*, 2000). The protein products of these ORFs remain to be conclusively identified, but the products of two of the ORFs, RPMS and RK-BARF0 interact with the Notch pathway when expressed exogenously. RPMS functions in a manner that is reminiscent of MINT/SHARP, by binding to CIR in the RBP-J κ /CBF1 repressor complex and mediating repression (Zhang *et al.*, 2001). RPMS affects the CIR-SKIP interaction in a way that prevents EBNA2/Notch1IC from displacing the co-repressor complex. SKIP facilitates SHARP function and thus SKIP is not only a key tethering point for EBNA2/Notch1IC binding, but also a key target for negative modulation of their activity (Hayward, 2004). The RK-BARF0 protein was found to interact with Notch4 in a yeast two-hybrid assay (Kusano and Raab-Traub, 2001). RK-BARF0 contains a potential signal peptide for ER targeting and interacts with unprocessed Notch4. RK-BARF0 appears to potentiate Notch function.

The BARTs are often expressed in circumstances in which the EBNA2 and EBNA3 proteins are not synthesised, such as epithelial cell infection and in EBV-associated malignancies in immuno-competent individuals. It is interesting that these transcripts should encode both a positive and a negative Notch regulator and this re-emphasises the importance of signal modulation of the Notch pathway responses. The existence of BARTs also suggests that there may be as yet unappreciated aspects of EBV biology where manipulation of Notch signalling occurs (Hayward, 2004). Another transcript that is generated from the *Bam*HI A region is *BARF1*, which encodes a 31 kDa protein that was originally identified as an early antigen expressed on induction of the EBV lytic cycle. Recent studies have shown that BARF1 is a secreted protein that is

expressed as a latent protein in EBV-associated NPC and gastric carcinoma (Decaussin *et al.*, 2000; zur Hausen *et al.*, 2000). BARF1 shares limited homology with the human colony stimulating factor 1 receptor (the *FMS* oncogene) and displays oncogenic activity when it is expressed in rodent fibroblasts and simian primary epithelial cells (Sheng *et al.*, 2001).

1.10 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 virus carriers to EBV naïve individuals. 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). 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 oriLyt (Hammerschmidt and Sugden 1988).

In vitro, lytic infection is induced mainly using chemicals, such as 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 interaction with AP-1 sites upstream of the immediate early viral genes (Farrell *et al.*, 1983; Farrell *et al.*, 1989). Lytic replication has been extensively studied in the Akata cell line. Viral replication can be induced in this cell line (which carries an LMP2A deleted virus), by cross-linking of surface immunoglobulins (Takada, 1984; Takada and Ono, 1989). Studies carried out using the Akata cell line, among others, have permitted the identification of the EBV replicative proteins.

The replicative proteins of the EBV lytic cycle have been classified as early antigens (EA), membrane antigens (MA), and viral capsid antigens (VCA) (Figure 1.16). 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 (Kieff, 1996). Following induction of the lytic cycle, cells that have become permissive to viral replication, undergo cytopathic changes characteristic of herpesviruses, including margination of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (Kieff, 1996). Lytic cycle gene expression follows a temporal and sequential order (Farrell, 1992; Takada and Ono, 1989). Some viral genes are expressed independently of new protein synthesis, early after induction, and are classified immediate early genes. The early lytic viral genes are expressed slightly later and their expression is not affected by inhibition of viral DNA synthesis. Late proteins are formally categorised as late based on a marked reduction in expression after inhibition of viral DNA synthesis (Kieff, 1996).

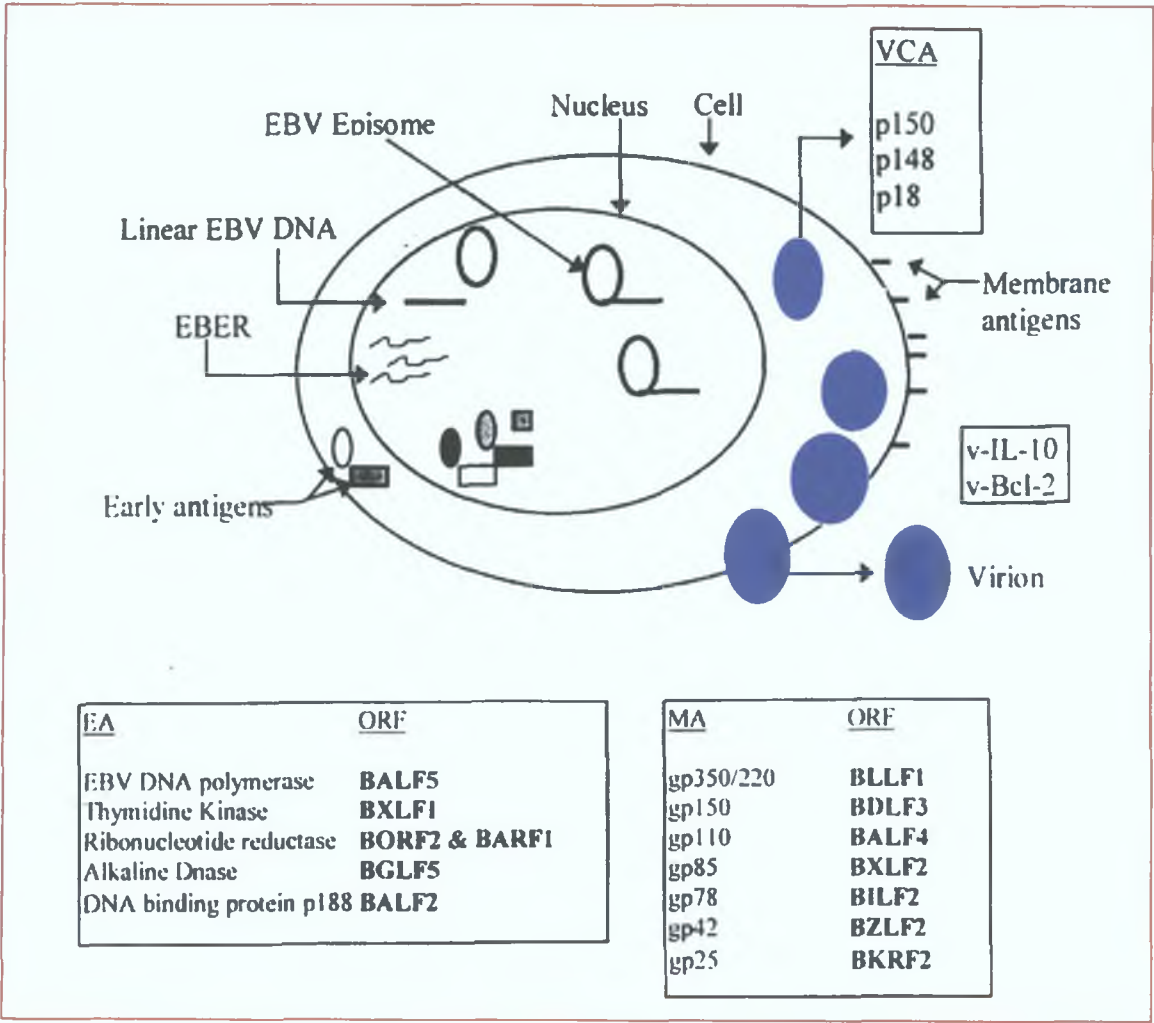


Figure 1.16 A schematic representation of early and late EBV gene expression The VCA, the MA and the EA are illustrated and their open reading frames are written in bold

1.10.1 Immediate early genes

After induction of the lytic cycle in cell lines, such as Akata, 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, 1996). BZLF1 also functionally and physically interacts with NF- κ B (Gutsch *et al.*, 1994), which is an important mediator of LMP1 effects during 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 pRb *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).

1.10.2 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, shows partial (25 %) sequence homology to the human *bcl-2* proto-oncogene. Both BHRF1 and Bcl-2 have been shown to protect human B lymphocytes from apoptosis (Henderson *et al.*, 1993). Several of the early genes are linked to DNA replication. 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 (Kieff, 1996).

1.10.3 Late genes

The late genes encode 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 1.16 above. The late BCRF1 gene, which is located in the middle of the EBNA regulatory domain between oriP and Cp, shows 84 % sequence homology to the human IL-10 (Vieira *et al.*, 1991). IL-10 was first recognised for its ability to inhibit activation and effector function of monocytes, macrophages and T cells. EBV-derived IL-10 is thought to play a role in the establishment of latent infection by suppression of the host immune system (Helminen *et al.*, 1999; Kurilla *et al.*, 1993; Rousset *et al.*, 1992).

1.11 EBV-mediated regulation of cell growth and survival

Cell proliferation and survival are two essential features of immortalisation. The immortalisation of B cells by EBV requires the co-operative action of many latent genes. EBV genes manipulate the cell survival and proliferation machinery by (i) regulating components of the cell cycle, (ii) influencing pathways that regulate anti-apoptotic proteins and (iii) expressing viral homologues to anti-apoptotic proteins. While characterisation of the mechanism of action of individual latent genes is essential, it is also important to understand how latent genes can co-operate and may have overlapping functions for B cell immortalisation. This section attempts to summarize the complex interactions of EBV with the network of proteins involved in cell proliferation and survival.

1.11.1 EBV regulates components of the cell cycle machinery

1.11.1.1 The cell cycle

The cell cycle (Figure 1.17) is regulated in four distinct phases called G1, S-phase, G2 and M-phase (Scherr, 1996). The G1 phase of the cell cycle is the longest and occurs prior to DNA synthesis, which occurs during S-phase. During M-phase, cell division takes place. The progression through the cell cycle is regulated by many different proteins, some of which are illustrated in Figure 1.17. The first biochemical event observed, in early G1 phase, is the induction of the D-type cyclins and their partners, cyclin dependent kinases (cdks), cdk4 and cdk6. One of either cd4 or cd6 partners a D-type cyclin, in the absence of cyclin dependent kinase inhibitors (CDKIs), to become catalytically active. The induction of D-type cyclins is paralleled by a loss in CDKIs (Brennan, 2001). The most important substrate of the cyclin D/cdk4/6 complex is the family of pocket proteins, typified by pRb, the retinoblastoma susceptibility gene product (Nevins *et al.*, 1997). Other members of the pocket protein family include p130 and p107. These pocket proteins repress a family of transcription factors called E2F, and the E2F-pocket protein complexes also act as gene suppressors (Nevins *et al.*, 1997). Phosphorylation of pRb by the cyclin D/cdk4/6 complex results in the release of E2F transcription factors and transcription of E2F-responsive genes. E2F binding sites have been identified in the promoters of many genes important for cell cycle regulation and the promoters of proteins involved in DNA synthesis. In many systems, the induction of E2F transcriptional activity results in entry into the cell cycle (Johnson *et al.*, 1993).

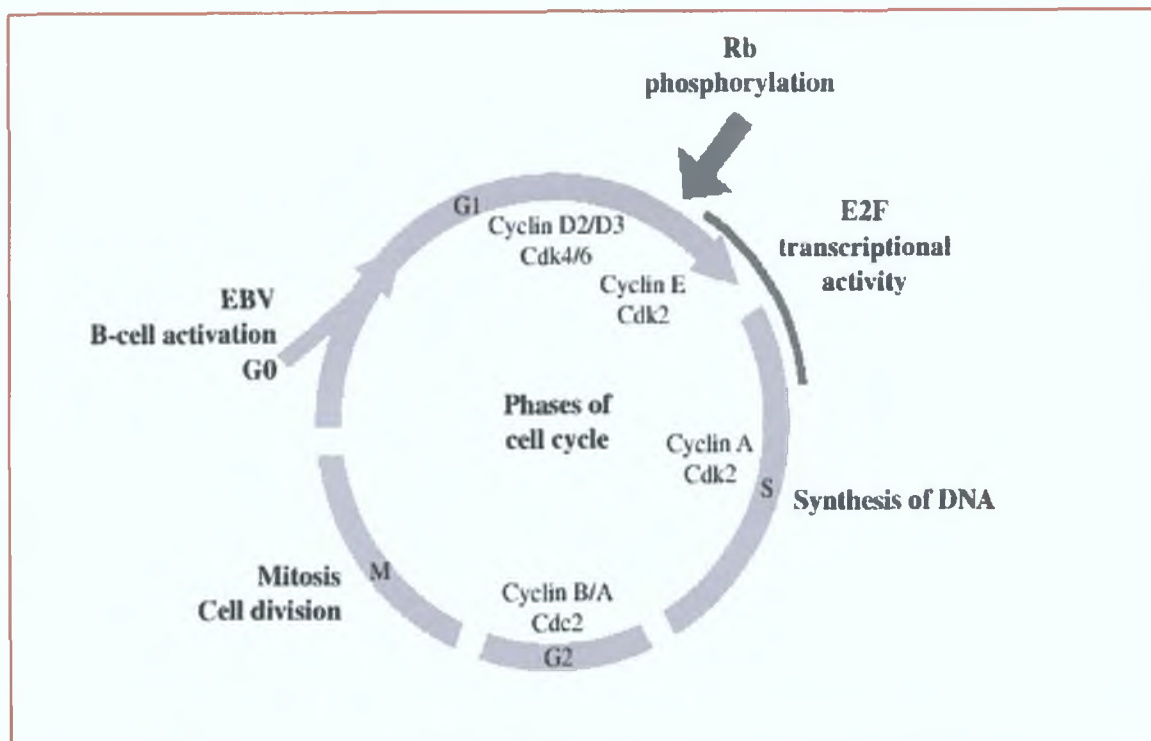


Figure 1.17. Phases of the cell cycle. (adapted from Brennan, 2001).

The induction of D-type cyclins during early G1 phase is followed by the induction of cyclin E during late G1 phase, and the entry into S-phase, whereupon DNA synthesis occurs. Cyclin E partners cdk2 and also phosphorylates pocket proteins. Cyclin E/cdk2 has a wider range of substrates than the cyclin D/cd4/6 complex but the importance of these has not yet been characterized (Brennan, 2001).

1.11.1.2 Interactions of EBV proteins with the cell cycle

Cyclin D2 is the first cell cycle protein to be induced following either EBV infection or mitogenic stimulation of B cells (Spender *et al.*, 1999). It is accompanied by a loss of the CDKI p27kip1. A phosphorylation of the pocket protein, pRb, is observed approximately 6 hours later. All these events parallel those seen during normal B cell proliferation, leading to the proposal that EBV exploits normal cell pathways to regulate pRb phosphorylation during the cell cycle (Cannell *et al.*, 1996; Kempkes *et al.*, 1995b). The genes involved in the regulation of cyclin D2 by EBV have been studied. It has been shown that vectors expressing EBNA2 and EBNA-LP are sufficient to activate the expression of cyclin D2 during immortalisation of resting human B lymphocytes. Neither EBNA2 nor EBNA-LP alone induced cyclin D2 (Sinclair *et al.*, 1994). Subsequent studies have further demonstrated the importance of EBNA2 in cell cycle

progression, with recent data indicating that EBNA2 has the ability to trans-activate a cyclin D2 promoter reporter construct (Spender *et al.*, 2001). However, the evidence to date suggests that EBNA2 induction of cyclin D2 is indirect (Spender *et al.*, 2001; Kaiser *et al.*, 1999) and the mechanism by which EBNA2 links to cyclin D2 remains to be characterised. The *c-myc* oncogene has been shown to be a direct target of EBNA2 (Spender *et al.*, 2001; Kaiser *et al.*, 1999) and c-Myc has also been shown to trans-activate the D2 promoter (Bouchard *et al.*, 1999). This was shown to be via an E-box located outside the region of the promoter trans-activated by EBNA2, suggesting that *c-myc* is not the cross-link between EBNA2 and cyclin D2. The co-operation of EBNA2 with EBNA-LP to induce cyclin D2 has not yet been further investigated. There is one report that LMP1 can also regulate D2 (Arvanitakis *et al.*, 1995), suggesting a third protein that may play a role in cell cycle induction.

The induction of cyclin D2 is paralleled by the loss of the CDKI p27kip1 in response to EBV and many other systems (Spender *et al.*, 2001; Slingerland and Pagano, 2000). The regulation of p27kip1 in other systems has been investigated, and it has been shown to be ubiquitinated and degraded in response to phosphatidylinositol 3-kinase (PI3K)-mediated pathways and is also regulated transcriptionally. The only EBV protein that has been shown to play a role in the regulation of p27kip1 is EBNA3C (Parker *et al.*, 2000), where a cell line expressing EBNA3C in a regulated fashion, showed an inverse correlation between EBNA3C induction and the presence of p27kip1. Since cyclin D2 and p27kip1 together regulate cdk4 and cdk6 activity, the link between EBNA3C and p27kip1 implicates a fourth EBV protein in the progression through the G1 phase of the cell cycle (Brennan, 2001). Furthermore, both EBNA3C and EBNA-LP have been found to interact with pRb (Parker *et al.*, 1996, Szekely *et al.*, 1993), once again implicating these latent proteins in the direction of cell cycle progression.

1.11.1.3 The role of cytokines in EBV-mediated immortalisation

Following B lymphocyte activation, EBV induces the synthesis of various cytokines. While a diverse range of cytokines is expressed, for example TNF and IL-1, perhaps the most interesting in terms of cell growth factors, are IL-6 and IL-10. Both of these cytokines play an important role in the growth of EBV-transformed cells (Haddad *et al.*, 2001; Kitagawa *et al.*, 2000; Beatty *et al.*, 1997). EBV also encodes a viral homologue

of IL-10 (from the BCRF1 ORF), albeit expressed in the lytic cycle, which enhances the growth transformation of a B cell infected with EBV (Stuart *et al.*, 1995). IL-6 and IL-10 share signalling pathways. The receptor molecules that IL-6 and IL-10 use to mediate their activities both activate the JAK family of tyrosine kinases, and downstream signalling cascades including STAT proteins and the PI3K pathway (Brennan, 2001). IL-6 is known to be pro-inflammatory, whereas IL-10 is known to be anti-inflammatory (Moore *et al.*, 2001). However, IL-10 also acts as a co-factor for B cell growth (Moore *et al.*, 2001), and it has been demonstrated that PI3K is important for IL-10-mediated cell growth of both primary monocytes and a murine mast cell line (Crawley *et al.*, 1996)

Recent reports suggest that EBV uses both common and distinct mechanisms for the regulation of IL-6 and IL-10. Both cytokines have been shown to be regulated by LMP1 via NF- κ B and the p38 stress activated protein kinase (Eliopoulos *et al.*, 1997; Eliopoulos *et al.*, 1999b; Nakagomi *et al.*, 1994; Vockerodt *et al.*, 2001). However, new observations have implicated an alternative way in which IL-10 may be regulated by the EBERs. Transfection of EBERs into EBV-negative BL cells resulted in the induction of IL-10, while EBER1 and EBER2 was sufficient, the optimal IL-10 expression was observed when both were introduced (Kitigawa *et al.*, 2000).

1.11.1.4 Signalling molecules that link to the cell cycle

Active signalling pathways are a feature of many malignant cells. Thus, it is important to characterize the potential role of signalling molecules that are activated by EBV-induced cytokines and by EBV genes themselves. The pathways activated by IL-6 and IL-10 (for example the JAK family of tyrosine kinases, PI3K and STATs) have been shown to play a role in lymphocyte growth and transformation (Bowman *et al.*, 2000; Ihle, 2001; Imada and Leonard, 2000; Brennan *et al.*, 1997), although some of the data is controversial. The pattern of tyrosine phosphorylation seen in EBV-immortalised B cells is distinct from that seen in matched cells that lack expression of the EBV latent genes (Brennan, 2001). This suggests that tyrosine kinases, or phosphatases, have altered activity in EBV-immortalised cells, although the identity of the important kinases and their substrates remains unknown.

The STAT family of transcription factors are one substrate of the JAK family of tyrosine kinases. STATs have been shown to be active in EBV-immortalised cells (Weber-Nordt *et al.*, 1996) and may play a role in oncogenesis (Bowman *et al.*, 2000). It has been reported that LMP1 can activate JAK3 and STAT1 (Gires *et al.*, 1999). STAT1 is predominantly growth inhibitory so it is unlikely that it would contribute directly to malignancy (Bowman *et al.*, 2000). LMP1 can also activate a STAT reporter that is known to bind STAT1, STAT3 and STAT5 (Brennan *et al.*, 2001), but the molecular basis of this is unknown. Over-expression of STAT3 has been shown to contribute to transformation, and STAT5 plays a role in cytokine-induced proliferation, so perhaps these are better candidates for STATs involved in EBV-induced proliferation (Ihle, 2000). There is also evidence of functional STAT binding sites in the promoters for the *LMP1* and *EBNA1* genes, demonstrating that another role may be played by this family of transcription factors in EBV biology (Chen *et al.*, 1999a; Chen *et al.*, 2001).

There is some evidence that pathways downstream of the small G protein Ras are active in EBV-immortalised cells and that MAP-ERK can be activated by LMP1 (Roberts and Cooper, 1998). However, the MAP-ERK pathway does not appear to be required for EBV-induced proliferation, although it is required for normal B cell proliferation (Fenton and Sinclair, 1999). PI3K, a lipid kinase that is involved in lymphocyte survival (Pogue *et al.*, 2000; Carey and Scott, 2001) and proliferation (Brennan *et al.*, 1997; Craddock *et al.*, 1999), can be activated by oncogenic Ras (Vanhaesebroeck and Alessi, 2000). An active pathway regulated by PI3K in EBV-immortalised cells has been identified and inhibition of PI3K using a chemical inhibitor, inhibits the proliferation of EBV-immortalised cells (Brennan, 2001). Further evidence for the importance of this pathway was provided by a study where LMP2 was found to activate a PI3K-mediated pathway, Akt (Swart *et al.*, 2000; Scholle *et al.*, 2000). Akt, in response to PI3K, has been shown to modulate the forkhead family of transcription factors involved in p27kip1 regulation, suggesting a mechanism by which Akt might mediate the requirement of PI3K for cytokine induced proliferation (Dijkers *et al.*, 2000). Furthermore, PI3K has also been shown to affect D-type cyclin expression in EBV-immortalised B cells (Brennan, 2001), suggesting a mechanism for its role in EBV-induced lymphocyte growth.

1.11.2 EBV and cell survival

In addition to proliferation, cell survival is also an important a feature of malignancy and transformation. A central component of the overall EBV strategy and its role in the development of related malignant disease is the ability of the viral proteins to suppress the cellular apoptotic program (Allday, 1996; Klein, 1994). Apoptosis is a genetically controlled pre-programmed form of cell suicide involving dramatic morphological changes including cell shrinkage, nuclear re-organization, plasma membrane blebbing and eventual fragmentation of the cell into membrane bound apoptotic bodies (Allday, 1996). While the process of apoptosis is responsible for shaping organs during embryogenesis and maintaining tissue homeostasis within the adult organism, it also functions as an emergency response to aberrant growth induced by the activation of oncogenes and viral infection. In the case of virus-infected cells, the induction of early cell death would severely limit virus production and reduce or eliminate spread of virus progeny in the host (Shen and Shenk, 1995). In order to maximize their replicative capacity many viruses have evolved mechanisms that suppress the apoptotic programme (Shen and Shenk, 1995). EBV utilises such mechanisms to (i) delay cell death during the lytic stage of infection until virion numbers have been sufficiently amplified to transmit infection to other individuals via saliva and (ii) to ensure persistence in the host memory B cell compartment.

1.11.2.1 EBV up-regulates expression of the anti-apoptotic cellular gene *bcl-2*

Group I BL cell lines (which display type I latency, thus only express EBNA1, EBERs and BARTs) and many EBV-negative BL lines can readily be triggered into apoptosis, whereas Group III BL cell lines, which express the complete set of EBV latent proteins, are relatively resistant to a variety apoptotic triggers, including growth factor withdrawal, Ca^{2+} ionophore treatment and over-expression of the p53 tumour suppressor gene (Henderson *et al.*, 1991; Okan *et al.*, 1995). EBV-negative BL cells converted to the type III latency state by infection with the B95-8 strain of EBV also display elevated thresholds of resistance to apoptotic stimuli (Gregory *et al.*, 1991), thus implicating EBV latent genes in cell survival.

In addition to being resistant to apoptotic stimuli, group III BL cell lines display high levels of the Bcl-2 protein (Henderson *et al.*, 1991). Bcl-2 is a prototype anti-apoptotic protein that interacts with the mitochondrial membrane and inhibits the action of caspases (Adams and Cory, 2001). Transfection of individual EBV latent genes into EBV-negative BL cell lines has shown that up-regulation of Bcl-2 expression correlates with the expression of LMP1 (Liu *et al.*, 1991; Henderson *et al.*, 1991; Rowe *et al.*, 1994) and possibly EBNA2 (Finke *et al.*, 1992) and EBNA3B (Silins and Sculley, 1995). Several lines of evidence, however, indicate that in addition to Bcl-2, other survival mechanisms play a role protecting type III BL cells from apoptosis. Firstly, over-expression of the Bcl-2 protein in group I cells (well beyond the level of group III cells) is necessary to attain the high levels of protection observed in group III cells (Milner *et al.*, 1992). Secondly, prolonged culture of group I lines *in vitro* has been shown to result in enhanced survival in the absence of Bcl-2 up-regulation (Milner *et al.*, 1992). Also, Bcl-2 is not detectable in germinal centre B cells *in vivo*, the normal equivalent of BL cells, and only appears when the cells reach the follicular mantle (Pezzella *et al.*, 1990; Aiello *et al.*, 1992; Akagi *et al.*, 1994). Finally, the induction of Bcl-2 by LMP1 is a delayed response with maximal levels detectable after 2 days of LMP1 induction (Rowe *et al.*, 1994). Subsequent studies have shown that LMP1 can also up-regulate other anti-apoptotic proteins, including A20, Mcl-1, cIAPs and Bfl-1 (Laherty *et al.*, 1992; Wang *et al.*, 1996; Hong *et al.*, 2000; D'Souza *et al.*, 2000).

1.11.2.2 EBV up-regulates expression of the anti-apoptotic cellular gene A20

A20 is an inducible zinc finger protein that confers resistance to TNF α cytotoxicity (Laherty *et al.*, 1992). The 790 amino acid protein is induced in response to inflammatory cytokines and cellular activators in many cell types, including fibroblasts, lymphocytes and endothelial cells (Beyaert *et al.*, 2000). Transfection experiments have demonstrated that LMP1 induces the expression of A20 in both lymphocytes and epithelial cells, and A20 is constitutively expressed in EBV-immortalised B cells (Laherty *et al.*, 1992; Fries *et al.*, 1996; Spender *et al.*, 1999). Studies of the A20 promoter have shown that LMP1 transcriptionally activates the A20 gene through cis-acting NF- κ B binding sites. Furthermore, the electrophoretic mobility shift assay (EMSA) confirmed LMP1-inducible binding of an NF- κ B-like factor to the NF- κ B binding sites within the A20 promoter. A20 has also been identified as functioning in a

negative feedback loop by inhibiting the activation of NF- κ B from both CTAR1 and CTAR2 of the LMP1 protein (Eliopoulos *et al.*, 1999b). This effect is mediated by the binding of A20 to TRAF2. This effect is also evident in CD40 signalling which also induces expression of A20 in BL cells (Sarma *et al.*, 1995).

1.11.2.3 EBV up-regulates expression of the anti-apoptotic cellular gene *mcl-1*

The maximum induction of Bcl-2 by LMP1 takes about 48-72 hours (Rowe *et al.*, 1994). However, induction of a Bcl-2 homologue, Mcl-1, by LMP1 precedes induction of Bcl-2 (Wang *et al.*, 1996). Mcl-1 is an anti-apoptotic protein that has been shown to contribute to the longevity of chronic lymphotropic leukaemia B cells and its higher expression is linked with resistance to chemotherapy (Moshynska *et al.*, 2004). Up-regulation of Mcl-1 in response to LMP1 expression is transient, with Mcl-1 levels decreasing as Bcl-2 levels increase. These findings support the hypothesis that Mcl-1 functions as a rapidly inducible, short-term effector of cell viability. It has also been demonstrated that LMP1 blocks the decline in Mcl-1 levels in response to apoptotic stimulation by cyclic AMP. This effect of LMP1 is associated with delayed cell death in the EBV-negative 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 such time that Bcl-2 is up-regulated (Wang *et al.*, 1996).

1.11.2.4 EBV up-regulates expression of cellular inhibitor of apoptosis protein 2 (c-IAP2)

c-IAP2 is a member of the cellular inhibitor of apoptosis protein family (c-IAP), which has been found to be up-regulated by LMP1 (Hong *et al.*, 2000). c-IAP2 was initially identified as a molecule that is recruited to the TNF receptor via TRAF1 and TRAF2 (Rothe *et al.*, 1995). It has been suggested that c-IAP2 can inhibit apoptosis by modulating the TNF-induced activation of NF- κ B (Chu *et al.*, 1997). Other stimuli that activate NF- κ B, including IL-1, LPS, and CD30 stimulation, have also led to an up-regulation in the transcription of c-IAP2 (Stehlik *et al.*, 1998; Craxton *et al.*, 1998). This prompted studies into the regulation of the c-IAP2 gene, which resulted in the

finding that expression of EBV LMP1 trans-activates the c-IAP2 promoter (Hong *et al.*, 2000).

1.11.2.5 EBV up-regulates expression of the anti-apoptotic cellular gene *bfl-1*

Recent studies in our laboratory have identified another anti-apoptotic gene, *bfl-1* (also known as *A1*), which is up-regulated by LMP1 expression. Expression of LMP1 in EBV-negative BL cell lines coincides with a dramatic increase in *bfl-1* mRNA (D'Souza *et al.*, 2000). In this study, *bfl-1* was shown to protect cells against serum depletion-induced apoptosis. Moreover, expression of LMP1 in EBV-negative cell lines was found to trans-activate the *bfl-1* promoter (D'Souza *et al.*, 2004). Bfl-1 was originally identified from granulocyte macrophage-colony stimulating factor (GM-CSF)-induced mouse bone marrow as a novel hemopoietic specific early response gene with sequence similarity to Bcl-2 (Lin *et al.*, 1993). Its human homologue was cloned by three independent approaches from fetal liver, activated endothelium and myeloid leukaemia (Choi *et al.*, 1995; Karsan *et al.*, 1996a; Kenny *et al.*, 1997).

bfl-1 gene expression is generally confined to immune cells and tissues, in a pattern similar to that of NF- κ B, and is strongly induced by cytokine simulation of leukemic, endothelial and hemopoietic cells. Constitutively elevated levels of *bfl-1* transcripts are seen in mature neutrophils and are selectively induced in long-lived peripheral B cells (Tomayko *et al.*, 1998). These observations suggest an important protective role for Bfl-1 in the survival and selection of distinct subsets of cells in the immune system. Consistent with this observation, Bfl-1 suppresses apoptosis induced by the pro-inflammatory cytokine TNF α (Karsan *et al.*, 1996b; Zong *et al.*, 1999; Duriez *et al.*, 2000), tumour suppressor p53 (D'Sa-Eipper *et al.*, 1996), B cell receptor aggregation (Kuss *et al.*, 1999; Grumont *et al.*, 1999; Craxton *et al.*, 2000), the pro-apoptotic factors Bax and Bad (Zhang *et al.*, 2000; Holmgreen *et al.*, 1999). It has also been found to suppress chemotherapy-induced apoptosis (Wang *et al.*, 1999; Cheng *et al.*, 2000; Kim *et al.*, 2004). Stimuli inducing Bfl-1 expression share the capacity to activate the transcription factor NF- κ B, which regulates the *bfl-1* gene (Zong *et al.*, 1999; Grumont *et al.*, 1999; Lee *et al.*, 1999; Wang *et al.*, 1999).

1.11.2.5.1 Mechanism of action of Bfl-1

Bfl-1 is a member of the Bcl-2 family of proteins, which consists of approximately 20 homologues of pro- and anti-apoptotic regulators of programmed cell death (Kirkin *et al.*, 2004). Whether induced by death receptors or other stimuli, apoptosis signalling generally involves cytochrome c (Cyt c) release from mitochondria (Li *et al.*, 1997). Upon release, Cyt c, together with dATP or ATP, binds to the adapter protein APAF1. This molecule then oligomerises and forms the apoptosome complex, which recruits and activates caspase 9, resulting in the initiation of effector caspase activation and the execution of apoptosis (Kirkin *et al.*, 2004). The mode of function of the individual Bcl-2 family members, therefore, is to either preserve or disturb mitochondrial integrity, thereby inducing or preventing release of apoptogenic factors, like Cyt c, from the mitochondria. An understanding of how the Bfl-1 protein functions to inhibit apoptosis necessitates examination of how individual members of the Bcl-2 family interact with each other in order to regulate cell death. Bcl-2 family proteins are defined by inclusion of at least one of four distinct Bcl-2 homology (BH) domains and the variable presence of a putative membrane-spanning region at the C-terminus (Cory and Adams, 2002) (Figure 1.18).

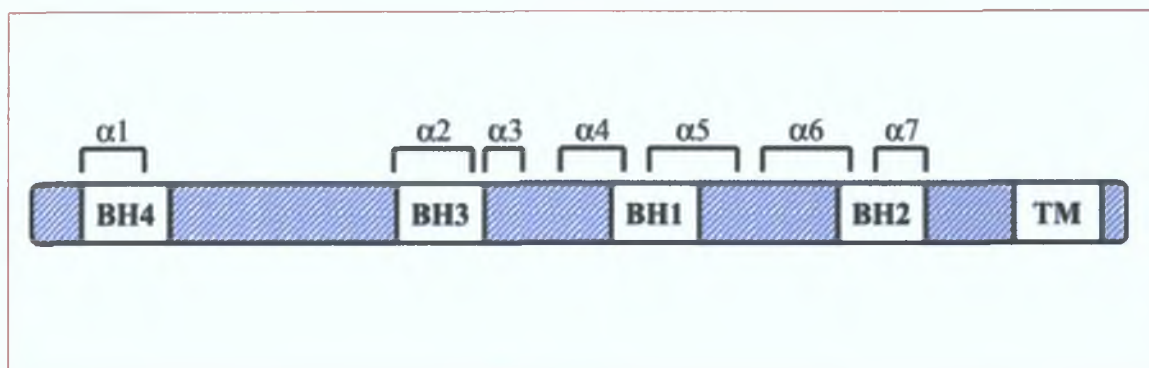


Figure 1.18: A schematic representation of Bcl-2 protein domain organisation. The locations of the four Bcl-2 homology (BH1 to BH4) domains are indicated, along with the trans-membrane domain (TM) and the known alpha-helical regions ($\alpha 1$ -7) (adapted from Kirkin *et al.*, 2004).

The Bcl-2 family of proteins can be sub-divided into three groups. The first group consists of the anti-apoptotic Bcl-2 type proteins, which include Bcl-2, Bcl-xL, Bcl-w, Bfl-1, Boo/Diva/Bcl-B and Mcl-1 (Kirkin *et al.*, 2004). Members of this group contain three to four BH domains, which are required for their anti-apoptotic function. The BH

domains mediate interactions between the Bcl-2-like proteins and other members of the Bcl-2 family. The second group of the Bcl-2 family consists of the Bax-like pro-apoptotic proteins, including Bax, Bak and Bok (Kirkin *et al.*, 2004). While Bax and Bak are widely expressed (Krajewski *et al.*, 1994; Krajewski *et al.*, 1996), Bok appears to be restricted to the reproductive tissues (Hsu *et al.*, 1997). The third group of Bcl-2 proteins is the pro-apoptotic BH3 domain-only group. This group has many members, which differ strikingly in their responsiveness to stimuli and thereby in the pathways they regulate (Huang and Strasser, 2000). The BH3 domain-only family members share only the short BH3 domain with the other Bcl-2 family members (and with each other).

A model for the mechanism of action of Bcl-2 family members, prominent until recently, predicted that Bcl-2 type proteins autonomously inhibit apoptosis. Heterodimerisation with pro-apoptotic members would abrogate their protective function and thus provoke apoptosis (Huang and Strasser, 2000; Kelekar and Thompson, 1998). However, recent findings indicate that death induction is independent of Bcl-2 type proteins, but involves collaboration between Bax-type proteins and BH3 domain-only family members. Studies using Bak and Bax knockout mice have provided insight into how pro-apoptotic Bcl-2 family members induce cell death. Cells lacking both Bax and Bak did not die upon expression of BH3 domain-only proteins, in contrast to cells expressing either one of these molecules. This indicated that BH3 domain-only proteins require at least one Bax-type partner to induce cell death (Zong *et al.*, 2001; Wei *et al.*, 2001). Both Bax and Bak undergo a conformational change in response to apoptotic stimuli (Werner *et al.*, 2002). Moreover, they assemble into homo-multimers in the mitochondrial membrane and upon oligomerization, they actively induce Cyt c release (Borner, 2003), possibly by (i) forming a new channel (Schlesinger *et al.*, 1997); (ii) by enlarging an existing permeability transition pore (Tsujimoto and Shimizu, 2000; Zamzami and Kroemer, 2001) or (iii) forming supramolecular openings in the outer membrane which are different from discrete protein channels (Kuwana *et al.*, 2002). Anti-apoptotic family members like Bcl-2, Bcl-xL, hold Bax and Bak in check, possibly forming hetero-dimers, thereby preserving mitochondrial integrity (Borner, 2003). The conformational change and multimerisation of Bax or Bak is inhibitable by Bcl-2 (Antonsson *et al.*, 2001; Mikhailov *et al.*, 2001) and inducible by the BH3 domain-only protein Bid (Wei *et al.*, 2000; Antonsson *et al.*, 2001). In healthy cells, BH3-only proteins are kept inactive (Cory and Adams, 2002). In response to pro-apoptotic signals, they become transcriptionally up-regulated and/or post-translationally modified to gain

their full apoptotic potential (Kirkin *et al.*, 2004). The Bid protein is cleaved by caspase 8 in response to death receptor activation (Li *et al.*, 1998; Luo *et al.*, 1998). Cleavage results in exposure of the Bid BH3 domain, which is normally buried within the full-length protein (Chou *et al.*, 1999; McDonnell *et al.*, 1999). The cleaved C-terminus complex, truncated Bid (tBid), is then myristoylated and translocated to the mitochondria (Zha *et al.*, 2000). Bid seems to form hetero-trimers in the mitochondrial membrane, which then may induce mitochondrial Bax or Bak to oligomerise (Wei *et al.*, 2000), promoting Cyt c release (Luo *et al.*, 1998; Li *et al.*, 1998).

The novel model emerging from these findings, is that Cyt c release depends on interaction between a BH3 domain-only protein and a Bax-type partner, which allows the formation of a Bax-type trans-membrane pore (Werner *et al.*, 2002). Bfl-1 has been shown to exert its anti-apoptotic activity by inhibiting the collaboration between the BH3 domain-only protein Bid and its pro-apoptotic partners Bax or Bak in the induction of Cyt c release (Werner *et al.*, 2002). Bfl-1 does this by binding to the full length Bid via the Bid BH3 domain. It does not interfere with the proteolytic activation of Bid, nor with its mitochondrial insertion, but remains selectively complexed to tBid in the mitochondrial membrane, where it prevents the activity of a pro-apoptotic complex (Werner *et al.*, 2002).

1.11.2.6 EBV lytic proteins encode anti-apoptotic functions.

During the lytic cycle of virus replication, repressors of apoptosis can provide a selective advantage by protecting from cell death until sufficient virus progeny numbers have been reached. Consistent with this model, there are at least two known lytic cycle proteins, BHRF1 and BZLF1, which can provide a survival advantage to the EBV-infected cells. These lytic gene products can therefore delay apoptosis in order to maximize viral production and spread of virus progeny to other individuals. The BHRF1 protein shows partial (25 %) sequence homology to the human *bcl-2* proto-oncogene. Both BHRF1 and Bcl-2 have been shown to protect human B lymphocytes from apoptosis (Henderson *et al.*, 1993) and expression of BHRF1 in epithelial cells has been shown to confer enhanced resistance to cisplatin-induced apoptosis and 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).

The molecular interactions between EBV proteins and cell survival and proliferation machinery are summarised in Figure 1.19. It demonstrates the multiple genes encoded by EBV that interact with components of the cell cycle and the presence of cytokines IL-6 and IL-10. Taken together, these interactions account, at least in part, for the ability of EBV to induce cell proliferation and survival, which is essential for both EBV-induced immortalisation of B cells and establishment of life-long persistence in the memory B cell compartment.

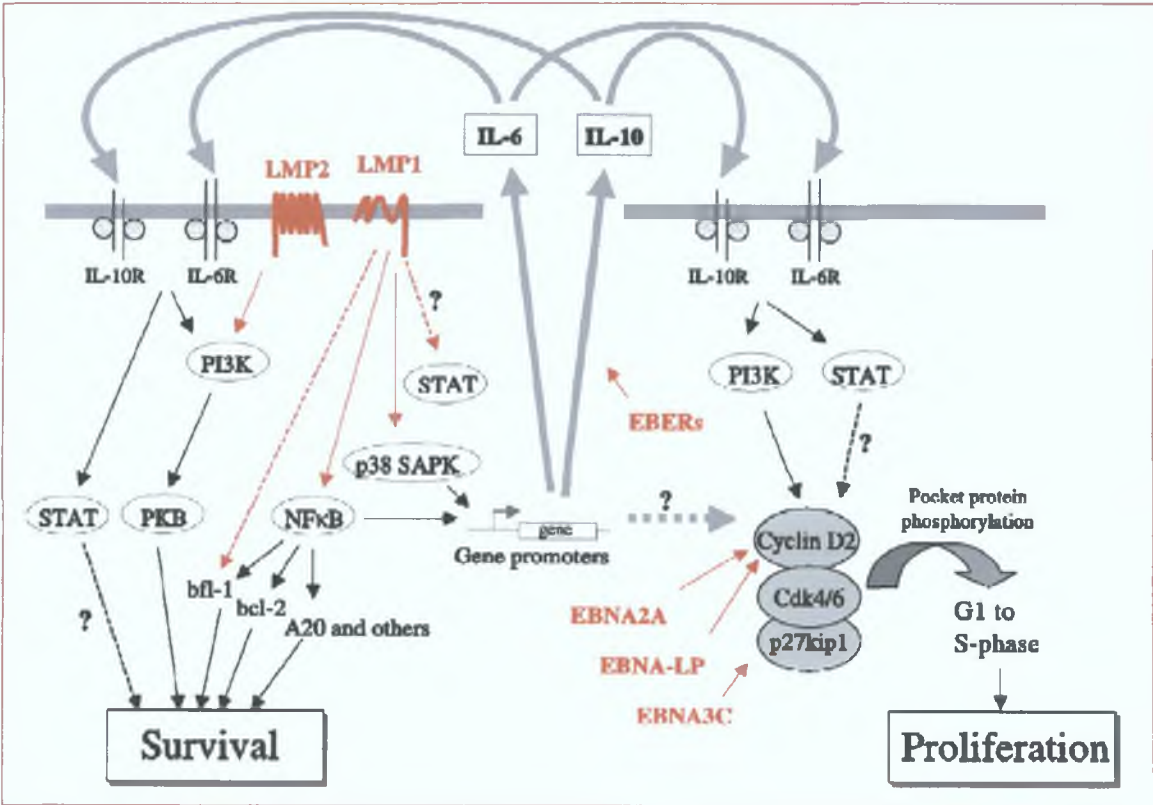


Figure 1.19: Molecular interactions between EBV proteins and cell survival and proliferation machinery. The molecular interactions between EBV proteins and the components of cell survival and proliferation are illustrated. The signalling pathways active in lymphoblastoid cell lines, PI3K and STATs, and some potential contributions of these pathways are illustrated. An outline of the role of LMP1, LMP2A and PI3K to cell survival are also shown (adapted from Brennan, 2001).

1.12 Objectives of the study

The up-regulation of anti-apoptotic proteins by EBV plays a central role in the overall virus strategy, together with the development of malignancy. It has recently been demonstrated in our laboratory that expression of LMP1 in EBV-negative BL-derived cell lines coincides with an increase in *bfl-1* mRNA levels, and that expression of *bfl-1* protected against serum depletion-induced apoptosis (D'Souza *et al.*, 2000). Furthermore, expression of LMP1 in EBV-negative cell lines was found to trans-activate the *bfl-1* promoter in an NF- κ B-dependent manner (D'Souza *et al.*, 2004). In a related study in our laboratory, it was observed that expression of EBNA2 as the sole EBV protein in an EBV-negative BL-derived cell line (DG75-tTA-EBNA2, Floettmann *et al.*, 1996) also led to an increase in *bfl-1* mRNA levels (Pegman *et al.*, submitted December, 2004), demonstrating for the first time that a second EBV protein activates transcription of the *bfl-1* gene. EBNA2 expression was subsequently found to trans-activate the *bfl-1* promoter in EBV-negative BL-derived cell lines, and the ability of EBNA2 to bind RBP-J κ /CBF1 is central for this effect (Pegman *et al.*, submitted December, 2004).

The induction of *bfl-1* by EBV in B cells has implications for the biology of EBV, as EBV-mediated up-regulation of this gene may contribute to the survival of EBV-infected B cells, since cells similar to LCLs are present in the circulation and during primary infection by the virus (Qu and Rowe, 1992; Tierney *et al.*, 1994). Additionally, the effect of *bfl-1* may contribute to the development of EBV-associated B cell malignancies, such as post-transplant lymphoproliferative disorders and BL tumours. Mechanistic studies of the contribution of *bfl-1* to cell survival will provide important information about both normal B cell development and potential routes to B cell malignancy. The main objectives of the current study were to further investigate the molecular basis of *bfl-1* regulation by LMP1 and EBNA2 when expressed individually in BL-derived cell lines and when present together in EBV-infected cell lines. In summary, the following conclusions may be drawn from the experiments below:

- NF- κ B composed of p65 subunits trans-activates the *bfl-1* promoter in the EBV-negative BL-derived cell line DG75, and an NF- κ B-like binding site at position -52 to -43 relative to the transcription start site is essential for this effect.

- EBNA2 trans-activates the *bfl-1* promoter in DG75 cells and the DNA binding protein RBP-J κ /CBF1 is essential for this effect.
- EBNA2-mediated trans-activation of *bfl-1* in DG75 cells does not lead to the activation of NF- κ B, and therefore differs from LMP1-mediated trans-activation.
- The cellular functional equivalent of EBNA2, NotchIC, does not trans-activate the *bfl-1* promoter in DG75 cells, and this is not due to the fact that the NotchIC protein has a weaker trans-activation domain than that of EBNA2.
- An EBNA2 mutant, with a trans-activation domain deletion, significantly inhibits wild type EBNA2-mediated trans-activation of both the *LMP1* and *bfl-1* promoters in DG75 cells
- Blocking either LMP1- or EBNA2-mediated signalling alone in the EBV-infected cell lines Ag876, IB4 or EREB 2.5 does not lead to a decrease in *bfl-1* promoter activity.
- Blocking both LMP1- and EBNA2-mediated signalling simultaneously in the EBV-infected cell lines Ag876, IB4 and EREB 2.5 leads to significant inhibition in *bfl-1* activity.
- Induction of EBNA2 in a panel of estrogen-responsive EBNA2 (EREB) cell lines, and the resulting LMP1 expression, leads to a transient increase in the level of *bfl-1* mRNA.
- In the absence of functional EBNA2 and LMP1, *bfl-1* mRNA levels significantly decrease in the panel of EREB cell lines.
- LMP1 maintains elevated *bfl-1* mRNA levels in the absence of functional EBNA2 in the panel of EREB cell lines.

- Induction of functional EBNA2 alone in the panel of EREB cell lines leads to an increase in the level of *bfl-1* mRNA.

CHAPTER 2

Materials & Methods

2.1 BIOLOGICAL MATERIALS

2.1.1 Cell lines

Table 2.1: Cell lines used in the study

Cell Lines	EBV Status	Cell Classification	Description
Ag876	+	Type III BL	Type III BL cell line expressing all of the EBV latent genes.
DG75	-	EBV negative BL	Lymphoid B cell line derived from an Israeli Burkitt-like lymphoma case (Ben-Bassat <i>et al.</i> , 1977).
EREB 2.5	+	LCL	An LCL established by co-infecting B-cells with a 28 kb mini-EBV plasmid that only expresses estrogen-responsive EBNA2, together with the EBV P3HR1 strain in which it's own EBNA2 gene is deleted. (Kempkes <i>et al.</i> , 1995b).
EREB 2.5 pHEBo	+	LCL	EREB 2.5 cells stably transfected with an episomal oriP vector, pHEBo, expressing hygromycin phosphotransferase (Zimber-Strobl <i>et al.</i> , 1996).
EREB 2.5 SV LMP clone 2C	+	LCL	EREB 2.5 cell line stably transfected with an oriP vector expressing LMP1 from the SV40 early promoter/enhancer and hygromycin phosphotransferase (Zimber-Strobl <i>et al.</i> , 1996).

EREB 2.5 SV LMP clone 11C	+	LCL	A second EREB 2.5 cell line stably transfected with an oriP vector expressing LMP1 from the SV40 early promoter/enhancer and hygromycin phosphotransferase (Zimber-Strobl <i>et al.</i> , 1996).
EREB 2.5 SV LMP Mut2	+	LCL	EREB 2.5 cells stably transfected with an oriP vector expressing a truncated non-functional LMP1 and hygromycin phosphotransferase (Zimber-Strobl <i>et al.</i> , 1996).
EREB 2.5 Tet LMP 3A	+	LCL	EREB 2.5 cells stably transfected with a vector expressing tetracycline-regulatable LMP1.
IB4	+	LCL	An LCL established by infection of umbilical cord blood lymphocytes with the EBV strain B95.8 (King <i>et al.</i> , 1980).

The Ag876, DG75 and IB4 cell lines were obtained from Professor Martin Rowe, University of Wales, Cardiff, Wales. The estrogen-responsive cell lines EREB 2.5 and EREB 2.5 derivatives were gifts from Dr. Ursula Zimmer-Strobl, GSF-National Research Centre for Environment and Health, Munich, Germany.

2.1.2 Antibodies used in the study

Table 2.2: Antibodies used in the study

Antibody	Name	Description	Supplier
Goat anti-mouse IgG, Alkaline Phosphatase (AP) Conjugate	Anti-mouse IgG, AP conjugate (S3721)	An affinity purified goat anti-mouse antibody that reacts with all mouse IgG, and is conjugated to alkaline phosphatase.	Promega
Mouse anti-EBNA2	PE2	An affinity purified monoclonal antibody that reacts with the latent gene product EBNA2.	Professor Martin Rowe, University of Cardiff, Wales
Mouse anti-LMP1	CS1-4	An affinity purified monoclonal antibody that reacts with EBV-encoded latent gene product LMP1.	Professor Martin Rowe, University of Cardiff, Wales

2.1.3 Bacterial strains

E.coli JM109

endA1, recA1, gyrA96, thi, hsdR17 (r_k^- , m_k^+), *relA1, supE44, λ-*, $\Delta(lac-proAB)$, [F', *traD36, proA + B +, lacI*^q ZΔM15]

2.1.4 Plasmids

Table 2.3: Expression and reporter constructs used in the study

Plasmid	Source	Description
pSG5	Lindsey Spender, Ludwig Institute for Cancer Research, Imperial College School of Medicine, London.	pSG5-EBNA2 expresses the wild
pSG5-EBNA2		type B95.8 EBNA2 gene which has been cloned into pSG5 (Stratagene).
pSG5-EBNA2-WW323SR	Professor Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.	This plasmid is identical to pSG5- EBNA2 except that EBNA2 has had two tryptophan residues mutated to serine and arginine residues at positions 323 and 324 respectively. Thus pSG5-EBNA2 does not bind RBP-J κ /CBF1 (Ling <i>et al.</i> , 1993).
EBNA2 Mut (447-472)	Professor Martin Rowe, University of Wales, College of Medicine, Cardiff, UK.	Expression vector for an EBNA2 mutant protein with a trans- activation domain deletion (aa 447-472) in vector pJEF34 (pJEF4 with a β -globin intron upstream of the MCS).
EBNA2 Mut (321-323)	Evelyne Manet, INSERM, Saint Louis Hospital, Paris, France.	This is an expression vector for EBNA2 that contains a deletion from aa 321-323 sub-cloned into pSG5 (Waltzer <i>et al.</i> , 1995).
EBNA2 Mut (437-477)	Evelyne Manet, INSERM, Saint Louis Hospital, Paris, France.	This is an expression vector for EBNA2 that contains a deletion from aa 437-477 sub-cloned into pSG5 (Waltzer <i>et al.</i> , 1995).

pSG5-HA-mNotch1IC (1751-2294)	Professor Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.	Expression vector for mouse Notch1 IC (1751-2294) inserted after the Gal4 DNA binding domain of a modified pSG5 vector containing the hemagglutinin (HA) epitope at the 5' translation start site (Hsieh <i>et al.</i> , 1996).
pSG5-HA-mNotch1IC (1751-2294)-E2TANLS	Professor Diane Hayward, Johns Hopkins School of Medicine, Baltimore, Maryland 21231, USA.	A modified pSG5 vector containing the HA epitope at the 5' translation start site, followed by mouse Notch1IC and then sequences containing the EBNA2 trans-activation domain and nuclear localisation signal (Hsieh <i>et al.</i> , 1996).
pEFBOSneo pEFBOSneo-R218H	Professor Kenji Tanigaki, Department of Medical Chemistry, Kyoto University, Kyoto, Japan.	pEFBOSneo-R218H expresses a mutant RBP-J κ /CBF1 where an arginine residue at position 218 has been replaced with a histidine residue (Chung <i>et al.</i> , 1994) cloned into the empty vector pEFBOSneo (Kato <i>et al.</i> , 1997).
pSG5FLAG	Evelyne Manet, INSERM, Saint Louis Hospital, Paris, France.	An expression vector for the FLAG epitope sub-cloned into pSG5 (Stratagene) (Waltzer <i>et al.</i> , 1995).
pSG5FLAG-RBP	Evelyne Manet, INSERM, Saint Louis Hospital, Paris, France.	An expression vector for the full-length human RBP-J κ /CBF1 cDNA, sub-cloned into the pSG5FLAG vector (Waltzer <i>et al.</i> , 1995).

pSG5FLAG-RBP-VP16	Evelyne Manet, INSERM, Saint Louis Hospital, Paris, France.	An expression vector for a fusion protein between the transcriptional activation domain of VP16 and RBP-J κ /CBF1. (Waltzer <i>et al.</i> , 1995).
pSG5-LMP1	Professor Martin Rowe, University of Wales, College of Medicine, Cardiff, UK.	pSG5-LMP1 expresses wild type B95.8 LMP1 which has been cloned in front of the SV40 promoter contained in pSG5 (Stratagene).
pEFCX pEFCX-I κ B α DN	Dr. Peter Brodin, Umea University, Sweden.	pEFCX-I κ B α DN expresses a super-repressor mutant form of I κ B α in which the serine residues at positions 32 and 36 have been replaced with alanines. pEFCX is the empty vector (Liljeholm <i>et al.</i> , 1998).
pcDNA3.1HisC pCMV-p50 pCM.IN-p65(pCMV-p65) pCMV-CCR c-Rel (pCMV-c-Rel)	Celine Gelinas, Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, New Jersey, USA.	Expression vectors for the NF- κ B subunits (p50, p65, pc-Rel) sub-cloned into pCMV (D'Souza <i>et al.</i> , 2004).
pCMV-LacZ	Clontech	pCMV-LacZ contains <i>E.coli</i> β -galactosidase gene under the control of the CMV promoter-enhancer.
-1374/+81-Luc	Dr. Brendan D'Souza, Department of Biological Chemistry, UCLA School of Medicine, California, USA.	-1374/+81-Luc was generated by sub-cloning the <i>bfl-1</i> promoter sequence from a corresponding CAT reporter construct (Zong <i>et al.</i> , 1999) into pGL2-Basic (D'Souza <i>et al.</i> , 2004).

-1374/+81 mκB (-52)	Dr. Brendan D'Souza, Department of Biological Chemistry, UCLA School of Medicine, California, USA.	-1374/+81 mκB (-52) contains a mutation at the NF-κB-like binding site at position -52 to -43 (D'Souza <i>et al.</i> , 2004).
pGa (-129/-34) pGa (-129/-34 mκB)	Dr. Brendan D'Souza, Department of Biological Chemistry, UCLA School of Medicine, California, USA.	These reporter constructs contain <i>bfl-1</i> promoter sequences sub-cloned into the promoterless vector pGa50-7 (D'Souza <i>et al.</i> , 2004).
-1374/+81 (mCBF1)	Dr. Pamela Pegman, School of Biotechnology, DCU, Dublin, Ireland.	A luciferase reporter construct where the putative RBP-Jκ binding site at -243 to -249 on the wt <i>bfl-1</i> promoter has been mutated to an Xba I restriction site (Pegman <i>et al.</i> , submitted December, 2004).
3x enh κB luc	Professor Martin Rowe, University of Wales College of Medicine, Cardiff, U.K.	This reporter construct contains 3 κB elements upstream of a minimal conalbumin promoter linked to the firefly luciferase gene (Floettmann and Rowe, 1997).
pGa50-7 pGa981-6	Dr. Bettina Kempkes, Institute of Clinical Molecular Biology and Tumour Genetics, GSF, Munich, Germany.	The pGa981-6 reporter construct (Minoguchi <i>et al.</i> , 1997) was generated using a 50 bp oligonucleotide harbouring both CBF1 binding sites of the EBV <i>TP1</i> promoter, which was then ligated as a hexamer into plasmid pGa50-7 (Laux <i>et al.</i> , 1994a).

pLLO-luc	Dr. Gerhard Laux, Institute of Clinical Molecular Biology and Tumour Genetics, GSF, Munich, Germany.	This reporter construct contains the <i>LMPI</i> promoter and an upstream regulatory region driving expression of the luciferase gene (Laux <i>et al.</i> , 1994a).
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2.1.5 Oligonucleotides

Table 2.4: Oligonucleotides used in the study

Target	Primer Sequence	Product Size	Annealing Temperature
-367/-40 <i>bfl-1</i> promoter	5'-CGCGGATCCGGTGTATACCTATGAATG-3' 5'-CGCGGATCCTGATACATGGAGGCTGGT-3'	351 bp	60°C
-367/-129 <i>bfl-1</i> promoter	5'-CGCGGATCCGGTGTATACCTATGAATG-3' 5'-CGCGGATCCAAAATGTGAAAGAGGAAA-3'	240 bp	60°C
-367/-230 <i>bfl-1</i> promoter	5'-CGCGGATCCGGTGTATACCTATGAATG-3' 5'-CGCGGATCCGAAATTAGAATCCTTGTA-3'	151 bp	60°C
pGa50-7	5'-CTCAAGGATCTTACCGCTGT-3' (ampicillin forward) 5'-CTTCCAGCGGATAGAATGGC-3' (luciferase reverse)	Variable	60°C
<i>bfl-1</i>	5'-TTCATATTTTGTGCGGAGTTC-3' 5'-AGCATTTACAGATCTTTCCT-3'	150 bp	55°C
<i>gapdh</i>	5'-TGCACCACCAACTGCTTA-3' 5'-GATGATGTTCTGGAGAGC-3'	168 bp	55°C

All oligonucleotides were obtained from MWG Biotech.

2.2 CHEMICAL MATERIALS

BDH

Bromophenol blue (44385), EDTA (280254D), Glycine (444495D), Hydrochloric acid (28507BF), Isopropanol (296946H), Magnesium sulphate (29117), Methanol (29192BL), Nitrocellulose membrane (436107E), Potassium acetate (295814P), Sodium dihydrogen orthophosphate (30716), Sucrose (102745C), Tris base (271195Y).

Boehringer Mannheim

Calf intestinal phosphatase & 10 X dephosphorylation buffer (713023).

Gibco-BRL/Invitrogen

100 bp DNA ladder (15628-019), Foetal calf serum (10270-106), 1 Kb DNA ladder (15615-016), Restriction enzymes, RPMI 1640 (31870-025), Trypan blue (15250-061), Trypsin (25090-028).

Labscan

Chloroform (A3505E).

Merck

Calcium chloride (23821000), Glacial acetic acid (100632511), Magnesium chloride (1058321000), Potassium Hydroxide (50321000), Sodium carbonate (A654792), di-Sodium hydrogen phosphate (1065860500).

National diagnostics

Acrylagel (EC810), Bis-acrylagel (EC820).

Pierce

BCA Protein assay kit (23227).

Oxoid

Agar (L13), PBS tablets (BR14), Tryptone (L42), Yeast extract (L21).

Promega

dNTPs (U1330), Luciferase Assay System (E1501), Magnesium chloride 25mM (A3511), M-MLV reverse transcriptase & RT buffer (M1701), 5 X Reporter lysis buffer (E3971), RNasin (N2111), Wizard® PCR Preps DNA purification system (A7170).

Qiagen

Qiagen® Plasmid Purification Kit (12143), SYBR Green Kit (204143).

Roche

Taq polymerase & 10 X enzyme buffer (1146173), T4 DNA ligase & 10 X ligation buffer (481220), Hygromycin B (843555), Leupeptin (1017128).

Sigma-Aldrich Chemical Co.

3 M sodium acetate, pH5.2 (S7899), Agarose (A5093), Ampicillin (A9518), Aprotinin (A4529), APS (215589), BCIP-NBT (B1911), Bovine serum albumin (A9647), Chelating resin (C7901), Chloroform:isoamyl alcohol (24:1) (C0549), Cholera toxin (C3012), Colorburst electrophoresis markers (C4105), Coomassie Brilliant Blue R (B0149), DEAE-dextran (D9855), DEPC (D5758), DMEM:F12 Hams (D6421), DMSO (D8779), β -estradiol (E8875), Ethidium bromide (E4391), G418 (A1720), Glucose (G7528), Glycerol (G5516), Horse serum (C7901), Hydrocortisone (H4001), Hydrogen peroxide (H1009), Insulin (I5500), L-glutamine (G7513), Manganese chloride (M3634), MOPS (M3183), ONPG

(N1127), Penicillin/streptomycin (P0781), Phenol:chloroform:isoamyl alcohol (25:24:1) (P3803), PMSF (P7626), Ponceau S (P7170), Potassium chloride (P4504), Puromycin (P8833), RNase A (R6513), Rubidium chloride (R2252), SDS (L6026), Sodium chloride (S3014), Sodium hydroxide (S5881), Tetracycline (T7660), TEMED (T7024), Tri reagent (T9424), Tween 20 (P1379), Xylene cyanol FF (33594).

Upstate biotechnology

Epidermal growth factor (01-102).

2.3 DNA MANIPULATION

2.3.1 Storage of DNA samples

DNA samples were stored in Tris-EDTA (TE) buffer (pH 8.0) at 4°C. EDTA was used to chelate heavy metal ions that are needed for DNase activity while storage at pH 8.0 minimizes de-amidation. DNA was also stored in sterile distilled H₂O (dH₂O).

2.3.2 Phenol/chloroform extraction and ethanol precipitation

Phenol/chloroform extraction and ethanol precipitation were 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 minutes at 13,000 x g. The upper aqueous phase was removed, avoiding any material at the interphase, and placed in a sterile microfuge tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase, vortexed as before, and centrifuged for 5 minutes 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 followed by 2 volumes of 100 % (v/v) ethanol. This mixture was vortexed and incubated at -80°C for 5 minutes. The DNA samples were then centrifuged for 30 minutes 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 minutes at 10,000 x g, the supernatant was removed and pellets were air dried for approximately 10 minutes. Pellets were re-suspended in an appropriate volume of sterile TE (pH 8.0) or dH₂O.

2.3.3 Restriction digestion of DNA

Restriction digestion of DNA was carried out for identification purposes, plasmid linearisation or to cut particular fragments from a plasmid. Restriction enzymes specifically bind and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence, which is known as the recognition site. All restriction enzymes used were supplied with incubation buffers at a concentration of 10 X (working concentration 1 X). DNA digests were performed by addition of the reagents listed below:

200 ng - 1 µg of DNA (Final concentration of <300 ng/µl)

1 µl of enzyme/µg of DNA (10 U).

10 X buffer to a final concentration of 1 X

dH₂O to the final volume required

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

2.3.4 Dephosphorylation of linearised plasmid DNA

To minimize re-circulisation of linearised DNA required for ligation, treatment with calf intestinal phosphatase (CIP) is required. This dimeric glycoprotein prevents re-circulisation by removing the 5' phosphate groups on the linearised DNA molecule.

Digested DNAs (<100 ng/µl) were de-phosphorylated using CIP in a 100 µl volume (CIP was added 1 unit/100 pmoles for cohesive termini). The solution was vortexed, centrifuged briefly and incubated for 30 minutes at 37°C. This was followed by an enzyme denaturation

step achieved by heating to 75°C for 10 minutes. This DNA was purified by phenol/chloroform extraction and ethanol precipitation described in section 2.3.2. The purified linearised, CIP-treated DNA was stored at 4°C until required for ligation.

2.3.5 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) method was used to amplify target sequences of DNA for sub-cloning into linearised vectors, or for identification purposes. PCR involves the amplification of specific DNA sequences using DNA primers, which anneal to the DNA of interest. The primers are designed so that one anneals to the forward DNA strand and the other anneals to the reverse strand thus allowing polymerisation of both strands by the enzyme Taq DNA polymerase. This results in exponential amplification of the sequence of interest. PCR reactions were set up by addition of reagents in the following order:

dH ₂ O	37 µl
Template DNA (~500 ng)	1 µl
Forward primer (40 pmole)	2.5 µl
Reverse primer (40 pmole)	2.5 µl
10 X enzyme buffer (+15mM MgCl ₂)	5 µl
dNTP mix (each at 5 mM)	2 ul
Taq polymerase (5 U/µl)	1 µl

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

<u>First cycle</u>	95°C, 5 minutes	}	1 Cycle
<u>Second cycle</u>	95°C, 3 minutes		
	X°C, 2 minutes	}	30 Cycles
	72°C, 2 minutes		
<u>Final cycle</u>	72°C, 15 minutes	}	1 Cycle

X = Annealing temperature and was usually calculated by subtracting 5°C from the lowest melting temperature (T_m) of a primer pair. Annealing temperatures and primers are listed in Table 2.4.

2.3.6 Purification of PCR products

PCR products were purified from contaminants (including primer-dimers and amplification primers) using the Promega Wizard® PCR Preps DNA Purification System. 100 µl of completed PCR reaction was mixed with 100 µl direct purification buffer (provided with kit) and vortexed briefly. 1 ml of resin (provided with kit) was then added and the mixture was vortexed briefly 3 times over a 1-minute period. A Wizard® Minicolumn was prepared for each PCR product by removing the plunger from a 3 ml disposable syringe and attaching a Minicolumn to the syringe barrel. The resin/DNA mix was transferred into the barrel, the plunger replaced and the slurry was gently pushed into the Minicolumn. The syringe was detached from the Minicolumn and the plunger removed. The syringe barrel was reattached to the Minicolumn and 2 ml of 80 % isopropanol was transferred into the barrel. The plunger was inserted and the isopropanol was gently pushed through. The Minicolumn was detached from the syringe and inserted into a 1.5 ml microfuge tube, and centrifuged at 10,000 x g for 2 minutes to dry the resin. After centrifugation, the Minicolumn was transferred to a new microfuge tube, 50 µl of dH₂O was applied and left for 1 minute. The Minicolumn was then centrifuged for 20 seconds at 10,000 x g to elute the DNA fragment. The purified DNA was stored at 4°C until required for further use.

2.3.7 Ligation of DNA molecules

Cohesive end ligations of equimolar amounts of vector and insert DNA (1 µg) were generally carried out overnight at 16°C in a commercial ligation buffer (5 mM ATP) with 10 units of T4 DNA ligase/ml in a total volume of 10 µl. After ligation, the samples were heated at 70°C for 10 minutes to inactivate the ligase and stored at -20°C until required for transformation.

2.3.8 Preparation of competent cells

A modified Rubidium chloride (RbCl_2) method was employed to prepare competent cells. An *E.coli* strain (JM109) was streaked from a glycerol stock on to a Luria-Bertani (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 broth (Appendix). This culture was incubated at 37°C in a shaking incubator at 200 rpm overnight. 2.5 ml of the resulting culture was then used to inoculate 250 ml of sterile LB broth supplemented with 20 mM MgSO_4 . This 250 ml culture was incubated in a 1 L flask at 37°C until the O.D. of the culture at 640 nm had reached 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 minutes. The resulting pellets were re-suspended in 0.4 original volume ice cold TFB1 (Appendix) (100 ml for 250 ml culture). Cells were kept on ice for all subsequent steps and pipettes, tubes and flasks were chilled. The re-suspended cells were then incubated on ice at 4°C for 5 minutes followed by centrifugation at 4,500 x g at 4°C for 5 minutes. Cells were then gently re-suspended in 1/25 of the original volume of ice-cold TFB2 (Appendix) (10 ml for a 250 ml culture). Cells were divided into 100 µl aliquots and incubated on ice for 1 hour. Prior to storage the aliquoted cells were snap frozen in a dry ice/isopropanol bath. JM109 competent cells prepared by this method and stored at -80°C are stable for 1 year.

2.3.9 Transformations

100 µl of competent cells were placed in a pre-chilled microfuge tube containing 5 µl DNA. The contents were mixed gently and incubated on ice for 30 minutes, during which time an aliquot of SOC medium (Appendix) was pre-heated at 42°C. After 30 minutes on ice the cells were heat-shocked in a water bath at 42°C for 55-65 seconds, followed by incubation on ice for a further 2 minutes. 1 ml of preheated SOC was then added to the cells and incubated at 37°C in a shaking incubator for 1 hour 10 minutes. The cells were pelleted by centrifugation for 3 minutes at 6,000 x g. Following centrifugation, 800 µl of supernatant was removed and discarded. The cells were re-suspended in the remaining supernatant and plated out, along with controls, on LB plates containing the appropriate antibiotic (usually

ampicillin at a concentration of 100 µg/ml) and incubated overnight at 37°C. Transformed cells become resistant to the antibiotic, thus only transformed cells will yield colonies. These colonies were subsequently used to prepare broth cultures for DNA mini-preparations.

2.3.10 Small scale preparation of plasmid DNA (Miniprep)

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 transferred to a sterile microfuge tube and centrifuged for 30 seconds at room temperature; the remainder was stored at 4°C. The supernatant was removed from the tube, leaving the pellet as dry as possible. The pellet was re-suspended thoroughly in 100 µl of solution I (Appendix) by vortexing. 200 µl of freshly prepared solution II (Appendix) was added, the tube contents were mixed by inverting the tube rapidly a number of times. 150 µl of ice-cold solution III (Appendix) was added and the tubes were vortexed gently for 10 seconds. The lysate was centrifuged for 5 minutes 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 minutes at 12,000 x g. The upper aqueous phase was removed to a fresh tube, to which 2 volumes of 100 % (v/v) ethanol were added, the solution was vortexed and centrifuged for 5 minutes 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 re-suspended in 50 µl of TE (pH 8.0). 1 µl of DNase-free RNase A (20 µg/ml) was also added, and the mixture was vortexed, incubated at 37°C for 1 hour and then stored at 4°C. Glycerol stocks of all bacterial cultures were prepared at this stage by the adding 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.

2.3.11 Qiagen® plasmid DNA purification protocol (Maxiprep)

Plasmid DNA was purified using the QIAGEN® Plasmid Midi Kit from Promega. All buffers used are described in the Appendix. A glycerol stock of the bacteria of interest was streaked out on LB agar plate (containing the appropriate antibiotic) and incubated overnight at 37°C. An isolated colony from this plate was used to inoculate a 5 ml starter culture (with the appropriate antibiotic) and incubated in a shaking incubator at 225 rpm 37°C for 8 hours. 100 µl of the starter culture was used to inoculate 50 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 nm. The bacteria culture was transferred to a centrifuge tube and centrifuged by spinning at 6,000 x g for 15 minutes at 4°C using a JA-20 rotor in a Beckman centrifuge. The supernatant was removed and the bacterial pellet was re-suspended completely in 4 ml of Buffer P1 containing RNase A (100 µg/ml). The bacterial cells were then lysed by addition of 4 ml Buffer P2 and incubation at room temperature for 5 minutes. Following incubation, 4 ml of pre-chilled Buffer P3 was added (to precipitate genomic DNA, protein, cell debris and SDS), mixed gently by inverting the tube 5-6 times and incubated on ice for 20 minutes. The mixture was then centrifuged for 1 hour 30 minutes at 16,000 x g at 4°C and the supernatant saved.

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. Following equilibration, the supernatant from the previous centrifugation step was applied to the filter and allowed to flow through. The QIAGEN-tip was washed with 2 x 10 ml of Buffer QC. DNA was then eluted with 5 ml of Buffer QF. DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol, centrifuged immediately at 13,000 x g for 45 minutes 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 minutes and re-dissolved in a suitable volume of TE or dH₂O. DNA was then quantified by spectrophotometric analysis.

2.3.12 Spectrophotometric analysis of nucleic acids

DNA/RNA concentration was determined by measuring the absorbance at 260 nm, which is the wavelength at which nucleic acids absorb maximally (λ_{max}). A 50 $\mu\text{g/ml}$ preparation of pure DNA has an absorbance of 1 unit at 260 nm, while 40 $\mu\text{g/ml}$ 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 λ_{max} for nucleic acids) and at 280 nm (the λ_{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. Absorbances were read on the Shimadzu UV-160A spectrophotometer using a quartz cuvette and nucleic acid concentrations were determined according to the following equations:

$$\text{Concentration of DNA } (\mu\text{g}/\mu\text{l}) = \frac{\text{Absorbance (260 nm)} \times 50 \times \text{dilution factor}}{1000}$$

$$\text{Concentration of RNA } (\mu\text{g}/\mu\text{l}) = \frac{\text{Absorbance (260 nm)} \times 40 \times \text{dilution factor}}{1000}$$

2.3.13 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 was added to 100 ml 1 X TAE buffer based on the percentage agarose gel required. Increasing the percentage agarose (1.8-2 %) in the gel was generally used to improve resolution of smaller DNA fragments while separation of larger DNA molecules was best achieved by using lower percentage gels (0.7-0.8 %). The agarose was completely dissolved by boiling and after sufficient cooling ($\sim 60^{\circ}\text{C}$) the gel was cast into the Hybaid horizontal gel electrophoresis system. A comb was inserted for formation of the wells. The gel was left to set for at least 1 hour before filling the chamber with 1 X TAE and removing the comb. Sample buffer,

containing bromophenol blue as a tracking marker (Appendix), was added to each sample before loading up to 20 µl per well. DNA sample buffer was also added to 500 ng of a 1 Kb or 100 bp ladder, which was loaded as a size marker. The gel was run at constant voltage (5 V/cm), for 1 to 2 hours. After completion, the gel was stained in ethidium bromide (0.5 mg/ml) for 30 minutes, destained in dH₂O for 15 minutes and viewed under UV illumination.

2.4 CELL CULTURE METHODS

All cell culture techniques were performed in a sterile environment using a Holten laminar flow cabinet. Cells were visualized 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 the Appendix. The cell lines DG75, IB4, Ag876, EREB 2.5, and the EREB 2.5 derivatives (pHEBo, SV LMP clone 2C, SV LMP clone 11C, SV LMP Mut 2, Tet LMP clone 3A) were maintained in RPMI 1640 supplemented with 10 % foetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin (Appendix). Additional supplements were added to some culture media as described in section 2.4.2. Cultures were seeded at a density of 2×10^5 to 5×10^5 cells per ml in 25 cm² flasks and expanded in 75 cm² flasks. Cells were sub-cultured two or three times per week by harvesting into a sterile centrifuge tube and pelleting at 1000 x g for 5 minutes at room temperature. The cell pellet was re-suspended gently in an appropriate volume of fresh supplemented media and replaced into the tissue culture flask. All cell lines were incubated in a humid 5 % CO₂ atmosphere at 37°C in a Heraeus cell culture incubator.

2.4.2 Media supplements

Supplements were added to the growth media of certain cell lines to (a) select cells containing transfected plasmids or (b) activate function of a gene or (c) induce expression of a gene of interest.

In the case of the estrogen responsive EREB 2.5 cell line and its derivatives (pHEBo, SV LMP clone 2C, SV LMP clone 11C, SV LMP Mut 2 and Tet LMP clone 3A), the EBNA2 activation domain is fused to the estrogen receptor binding domain, such that the activity of EBNA2 is dependent on the presence of estrogen. These cell lines were maintained in supplemented medium containing 1 μ M estrogen (β estradiol). To abolish functional EBNA2 activity, EREB 2.5 cells were washed 4 times in PBS, with a further 2 washes 24 hours later. Hygromycin was added to the EREB 2.5 derivatives to select for stably transfected cells, at the following concentrations:

pHEBo	150 μ g/ml hygromycin
SV LMP clone 2C	75 μ g/ml hygromycin
SV LMP clone 11C	75 μ g/ml hygromycin
SV LMP Mut 2	75 μ g/ml hygromycin
Tet LMP clone 3A	100 μ g/ml hygromycin

The Tet LMP 3A clone has been transfected with a tetracycline-regulatable construct. LMP1 expression is off in the presence of 1 μ g/ml tetracycline and can be switched on by removing tetracycline from the culture medium.

2.4.3 Cell counts

Cell counts were performed using an improved Neubauer haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viability. 10 μ l of trypan blue was added to 90 μ l 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 (of 3 individual counts) by the dilution factor (usually 1.1) and again by the volume of the haemocytometer chamber (1×10^4 cells/ml). Thus, cell counts were expressed as the number of cells per ml.

2.4.4 Cell storage and recovery

In order to prepare stocks of suspension cells for long-term storage, 1×10^7 cells in exponential phase were pelleted and re-suspended in 750 μ l of supplemented RPMI to which 150 μ l of FBS was added, then placed on ice for 10 minutes. DMSO was added to a final concentration of 10 % (v/v), mixed gently and transferred 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 from liquid nitrogen by thawing rapidly at 37°C and transferring to a sterile centrifuge tube containing 5 ml of pre-warmed supplemented media. The cells were centrifuged at 1000 x g for 5 minutes, the pellet was re-suspended in 5-10 ml of fresh supplemented medium, transferred to a culture flask and incubated at 37°C in 5 % CO₂.

2.4.5 Transient transfections

Transient transfection of cells was performed either by electroporation or the DEAE-dextran protocol. In all cases, cells were seeded at a density of 5×10^5 per ml of medium 24 hours prior to transfection. After 24 hours 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, allowing for optimal DNA uptake during transfection. The same quantity of total DNA was used per transfection.

2.4.5.1 Electroporation of B lymphocytes

Transfection of IB4, Ag876 and the EREB 2.5 cell line was carried out by electroporation. During the electroporation method of transfection, the application of brief high voltage electric pulses to the cells leads to the formation of nanometer-sized pores in the plasma membrane. DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of redistribution of membrane components that accompanies the closure of the pores.

Total DNA (10-20 µg per transfection) dissolved in 30 µl TE buffer (pH 8.0) was transferred into a sterile electroporation cuvette. 1×10^7 cells were used per transfection. Cells were centrifuged at $1,000 \times g$ for 5 minutes and the supernatant discarded. The cells were then washed in PBS, centrifuged at $1,000 \times g$ for 5 minutes and the supernatant again discarded. For each 1×10^7 cells, 220 µl of fresh medium was used for re-suspension and the cells transferred to the DNA-containing electroporation cuvettes. The cell/DNA mix was incubated at RT for 5 minutes. Each cell/DNA mix was then pulsed at 250 V with a capacitance of 960 µF (using a capacitance extender) and resistance set at infinity in a Biorad Gene Pulser. Immediately after electroporation the cuvettes were placed on ice until the cell/DNA mix was transferred into 5 ml supplemented medium in a 6 well dish. Transfected cells were harvested 48 hours later.

2.4.5.2 DEAE-Dextran-mediated transfection

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

A total of up to 25 µg of DNA dissolved in dH₂O was used per transfection. Total DNA was prepared in a sterile microfuge tube and brought to a volume of 600 µl with TBS

(Appendix). Then, 600 μ l of 1 mg/ml DEAE dextran solution made up in TBS was mixed with the DNA solution.

1.4×10^7 cells were used for each transfection. On the day of transfection, cells were centrifuged at 1,000 x g for 5 minutes and the supernatant was discarded. The cells were washed in PBS, centrifuged at 1,000 x g for 5 minutes and the supernatant again discarded. The cell pellet was gently re-suspended in the DNA/DEAE-dextran mix. The transfection cocktails were incubated at room temperature for 30 minutes with gentle swirling every 5-10 minutes to allow homogenization. Transfections were terminated by adding 10 ml supplemented medium, and the cells pelleted by centrifugation at 1,000 x g for 5 minutes. The cells were then washed in PBS as before and transferred to 25 cm² cell culture flasks containing 10 ml supplemented medium for incubation. Cells were harvested at 48 hours post-transfection.

2.4.5.3 Harvesting cells post-transfection

Cells were pelleted by centrifugation at 1,000 x g for 5 minutes at room temperature. The cells were washed once in sterile PBS and centrifuged again at 1,000 x g for 5 minutes. The supernatant was discarded, insuring all traces of PBS were removed from the pellet. The pellet was re-suspended in 50 μ l of 1 X reporter lysis buffer (diluted with water from a 5 X stock, Promega) and the cell suspension transferred to a microfuge tube. The tubes were vortexed for 10-15 seconds. The lysates were clarified by centrifugation at 13,000 x g for 5 minutes and the supernatant saved in a fresh tube. Samples were stored at -80°C.

2.4.5.4 Luciferase assay

During transfections, promoter activity was determined by means of the luciferase assay. Firefly luciferase, a monomeric 61 KDa protein, catalyses luciferin oxidation using ATP-Mg⁺⁺ as a co-substrate. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. In the

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

Luciferase assay reagent was prepared by reconstituting luciferase assay substrate with luciferase assay buffer (Promega) and stored in aliquots in the dark at -80°C. At the time of assay, it was important to allow sufficient time for the luciferase detection reagent to equilibrate to room temperature. 20 µl aliquots of cell lysate were dispensed into individual wells in a white 96 well plate, along with 20 µl of 1 X lysis buffer to act as a blank. Subsequently, 100 µl of detection reagent was added to the lysate to initiate enzyme activity, mixed by repetitive pipetting (3 times) and light emission integrated over a period of 60 seconds, after lag period of 10 seconds, was measured on a luminometer (Labsystems Luminoskan 391A). Luciferase activity levels were adjusted for transfection efficiencies, estimated using β -galactosidase assay.

2.4.5.5 β -galactosidase assay

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

The β -galactosidase assay was performed using a sample from the same lysates used during the luciferase activity. Cell extract (30 µl) was added to 3 µl 100 X Mg solution, 66 µl 1 X ONPG and 201 µl 0.1 M sodium phosphate (Appendix) and incubated at 37°C for 30 minutes or until a faint yellow colour developed. This yellow colour development is the result of *o*-nitrophenyl- β -D-galactopuranside (ONPG) hydrolysis by β -galactosidase to

form *o*-nitrophenyl. A reaction tube was included containing 1 X lysis buffer instead of cell lysate in order to obtain a background reading. Reactions were terminated by adding 500 μ l 1 M Na₂CO₃. Optical densities were read at 420 nm over a linear range of 0.2-0.8.

2.5 RNA ANALYSIS

2.5.1 RNase-free environment

Because RNA is easily degraded by ubiquitous RNases, a number of measures were employed to avoid this potential hazard. All glassware and metal spatulas were baked prior to use at 80°C for 16 - 24 hours in order to inactivate any RNases. Sterile disposable plastic-ware is generally considered RNase-free and therefore, did not require treatment. RNases are resistant to autoclaving but can be inactivated by the chemical diethylpyrocarbonate (DEPC). DEPC-treatment involves addition of the chemical to solutions at a final concentration of 0.1 % (v/v), incubation at room temperature for 18 hours followed by autoclaving. Solutions containing amines such as Tris cannot be DEPC-treated, as the DEPC is inactivated by these chemicals. Solutions containing amines were prepared using DEPC-treated dH₂O followed by autoclaving.

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 H₂O 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 upH₂O and allowed to dry. Because hands are a major source of RNase contamination, gloves were used at all times and changed frequently.

2.5.2 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 Tri reagent (Sigma). Cells grown in suspension were pelleted at $1,000 \times g$ for 5 minutes, washed once in PBS and the cell pellet lysed by repeat pipetting in Tri Reagent. 1 ml of Tri reagent was used per 1×10^7 cultured cells. The lysate was left at room temperature for five minutes after which the procedure may be halted by storing samples at -80°C .

Phase separation was achieved by adding 200 μl of chloroform per 1 ml of lysate. The samples were covered and shaken gently but thoroughly for 15 seconds or until completely emulsified. Samples were incubated at room temperature for 15 minutes. The resulting mixture was centrifuged at $13,000 \times g$ for 20 minutes at 4°C . During centrifugation the mixture separates into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase, which contains the RNA, was removed to a fresh tube and RNA was precipitated by adding 500 μl of ice-cold isopropanol per ml of Tri reagent used initially. The samples were incubated for 10 minutes on ice, then centrifuged at $13,000 \times g$ for 15 minutes 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 centrifuged at $13,000 \times g$ for 5 minutes at 4°C , and the 75 % (v/v) ethanol was removed. Pellets were air dried and dissolved in DEPC treated H_2O . The resulting RNA preparation was heated at 60°C and mixed gently to ensure a homogeneous solution prior to aliquoting. An aliquot was removed for spectrophotometric (section 2.3.12) and gel electrophoretic analysis (section 2.5.3).

2.5.3 RNA analysis by gel electrophoresis

In order to ascertain the integrity of RNA, isolated samples were run on 1.5 % (w/v) agarose gels. The appropriate amount of agarose was dissolved in DEPC-treated H_2O and prepared according to section 2.3.13. The RNA samples (1 μl) were prepared for electrophoresis by adding 3 μl of RNA sample buffer and made up to 15 μl in DEPC-treated H_2O . The samples were heated to 65°C for 10 minutes prior to loading on the gel. The gel was run in 1 X TAE as described in section 2.3.13. As ethidium bromide is included in the RNA sample buffer the gels did not require further staining and could be visualized directly on a UV trans-illuminator. The presence of two strongly stained 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.

2.5.4 Quantification of mRNA by reverse transcription real time PCR

The quantification of mRNA was performed in a two-step procedure. In the first step, cDNA was prepared from RNA by reverse transcription with random hexamers serving as primers. During the second step, cDNA was amplified by real time PCR. Real-time PCR is increasingly being adopted for RNA quantification based on its ability to detect the amount of PCR product present at every cycle (i.e. in real time), as opposed to the endpoint detection by conventional PCR methods, thus allowing the real-time progress of the reaction, especially its exponential phase, to be viewed. The real-time PCR approach is based on the detection and quantification of a fluorescent reporter, where the signal increases in direct proportion to the amount of PCR product in a reaction. SYBR green was the fluorescent reporter employed. SYBR green binds the double stranded PCR product in a sequence independent manner and will not bind single stranded DNA (i.e. primers). The real-time system was used for comparative gene expression analysis, normalising with GAPDH levels.

2.5.4.1 Reverse transcription (RT)

This is the process whereby mRNA is transcribed into cDNA using a reverse transcriptase, in this case Moloney Murine Leukemia Virus reverse Transcriptase (M-MLV RT). Initially, 2 µl of random hexamers was added to 2 µg RNA and the volume brought up to 10 µl with DEPC H₂O. The mixture was heated to 70°C for 5 minutes, to destabilize secondary mRNA structures, and then placed on ice. Then, the reagents listed below were added in the following order:

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

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

2.5.4.2 Real time PCR

cDNA generated by reverse transcription was quantified by real time PCR using the QuantiTect® SYBR® Green PCR kit (Qiagen). The kit provided a SYBR® Green master mix that contained Taq polymerase and dNTPs. Real time PCR reactions were set up as follows:

cDNA	2 µl
SYBR Green	12.5 µl
Nuclease Free H ₂ O	8.5 µl
Forward primer	1 µl
Reverse primer	1 µl

Samples were quantified using the Rotor Gene™ 3000 multiplex system (Corbett Research) under the following thermo-cycling conditions:

<u>First cycle (denature)</u>	95°C for 15 minutes	} 1 cycle
<u>Second cycle (cycling)</u>	95°C for 20 seconds 55°C for 30 seconds 72°C for 20 seconds	} 50 cycles
<u>Third cycle (hold)</u>	60°C for 1 minute	} 1 cycle
<u>Final cycle (melt)</u>	50-99°C rising by 1°C each step, waiting for 15 seconds on first step, then 5 seconds for each step afterwards.	

2.6 PROTEIN ANALYSIS

2.6.1 Preparation of cellular protein

Proteins were isolated from suspension cells for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and by western blotting. Prior to isolation, cells were washed in PBS. Cells were pelleted at 1,000 x g for 5 minutes and the supernatant removed. 10 ml of ice-cold PBS was added, the cells were centrifuged again at 1,000 x g and all of the supernatant removed. The cell pellet was re-suspended in ice-cold suspension buffer (Appendix) using 200 µl of suspension buffer for every 1×10^7 cells and the cell suspension transferred to a microfuge tube. An equal volume of 2 X SDS gel loading buffer (Appendix) 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 minutes and then subjected to sonication for 1 minute on full power to shear the DNA. The lysate was clarified by centrifugation at 12,000 x g for 10 minutes at room temperature. The supernatant was aliquoted and stored at -20°C until required for analysis.

2.6.2 Estimation of protein concentration

In order to standardize the amount of protein used in SDS-PAGE, the concentration of protein in the cell extracts was determined using the BCA (bicinchoninic acid) protein assay kit. In this assay, Cu^{++} reacts with the protein under alkaline conditions to give Cu^+ , which in turn reacts with BCA to give a coloured product. Two separate reagents were supplied in the commercially available kit (Pierce Chemicals): an alkaline bicarbonate solution (reagent A) and a copper sulphate solution (reagent B). Working solution was prepared by mixing 1 part reagent B with 50 parts reagent A. 200 μl of working solution was added to 10 μl of protein standard or unknown protein sample in wells of a microtitre plate. The plate was incubated at 37°C for 30 minutes in the dark. The absorbance of each well was read at 560 nm using a microtitre plate reader (Rosys Anthos 2010). Protein concentrations were determined from a bovine serum albumin (BSA) standard curve in the 0-2 mg/ml range. Standards were prepared as indicated in Table 2.5.

Table 2.5: Preparation of BSA standard solutions for BCA assay

Concentration of Standard ($\mu\text{g/ml}$)	Volume of 2 mg/ml BSA Stock Solution (μl)	Volume of dH_2O (μl)	Total Volume (μl)
2000	100	0	100
1800	90	10	100
1600	80	20	100
1400	70	30	100
1200	60	40	100
1000	50	50	100
800	40	60	100
600	30	70	100
400	20	80	100
200	10	90	100
0	0	100	100

2.6.3 SDS-polyacrylamide gel electrophoresis of proteins

During polyacrylamide gel electrophoresis (PAGE), proteins are driven through a gel composed of polyacrylamide that is cross-linked to form a molecular sieve, by an applied current. PAGE is usually carried out in the presence of the negatively charged detergent sodium dodecylsulphate (SDS), which binds in large numbers to all types of protein molecules. The electrostatic repulsion between the bound SDS molecules causes the proteins to unfold into a similar rod-like shape, thus eliminating differences in shape as a factor in separation. As the amount of SDS bound is proportional to the molecular weight of the polypeptide and is sequence independent, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

2.6.3.1 Preparation of SDS-polyacrylamide gels

SDS-PAGE was performed using 10 % polyacrylamide gels. The 10 % resolving gels and 5 % stacking gels were prepared as follows:

Resolving Gel		Stacking Gel	
<u>10 ml</u>	<u>10 % (ml)</u>	<u>2.5 ml</u>	<u>5 % (ml)</u>
acrylagel	3.33	acrylagel	0.42
bis-acrylagel	1.35	bis-acrylagel	0.168
1.5 M Tris (pH 8.8)	2.5	1 M Tris (pH 6.8)	0.312
dH ₂ O	2.61	dH ₂ O	1.5475
10 % (v/v) SDS	0.10	10 % (v/v) SDS	0.025
10 % (v/v) APS	0.10	10 % (v/v) APS	0.025
TEMED	0.01	TEMED	0.0025

An ATTO protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water, then with dH₂O and finally wiped in one direction with tissue soaked in 100 (v/v) ethanol. The gasket was placed around the ridged plate, the plates were assembled and secured with clamps. The 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 that had been wiped in 100 % ethanol was inserted and the gel was allowed to polymerise for at least 45 minutes. The electrophoresis tank was filled with 1 X Tris-glycine running buffer to a level of about 5 cm deep. After polymerisation, the gaskets and clamps were removed and the pre-poured gels were lowered into the buffer at an angle to exclude air bubbles from the gel buffer interface. The tank was completely filled with 1 X running buffer and the comb removed from the gel. Un-polymerised gel was removed by gently rinsing the wells with 1 X running buffer and the wells were then straightened using a loading tip. The gel plates were fixed firmly in place with the pressure platens. The chamber formed by the inner plates (notched plate facing inwards) was filled with 1 X running Buffer, the samples were loaded and the electrodes attached. The gels were electrophoresed at a constant current of 30 mA per gel until the blue dye front had reached the bottom of the gel. When complete, the plates were removed, separated and the gel was either placed in transfer buffer prior to western blotting or stained in Coomassie blue. Staining took place for 30 minutes, agitating constantly. The gel was then placed in destain (see Appendix) with constant agitation, until all background staining was removed. The destain was changed 4 or 5 times at 30 minute intervals, until all background stain was removed from the gel.

2.6.4 Western blotting

During western blotting, electrophoretically separated proteins are transferred from the polyacrylamide gel to a solid support, usually a nitrocellulose membrane, and probed with antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the solid support. The bound antibody is detected by a secondary immunological reagent, in this case a secondary antibody conjugated to the alkaline phosphatase enzyme.

2.6.4.1 Transfer of protein to *nitrocellulose* filters

Following gel electrophoresis, gels were equilibrated in transfer buffer (Appendix) for at least 30 minutes. Equilibration facilitates the removal of electrophoresis salts and detergents. If the salts are not removed, they increase the conductivity of the transfer and the amount of heat generated during transfer. Also, low percentage gels (<12 %) shrink in methanol-containing buffers. Equilibration allows the gel to adjust to its final size prior to electrophoretic transfer. Nitrocellulose membrane was cut to the dimensions of the gel, along with 6 pieces of 3 MM filter paper that were required for the gel/membrane sandwich (Figure 2.1). Protein transfer was carried out on the Bio-Rad Trans-Blot® SD semi-dry electrophoretic transfer cell. A pre-soaked sheet of filter paper was placed onto the platinum anode. A pipette was rolled over the surface of the filter paper to exclude all air bubbles. This step was repeated with two more sheets of filter paper. Then, the pre-wetted blotting membrane was placed on top of the filter paper and all bubbles rolled out. The equilibrated gel was carefully placed on top of the nitrocellulose membrane, aligning the gel on the centre of the membrane. Any air bubbles were again rolled out. 3 more sheets of pre-wetted filter were placed on top of the gel, with care being taken to remove air bubbles. The cathode was placed on top of the stack and the safety cover replaced on the transfer unit. Gels were transferred for 30 minutes at 17 V.

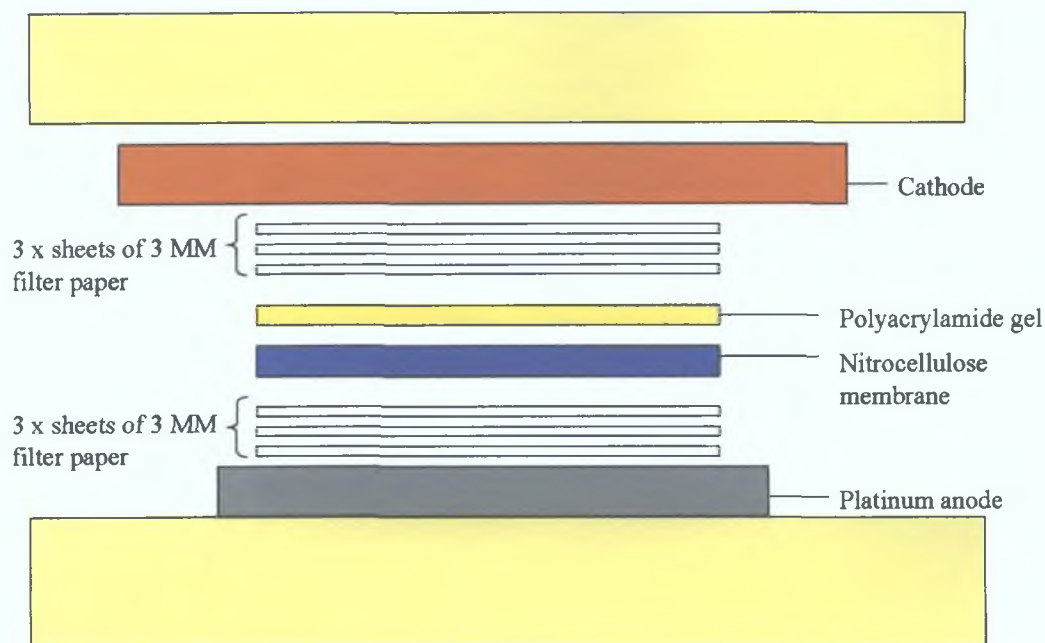


Figure 2.1: Semi-dry transfer of protein from polyacrylamide gel to nitrocellulose membrane.

2.6.4.2 Staining of proteins immobilized on nitrocellulose filters

Ponceau S staining was employed to determine whether uniform transfer of proteins to the nitrocellulose membrane had taken place. Transferred protein can be detected as red bands on a white background. This staining technique is reversible to allow further immunological analysis. Ponceau S is a negative stain, which binds to positively charged amino acid groups of the protein. It also binds non-covalently to non-polar regions in the protein. Following electrophoretic transfer, the nitrocellulose membrane was immersed in 20 ml Ponceau S solution (0.1 % Ponceau S (w/v), 5 % acetic acid (w/v), Sigma) and stained for 5 minutes with constant agitation. After proteins were visualised, the membrane was washed in several changes of dH₂O until all the stain had been washed away. The membrane is then used for immunological probing.

2.6.4.3 Immunological probing

Following Ponceau S staining, the membrane was incubated in blocking buffer (Appendix) for 2 hours at room temperature followed by incubation with the appropriate primary antibody (diluted in blocking buffer, see Table 2.6) 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.

Table 2.6: Incubation Conditions for Antibodies Used in Western Blotting

Primary Antibody	Name	Dilution In Blocking Buffer	Secondary Antibody	Dilution In Blocking Buffer
Anti-EBNA2	PE2	1/1000	AP-Conjugated Anti Mouse IgG	1/5000
Anti -LMP1	CS1-4	1/1000	AP-Conjugated Anti Mouse IgG	1/5000

After overnight incubation, the membrane was washed three times in TBS-T (0.1 % (v/v) Tween-20 in TBS) for 15 minutes. The filter was then incubated with the appropriate secondary antibody for 1 hour 30 minutes at room temperature, followed by three 15-minute washes in TBS-T. All of the above incubations were carried out with constant agitation. Membranes were then placed in a clean container and covered with 5-Bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium (BCIP/NBT, Sigma), which is used for the colourimetric detection of alkaline phosphatase-labeled molecules. This substrate system, when incubated with alkaline phosphatase, produces an insoluble NBT diformazan product that is easily observable with its purple colour. Therefore, proteins bound by antibody conjugated to alkaline phosphatase stain purple. The BCIP/NBT container was placed in the dark at room temperature without shaking for 30 minutes 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.

CHAPTER 3

Regulation of the Anti-Apoptotic Cellular *bfl-1* Gene in the EBV-Negative Burkitt's Lymphoma- Derived Cell Line DG75

3.1 INTRODUCTION

This chapter set out to investigate the regulation of the cellular *bfl-1* gene in the EBV-negative Burkitt's lymphoma (BL)-derived cell line DG75. Indeed, most non-EBV infected continuous B lymphocyte cell lines are derived from sporadically occurring EBV-negative BL and many can be infected with EBV *in vitro* (Calender *et al.*, 1990). The growth of BL cells *in vitro* is attributed to deregulated *c-myc* expression resulting from a specific translocation, in which *c-myc* is juxtaposed to a heavy or light-chain Ig locus (Dallas-Favara *et al.*, 1983), and to less characterised changes on chromosome 1 (Berger and Bernheim, 1985). Since BL cell lines exhibit phenotypic features reminiscent of germinal centre B cells (MacLennan *et al.*, 1988), the EBV-negative DG75 cell line provided a suitable system for determining the contribution of individual EBV genes to the regulation *bfl-1* in the context of the B lymphocyte. The EBV genes under investigation in the study included LMP1 and EBNA2, both of which are key effectors of EBV-mediated transformation.

3.2 RESULTS

3.2.1 NF- κ B plays a role in LMP1-mediated activation of the *bfl-1* promoter

A key role for the transcription factor NF- κ B in the regulation of the anti-apoptotic cellular gene *bfl-1* was established when it was shown that treatment of a variety of cell lines with the chemical agent phorbol-12-myristate 13-acetate (PMA), a well-known activator of NF- κ B, up-regulated *bfl-1* mRNA levels in BL cell lines (Moreb and Schweber, 1997). A study carried out in our laboratory demonstrated that EBV latent membrane protein 1 (LMP1) trans-activated the *bfl-1* promoter in EBV-negative BL-derived cell lines by a mechanism that is dependent upon NF- κ B (D'Souza *et al.* 2004). A previously identified NF- κ B binding site at position -833 to -823 relative to the transcription initiation site of the *bfl-1* promoter was shown to be essential for activation by the NF- κ B sub-unit c-Rel in HeLa cells (Zong *et al.*, 1999). However, experiments performed in our laboratory demonstrated that elimination of this binding site by site directed mutagenesis did not affect the level of LMP1-mediated promoter activity in EBV-negative BL-derived cell lines (D'Souza *et al.* 2004). Subsequently it was found that a 210 bp *bfl-1* promoter fragment (-129/+81-Luc, Figure 3.1A) containing a

deletion from the 5' end of the full-length *bfl-1* promoter (-1374/+81-Luc, Figure 3.1A) mediated NF- κ B-dependent trans-activation by both LMP1 and PMA. Using Transcription Element Search Software, an NF- κ B-like binding site at position -52 to -43 (Figure 3.1A) was identified in the region 5' to the transcription initiation site. When base substitutions were introduced into the core of this binding motif, complete loss of trans-activation by LMP1 was observed, thus demonstrating a direct role for the NF- κ B-like binding site at position -52 to -43 in LMP1-mediated activation of the *bfl-1* promoter (D'Souza *et al.* 2004).

The subunit composition of NF- κ B can greatly influence not only its ability to bind a particular DNA sequence motif, but also the extent of promoter activation (Perkins *et al.* 1992). To directly investigate the ability of Rel family members to drive transcription from the *bfl-1* promoter, transient co-transfections were performed using DG75 cells with vectors that express individual NF- κ B subunit proteins and *bfl-1* promoter reporter constructs (Figure 3.1A). It can be seen that the -129/+81-Luc reporter was trans-activated approximately 2.5-fold when co-transfected with the p65 expression vector (Figure 3.1B) and that trans-activation was completely abolished upon mutation of the NF- κ B-like binding motif at position -52/-43 [Figure 3.1B, -129/+81 (m κ B-52)]. The fact that p65 trans-activated this 210 bp *bfl-1* promoter fragment, which does not contain the -833 to -823 site, indicates that this previously identified NF- κ B binding motif is not essential for p65-associated activation of the promoter in this cell context. Co-transfection with either a p50 or c-Rel expression vector failed to significantly trans-activate the -129/+81-Luc construct (Figure 3.1B). It can also be seen that there was a lack of any synergistic when either p65/p50 or p65/c-Rel expression vector combinations were co-transfected the same reporter construct (Figure 3.1B).

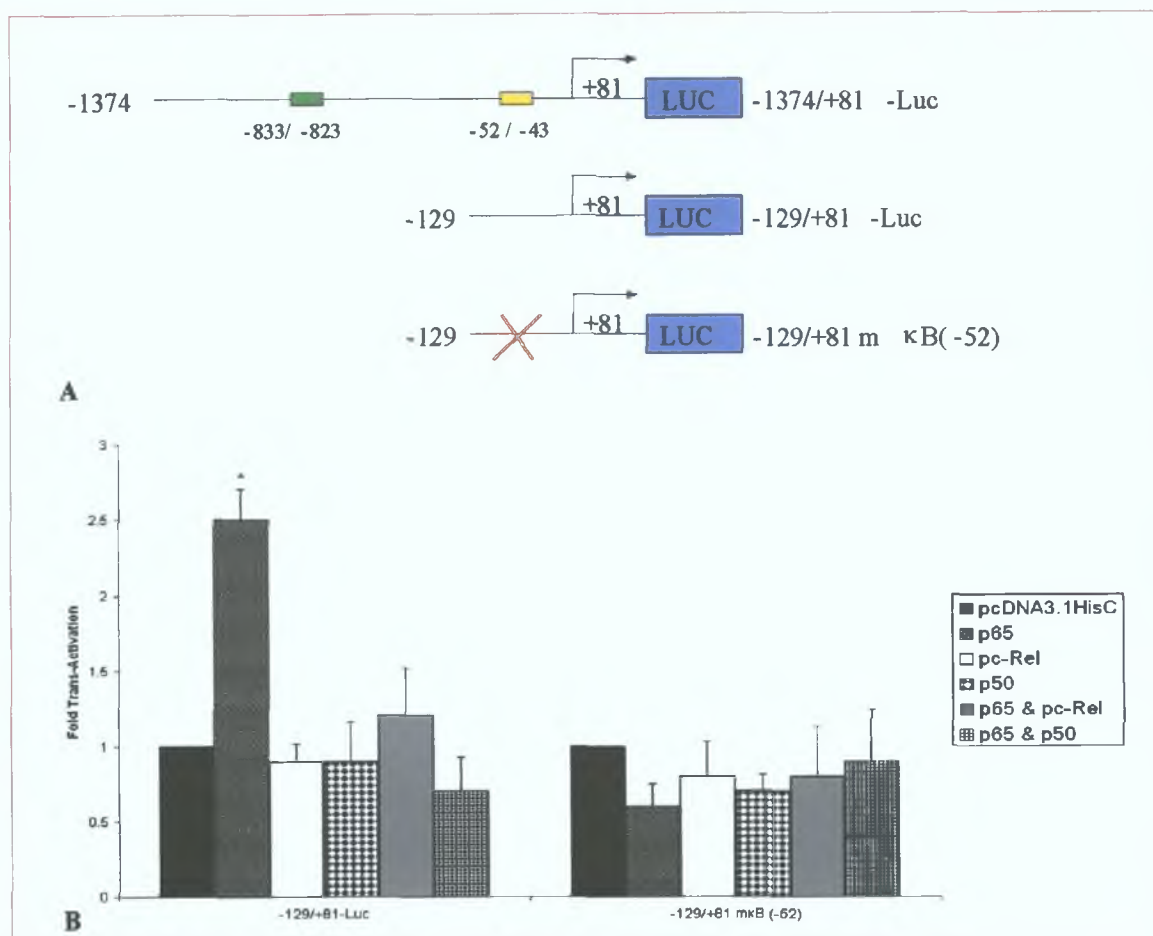


Figure 3.1: The NF- κ B-like binding site at -52 to -43 is essential for conferring p65-responsiveness on the *bfl-1* promoter reporter construct -129/+81-Luc. (A) Schematic representation of the *bfl-1* promoter-reporter constructs. The upper panel shows a 1.4 Kb DNA sequence from the 5' regulatory region of the *bfl-1* gene (from -1374 to +81 relative to the transcription initiation site). The position of a previously identified NF- κ B binding site at position -833 to -823 (Zong *et al.*, 1999) is indicated by the green box and the NF- κ B-like binding site at -52 to -43 is indicated by the yellow box. -129/+81-Luc contains a deletion from the 5' end of the *bfl-1* promoter sequence (co-ordinates of the 5' end are to the left of each construct). Base substitutions were introduced into the core of the NF- κ B-like binding motif at position -52 to -43 (marked with a red X) by site directed mutagenesis to generate the construct -129/+81 m κ B(-52) (Figure 1A, lower panel). They all share a 3' end terminus 81 bp downstream from the transcription initiation site (designated by the bent arrow) at which point they are joined to the luciferase gene (LUC). (B) DG75 cells were transfected with 2 μ g of vector expressing various NF- κ B subunits and 2 μ g of reporter construct indicated underneath the graph. Cells were harvested at 48 hours post-transfection and analysed for luciferase activities, which were then normalised for transfection efficiency (based on β -galactosidase activity measured from co-transfected pCMV-LacZ reporter, which was included in all transfections). Normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for each reporter construct when co-transfected with the empty vector (pcDNA3.1HisC), which was arbitrarily assigned a value of 1.0. Error bars indicate standard deviations of mean values from three independent experiments. This was the case for all subsequent transfection experiments. (* $p < 0.05$).

In order to determine whether this region of the *bfl-1* promoter could confer p65-responsiveness on a heterologous promoter, the -129/-34 portion of the sequence was sub-cloned upstream of the minimal β -globin promoter in the luciferase reporter construct pGa50-7 (D'Souza *et al.*, 2004, Figure 3.2A). Transient co-transfections of these reporter constructs were carried out with DG75 cells, and the results of the experiment are shown in Figure 3.2B. It can be seen that the pGa(-129/-34) reporter was trans-activated approximately 3.0 fold (over pGa50-7) when co-transfected with p65 expression vector and that trans-activation was again completely abolished upon mutation of the NF- κ B-like binding motif at position -52 to -43 (Figure 3.2B, pGa(-129/-34) mkB). The effect of p65 was mediated by the -129/-34 sequence of the *bfl-1* promoter since p65 expression did not affect the corresponding minimal promoter vector, pGa50-7. Co-transfection with either a p50 or c-Rel expression vector failed to significantly trans-activate the pGa(-129/-34) construct (Figure 3.2B). Once again it can be seen that there was a lack of any synergistic when either p65/p50 or p65/c-Rel expression vector combinations were co-transfected the same reporter construct. It may be concluded that the -129/-34 region of the *bfl-1* promoter functions as a p65-dependent transcriptional enhancer and that the NF- κ B-like binding site at position -52 to -43 is essential for this effect.

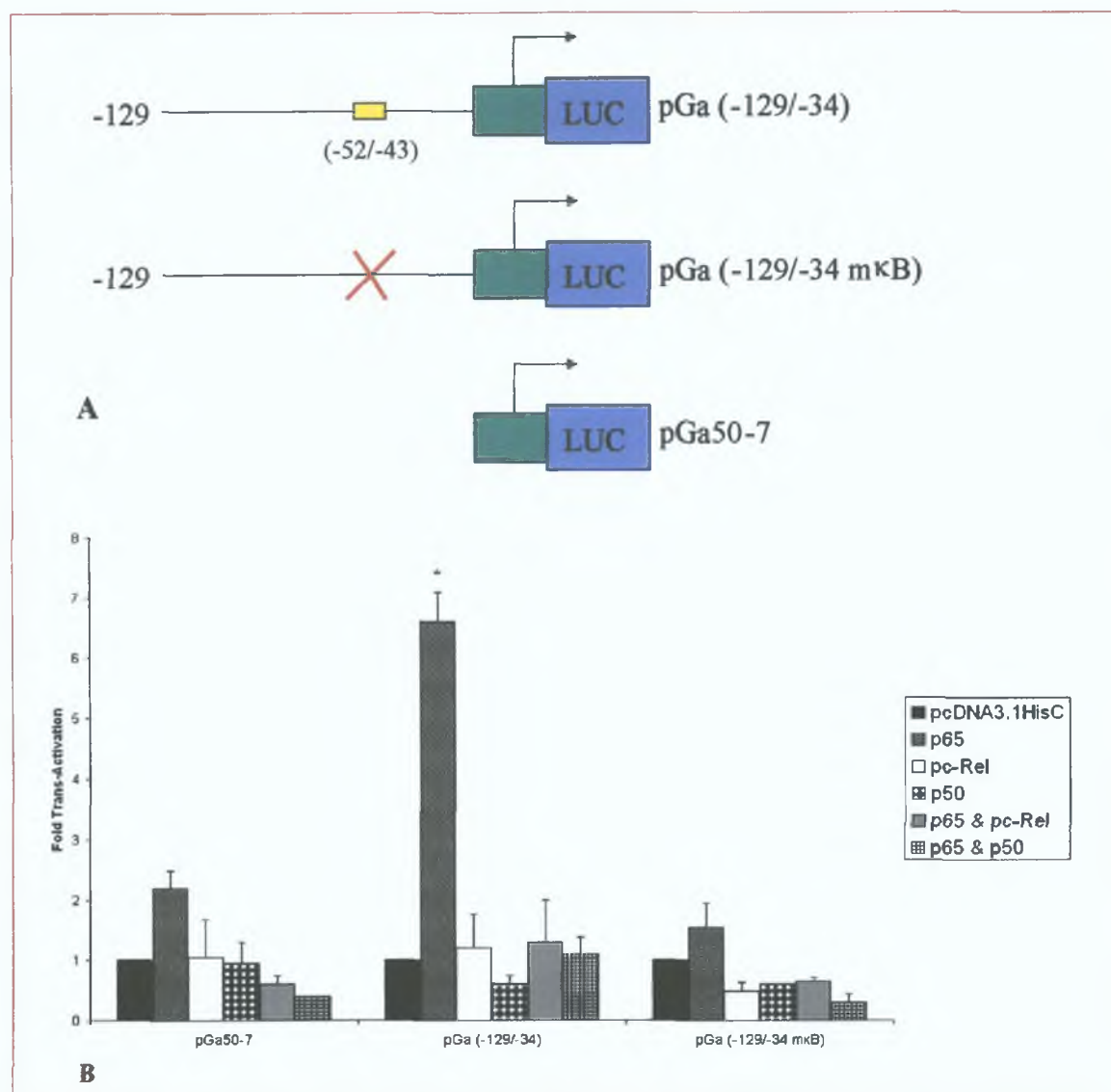


Figure 3.2: The NF-κB-like binding site at -52 to -43 is essential for conferring p65-responsiveness on a heterologous minimal promoter. (A) Schematic representation of luciferase reporter constructs generated after sub-cloning the -129/-34 portions of the *bfl-1* promoter upstream of the β-globin minimal promoter (green box with bent arrow) in pGa50-7. The name of each reporter construct includes the relevant 5' and 3' ends of the *bfl-1* sequence. The mutation introduced at the NF-κB-like site is indicated with a red X. (B) DG75 cells were transfected with 2 μg of vector expressing various NF-κB subunits along with 2 μg of luciferase reporter construct. Cells were harvested at 48 hours post-transfection and assayed for luciferase activity. Normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for each reporter construct when co-transfected with the empty vector (pcDNA3.1HisC). (* *p* < 0.05).

Taken together these experiments showed that p65 directly trans-activates the *bfl-1* promoter and that the NF-κB-like binding site at -52/-43 was essential both for this effect, and also for conferring p65-responsiveness on a heterologous minimal promoter in DG75 cells.

3.2.2 EBNA2-mediated activation of the *bfl-1* promoter is inhibited by co-expression of a dominant-negative mutant form of the Notch signalling pathway protein RBP-J κ /CBF1

It has already been observed in our laboratory that LMP1 up-regulates expression of the anti-apoptotic cellular gene *bfl-1* (D'Souza *et al.*, 2004). In a related study, elevated *bfl-1* mRNA levels were observed in an LMP1-negative derivative of the cell line MUTU-III (a group III/latency III BL-derived cell line) (Gregory *et al.*, 1990) relative to its isogenic group I/latency I counterpart MUTU-I (Pegman *et al.*, submitted December 2004). This prompted us to investigate if other EBV latent proteins were able to modulate the expression of *bfl-1*. As EBNA2 is a transcription factor and a key effector of phenotypic change, its contribution towards regulating the expression of *bcl-2* family members, including *bfl-1*, was firstly assessed by ribonuclease protection assay (RPA) using a tightly regulatable tetracycline-based system to express EBNA2 in the EBV-negative BL cell line DG75 (DG75-tTA-EBNA2) (Floettman *et al.*, 1996). In this experiment, the steady state level of *bfl-1* mRNA increased significantly in response to EBNA2 induction (Figure 3.3), demonstrating for the first time that a second EBV protein, EBNA2, when expressed at physiological levels, activated transcription of the *bfl-1* gene (Pegman *et al.*, submitted December 2004).

Transient transfections of the EBV-negative BL-derived cell lines DG75, BL41 and BJAB showed that co-transfection of the *bfl-1* promoter reporter construct -1374/+81-Luc (D'Souza *et al.*, 2004) with EBNA2 expression vector led to an increase in luciferase activity at 48 hours post-transfection (Pegman *et al.*, submitted December 2004). It is known that EBNA2 does not bind to DNA directly, but is tethered to responsive promoters by a ubiquitously expressed and highly conserved cellular DNA binding repressor protein RBP-J κ /CBF1 (Henkel *et al.*, 1994; Grossmann *et al.*, 1994; Waltzer *et al.*, 1994; Zimmer-Strobl *et al.*, 1994). This protein was characterised and cloned by virtue of its ability to bind the recombination signal of the murine immunoglobulin κ joining region (Matsunami *et al.*, 1989). In the absence of EBNA2, RBP-J κ /CBF1 acts as a transcriptional repressor by recruiting a histone deacetylase (HDAC) co-repressor complex to the promoter (Zimmer-Strobl and Strobl, 2001). EBNA2 has the ability to bind the transcriptional repression domain of RBP-J κ /CBF1, thereby relieving repression by replacing the co-repressor complex with its trans-activation domain. When transfections were carried out replacing EBNA2 with an

EBNA2 mutant that cannot bind RBP-J κ /CBF1 (EBNA2-WW323SR), a major decrease in the trans-activation of the *bfl-1* promoter in DG75 cells was observed (Pegman *et al.*, submitted December 2004). These results indicated that EBNA2 directly trans-activates the *bfl-1* promoter in EBV-negative BL-derived cell lines and that the ability of EBNA2 to bind RBP-J κ /CBF1 is central for this effect.

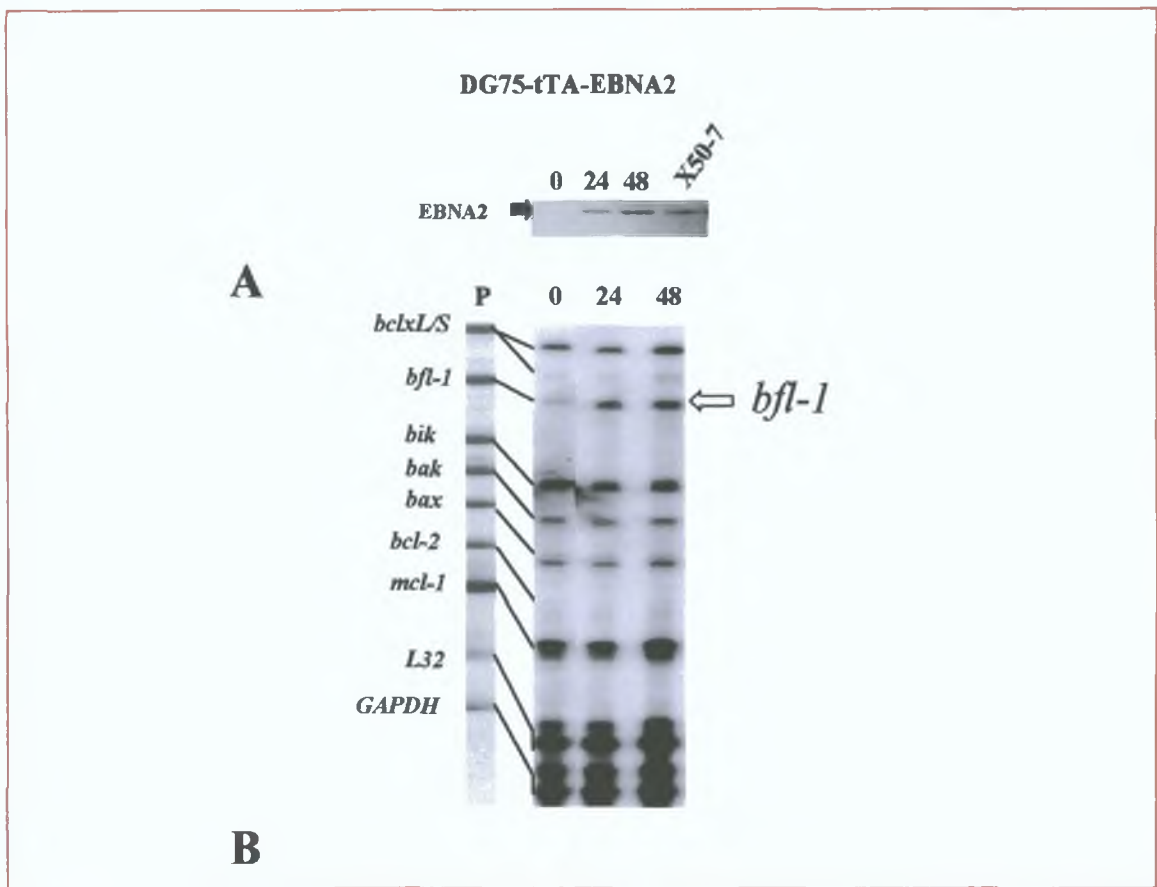


Figure 3.3: Induction of EBNA2 as the sole EBV protein leads to an increase in *bfl-1* mRNA levels in the EBV-Negative BL Cell Line DG75-tTA-EBNA2. EBNA2 expression was induced over a 72-hour time-course in the DG75-tTA-EBNA2 cell line, by the removal of tetracycline from cell culture medium. Total protein and RNA were prepared simultaneously from cells harvested at the indicated time-points post-EBNA2 induction (A) Western blotting was performed to detect EBNA2 in protein samples isolated during the 72-hour time-course. Protein isolated from the X50-7 (LCL) cell line was used as a positive control for EBNA2. (B) mRNA levels from the apoptosis-related genes *bclx-L*, *bclx-S*, *bfl-1*, *bik*, *bak*, *bax*, *bcl-2*, and *mcl-1* were analysed by RPA. Unprotected ^{32}P labelled antisense riboprobes (5000 cpm 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. (adapted from Pegman *et al.*, submitted December 2004).

In a control experiment to further investigate the role of RBP-J κ /CBF1 in EBNA2-responsiveness, co-transfections using DG75 cells were carried out with increasing amounts of an EBNA2 expression vector (pSG5-EBNA2) and the RBP-J κ /CBF1-regulated luciferase reporter construct pGa981-6, which contains a 50 bp oligonucleotide harbouring both RBP-J κ /CBF1 binding sites of the EBV *TP1* promoter sub-cloned as a hexamer into the plasmid pGa50-7 (Minoguchi *et al.*, 1997). EBNA2 trans-activated the RBP-J κ /CBF1-regulated reporter construct in a dose-dependent manner (Figure 3.4), with maximum trans-activation of approximately 4,500-fold observed upon co-transfection with 11 μ g of the effector plasmid pSG5-EBNA2.

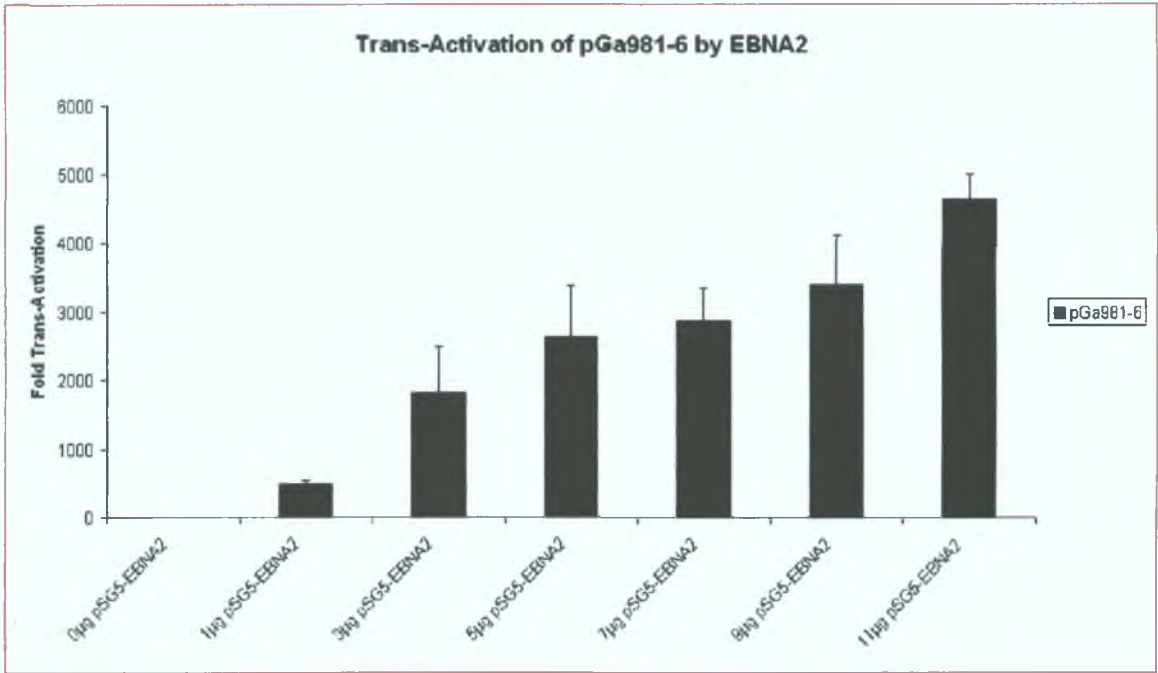


Figure 3.4: EBNA2 trans-activates the RBP-J κ /CBF1-regulated luciferase reporter construct pGa981-6. DG75 cells were co-transfected with increasing amounts of pSG5-EBNA2 together with 2 μ g of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection. Luciferase values were normalised as described earlier and expressed as activation (*n*-fold) relative to the corresponding value obtained for co-transfection with the empty vector (pSG5) and pGa981-6.

Based on the evidence that EBNA2 trans-activated the RBP-J κ /CBF1-regulated promoter pGa981-6, it may be deduced that RBP-J κ /CBF1 is present and functional in the EBV-negative BL-derived DG75 cell line.

The role of RBP-J κ /CBF1 in mediating EBNA2-mediated responsiveness was further investigated using an expression vector for a non-DNA binding mutant of RBP-J κ /CBF1 (pEFBOSneo-R218H, Chung *et al.*, 1994). To investigate if pEFBOSneo-

R218H could inhibit trans-activation of the luciferase reporter construct pGa981-6 by EBNA2, co-transfections were performed in DG75 cells with increasing amounts of pEFBOSneo-R218H and constant quantity of the expression vector for EBNA2. It can be seen from this experiment that expression of the mutant RBP-J κ /CBF1 (pEFBOSneo-R218H) efficiently inhibited EBNA2-mediated trans-activation of pGa981-6 (Figure 3.5). This effect was dose-dependent, with maximum inhibition of approximately 70 % when 12 μ g of pEFBOSneo-R218H was used. pEFBOSneo-R218H or the corresponding empty vector (pEFBOSneo) did not significantly alter the basal levels of promoter activity. It was also observed that pEFBOSneo did not affect EBNA2-mediated trans-activation of the luciferase reporter construct pGa981-6.

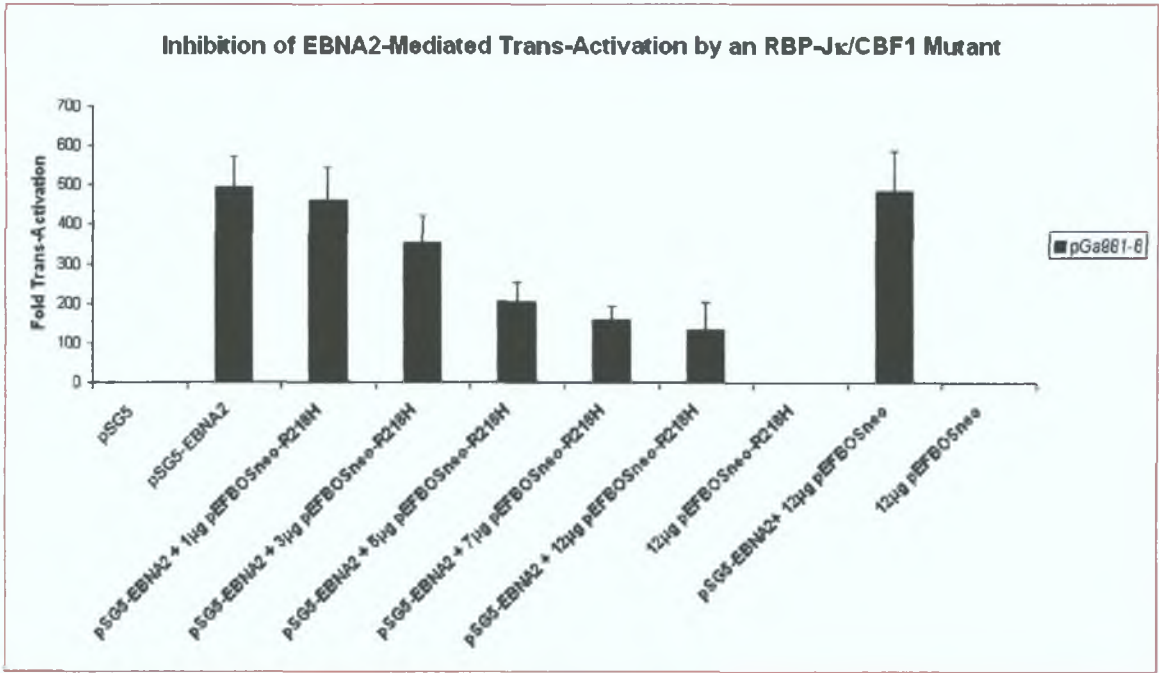


Figure 3.5: An RBP-J κ /CBF1 mutant inhibits EBNA2-mediated trans-activation of the luciferase reporter construct pGa981-6. DG75 cells were co-transfected with a constant amount of pSG5-EBNA2 (3 μ g) and increasing amounts of the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H), along with 2 μ g of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for co-transfection with the empty vector (pSG5) and the reporter. In a control transfection experiment, pSG5-EBNA2 was co-transfected with the empty vector for the RBP-J κ /CBF1 mutant, pEFBOSneo.

These results showed that expression of the RBP-J κ /CBF1 mutant, pEFBOSneo-R218H, effectively inhibits EBNA2-mediated responsiveness of the luciferase reporter construct pGa981-6 in DG75 cells.

As mentioned earlier, it has been found in our laboratory by transient transfections of the EBV-negative BL-derived cell lines DG75, BL41 and BJAB, that co-transfection of the *bfl-1* promoter reporter construct -1374/+81-Luc with EBNA2 expression vector led to an increase in luciferase activity at 48 hours post-transfection. Because EBNA2 trans-activated the RBP-J κ /CBF1-regulated promoter pGa981-6 in DG75 cells and this activity was effectively inhibited by the RBP-J κ /CBF1 mutant, tests were then performed to determine whether EBNA2-mediated trans-activation of the *bfl-1* promoter construct -1374/+81 could also be inhibited by expression of the mutant RBP-J κ /CBF1. Firstly, transient co-transfections were carried out with DG75 cell using various amounts of the expression vector for EBNA2 (pSG5-EBNA2) together with the full-length *bfl-1* promoter reporter construct -1374/+81-Luc (Figure 3.6). EBNA2 was shown to trans-activate the *bfl-1* promoter reporter construct in a dose-dependent manner with maximum trans-activation using 11 μ g of effector plasmid.

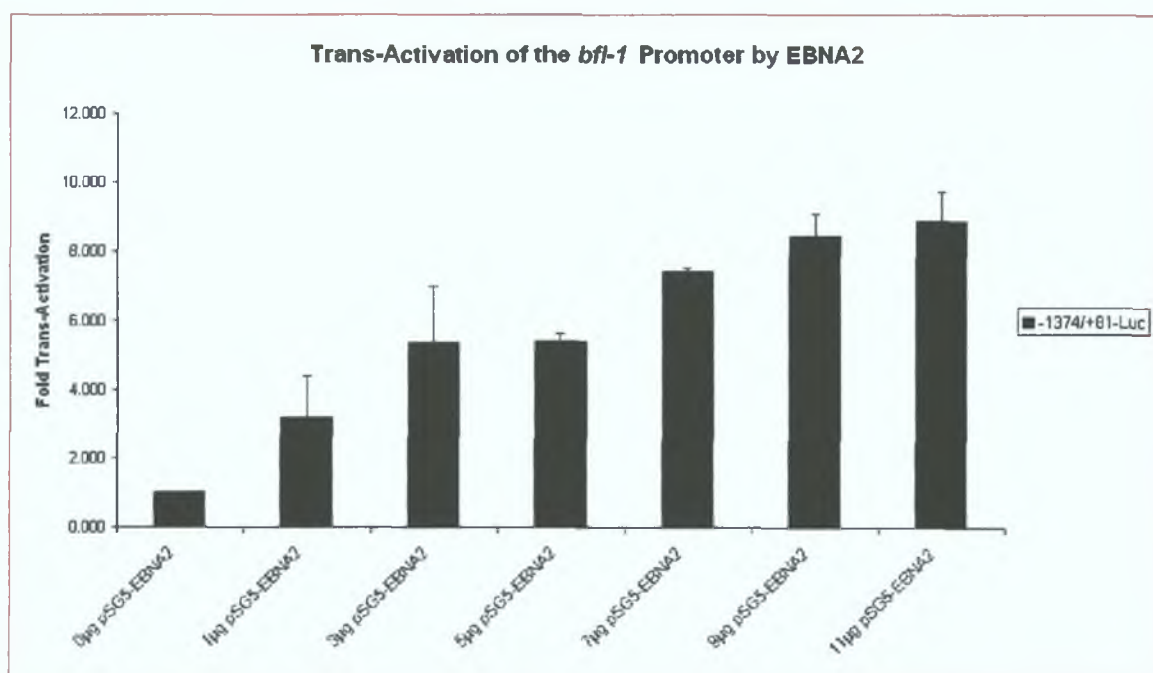


Figure 3.6: EBNA2 trans-activates the *bfl-1* promoter reporter construct -1374/+81-Luc. DG75 cells were co-transfected with increasing amounts of pSG5-EBNA2 along with 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for transfection with the empty vector (pSG5).

To determine whether the expression vector for the RBP-J κ /CBF1 mutant could be used as a tool to inhibit this EBNA2-mediated *bfl-1* promoter activation, co-transfections were performed as before using DG75 cells with the inclusion of various amounts of

pEFBOSneo-R218H. It can be seen from this experiment that co-expression of the mutant efficiently inhibited EBNA2-associated *bfl-1* promoter trans-activation in this cell context in a dose-dependent manner, to a maximum of 70 % when 12 μ g of pEFBOSneo-R218H was used (Figure 3.7). It was noted that inhibition was to a similar degree as for the control reporter construct pGa981-6 (Figure 3.5). Expression of pEFBOSneo-R218H or the corresponding empty vector (pEFBOSneo) did not significantly alter the basal levels of *bfl-1* promoter activity. It was also observed that pEFBOSneo did not affect EBNA2-mediated trans-activation of the promoter.

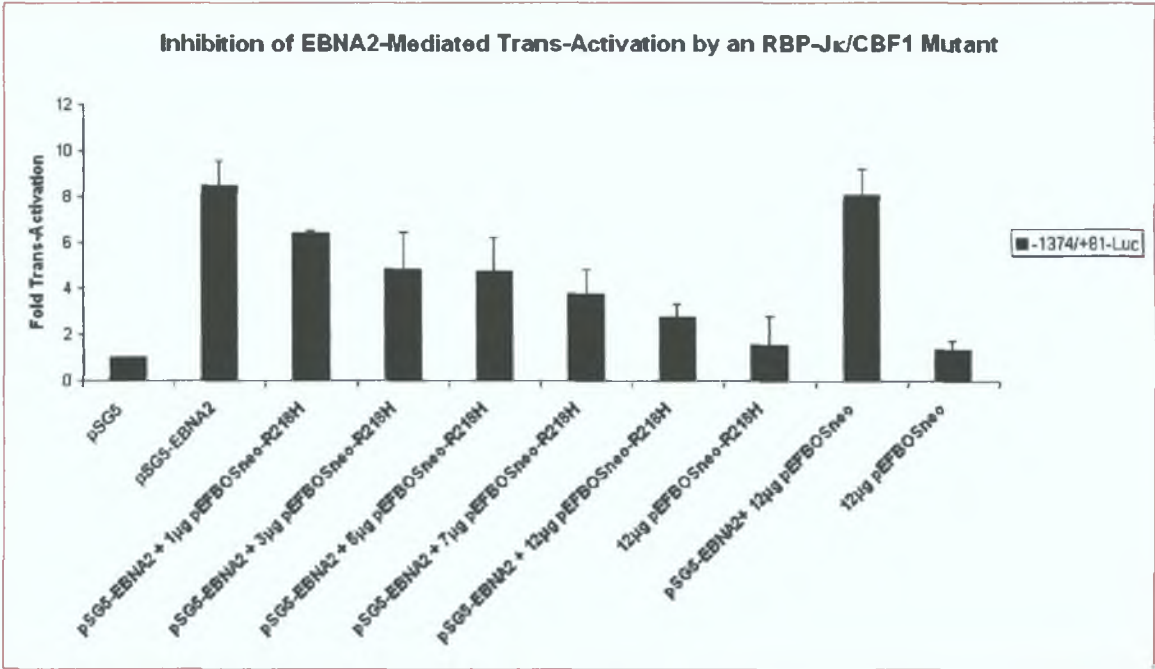


Figure 3.7: The RBP-J κ /CBF1 mutant blocks EBNA-2-mediated trans-activation of the *bfl-1* promoter reporter construct, -1374/+81-Luc. Co-transfections with DG75 cells were carried out using a constant amount of pSG5-EBNA2 (7 μ g) and an increasing amount of the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H), along with 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for co-transfection with the empty vector (pSG5) and the reporter. During control transfections, pSG5-EBNA2 was co-transfected with the empty vector for the RBP-J κ /CBF1 mutant, pEFBOSneo.

These results showed that co-expression of the RBP-J κ /CBF1 mutant effectively blocked EBNA2-mediated responsiveness on the *bfl-1* promoter reporter construct -1374/+81-Luc in the EBV-negative BL-derived cell line DG75.

3.2.3 EBNA2-mediated trans-activation of the *bfl-1* promoter does not lead to the activation of NF- κ B

It has well been established that *bfl-1* is an NF- κ B responsive gene (Moreb and Schweber, 1997, Zong *et al.*, 1999; Grumont *et al.*, 1999; Lee *et al.*, 1999; Wang *et al.*, 1999; D'Souza *et al.*, 2004) and there is an increasing body of evidence showing cell-context specific antagonistic or synergistic connections between NF- κ B and CBF1/Notch signal transduction pathways (Guan *et al.*, 1996; Bash *et al.*, 1999; Wang *et al.*, 2001). Furthermore, it has recently been shown that basal expression of I κ B α , and as a consequence NF- κ B activity, is under CBF1/Notch control (Oakley *et al.*, 2003). It was therefore important to monitor the level of activated NF- κ B in DG75 cells when either EBNA2 or the RBP-J κ /CBF1 mutant was expressed. To this end, co-transfections were carried out using a known NF- κ B-dependent reporter construct, 3 x enh κ B luc (Floettmann and Rowe, 1997), which contains 3 NF- κ B binding sites upstream of a minimal conalbumin promoter, linked to the luciferase gene. It can be seen from Figure 3.8 that there is a high basal level of activated NF- κ B present in this cell context. As expected, the NF- κ B-responsive promoter was activated by a further 2.7-fold upon co-transfection with the LMP1 expression vector (pSG5-LMP1). It was observed that the basal level of activated NF- κ B did not change significantly in response to co-transfection with expression vectors encoding EBNA2 or during co-transfections with EBNA2 in the presence of various amounts of the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H). However, some increase in the basal level of activated NF- κ B was found upon co-transfection with pEFBOSneo-R218H and the reporter construct in the absence of EBNA2. Co-transfections with the expression vector for EBNA2 and the empty expression vector for the RBP-J κ /CBF1 mutant (pEFBOSneo) did not significantly affect the levels of activated NF- κ B.

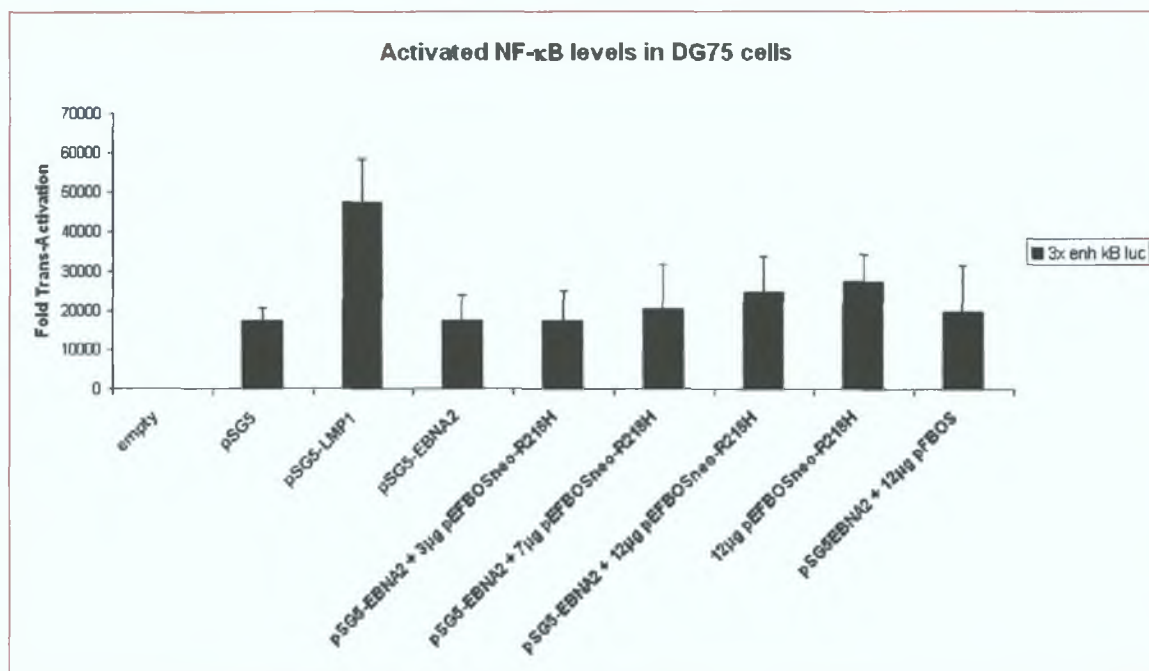


Figure 3.8: Activated NF- κ B levels are not dramatically affected by expression of EBNA2 or the RBP-J κ /CBF1 mutant. DG75 cells were co-transfected with the expression vector for LMP1 (2 μ g pSG5-LMP1), EBNA2 (7 μ g of pSG5-EBNA2) or EBNA2 along with various quantities of pEFBOSneo-R218H together with 2 μ g of the NF- κ B-responsive reporter construct 3 x enh kB luc. Cells were harvested 48 hours post-transfection and normalised luciferase values were expressed as activation (n-fold) relative to the value obtained during transfection with the empty vector (pSG5) in the absence of any reporter construct (empty).

It was next tested if inhibition of NF- κ B activation would affect EBNA2-mediated trans-activation of the *bfl-1* promoter. To this end, co-transfections were carried out using an expression vector for a mutant form of I κ B α (pEFCX-I κ B α DN), in which serine residues at positions 32 and 36 have been replaced with alanines (Liljeholm *et al.*, 1998). As a result, I κ B α can no longer be phosphorylated and subsequently proteolysed. Therefore, this mutant effectively retains NF- κ B in the cytoplasm, thus blocking its function as a regulator of transcription by preventing NF- κ B translocation to the nucleus. In a preliminary control experiment to demonstrate the functionality of the I κ B α mutant, co-transfections were performed using the NF- κ B-responsive reporter construct (3 x enh kB luc) together with increasing amounts of the super-repressor pEFCX-I κ B α DN. In the presence of the I κ B α super-repressor, a dramatic decrease in luciferase activity was observed (Figure 3.9A). This decrease in activated NF- κ B levels was observed upon co-transfection when just 1 μ g of pEFCX-I κ B α DN was used, with a decrease of approximately 75 %. When larger quantities of the expression vector for the I κ B α mutant were used in transfection experiments, luciferase activity decreased even further. In order to investigate if the I κ B α mutant could inhibit EBNA2-mediated

trans-activation of the *bfl-1* promoter, co-transfections were performed using various amounts of the mutant, along with the *bfl-1* promoter reporter construct -1374/+81-Luc. Over-expression of the I κ B α super-repressor only resulted in a marginal decrease (< 10 %) in luciferase activity, even when up to 12 μ g of expression vector was used (Figure 3.9B).

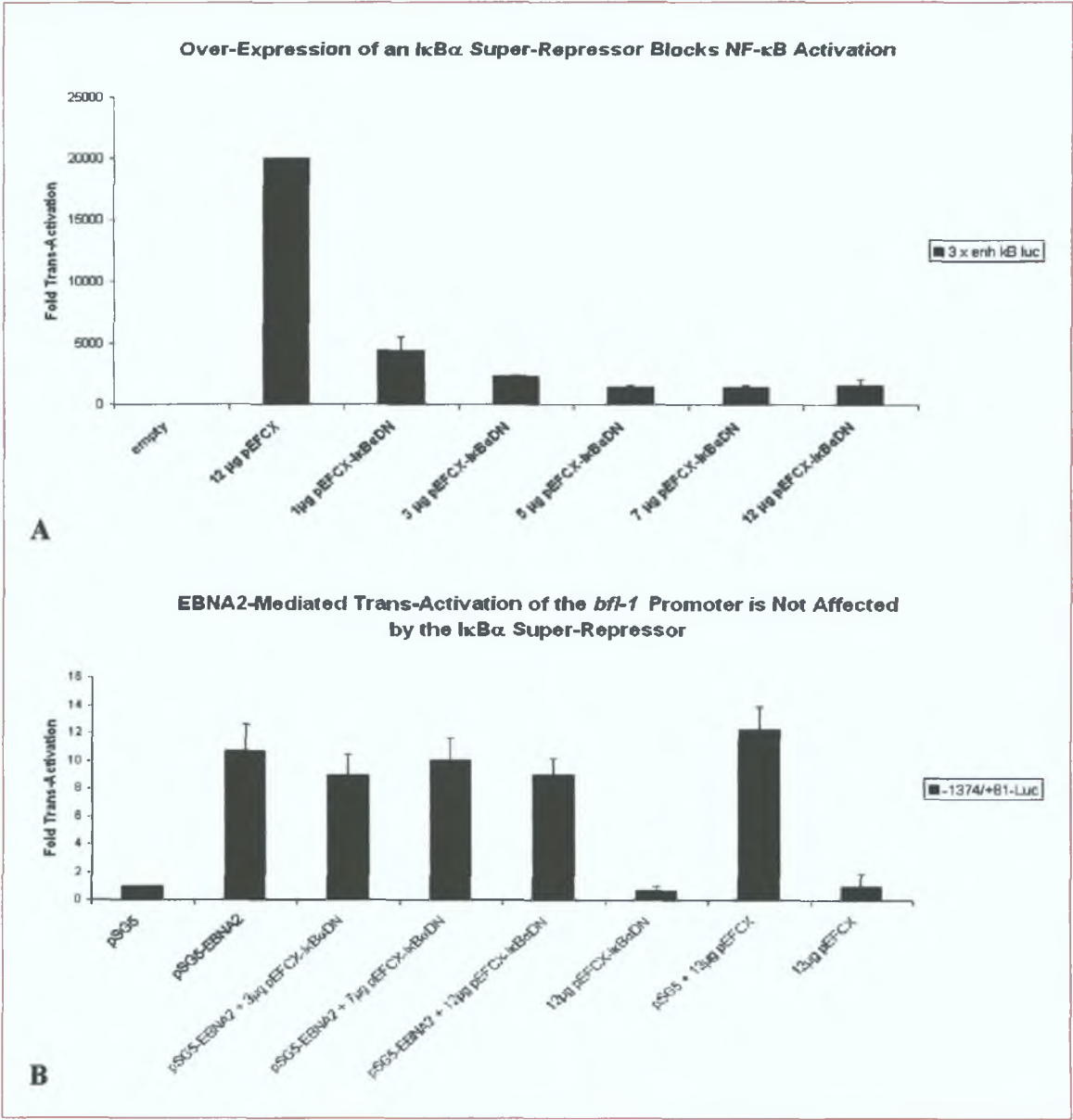


Figure 3.9: Over-expression of an I κ B α super-repressor inhibits NF- κ B activation but does not affect EBNA2-mediated trans-activation of the *bfl-1* promoter. (A) Co-transfections were performed using 2 μ g of the activated NF- κ B-responsive reporter construct 3 x enh κ B luc, together with increasing quantities of the expression vector for the I κ B α super-repressor (pEFCX-I κ B α DN). (B) Co-transfections were performed using a constant amount of pSG5-EBNA2 (7 μ g), various amounts of the I κ B α super-repressor and 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc. Transfected cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained upon co-transfection with the empty vector.

These results suggested that the activation of NF- κ B does not play a role in EBNA2-mediated trans-activation of the *bfl-1* promoter in DG75 cells, based on evidence that EBNA2-mediated *bfl-1* promoter activity is not associated with an increase in the level of activated NF- κ B and over-expression of an I κ B α mutant does not affect EBNA2-mediated trans-activation of the *bfl-1* promoter in DG75 cells.

3.2.4 EBNA2-mediated trans-activation of the *LMP1* promoter is inhibited by co-expression of a dominant-negative mutant form of the Notch signalling pathway protein RBP-J κ /CBF1.

Regulation of expression of the *LMP1* gene has been well studied and it has previously been shown that the *LMP1* promoter is EBNA2-responsive (Abbot *et al.*, 1990; Fahraeus *et al.*, 1990; Wang *et al.*, 1990; Zimmer-Strobl *et al.*, 1991; Laux *et al.*, 1994a). This responsiveness is demonstrated in Figure 3.10, which shows the results of co-transfections performed using increasing quantities of the expression vector for EBNA2 (pSG5-EBNA2) and the *LMP1* promoter reporter construct (pLLO-luc). It was observed that EBNA2 trans-activated the promoter in a dose-dependent fashion, reaching a peak when 7 μ g of expression vector were used. Using this quantity a 22-fold trans-activation was achieved. When higher quantities of expression vector were used this maximum activation decreased somewhat.

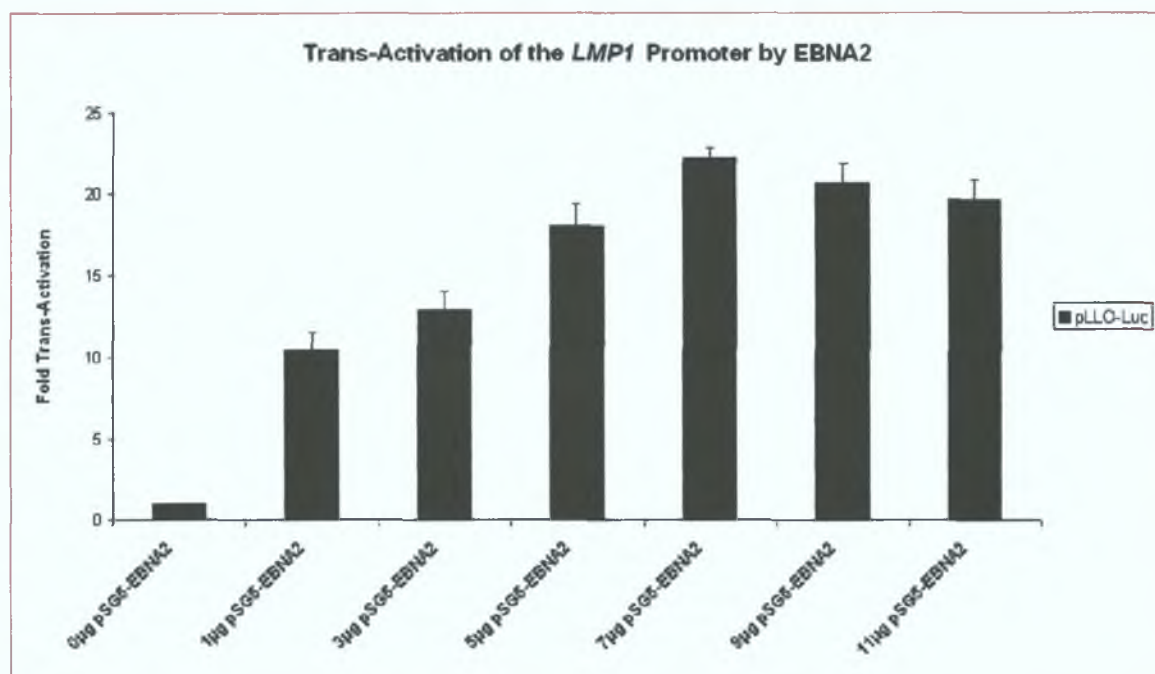


Figure 3.10: EBNA2 trans-activates the *LMP1* promoter reporter construct pLLO-luc. Transient transfections were performed using DG75 cells with various quantities of the expression vector for EBNA2 (pSG5-EBNA2) and 2 μ g of the *LMP1* promoter reporter construct pLLO-luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained upon co-transfection with the empty vector (pSG5).

To investigate the role of RBP-J κ /CBF1 in mediating EBNA2-responsiveness on the *LMP1* promoter, co-transfections were performed using DG75 cells with the inclusion of various amounts of pEFBOSneo-R218H along with the *LMP1* promoter reporter construct pLLO-luc. It can be seen from this experiment that expression of the mutant efficiently inhibited EBNA2-associated *LMP1* promoter trans-activation in a dose-dependent manner, with almost complete inhibition when 12 μ g of pEFBOSneo-R218H was used (Figure 3.11). It was noted that inhibition EBNA2-mediated activation of the *LMP1* promoter by the RBP-J κ /CBF1 mutant was to a greater degree than that of the *bfl-1* promoter (Figure 3.7). In the case of the *bfl-1* promoter, it is possible that the remaining residual *bfl-1* promoter activity may be due to the high basal level of activated NF- κ B present in DG75 cells. Co-transfection with pEFBOSneo-R218H or the corresponding empty vector (pEFBOSneo) did not significantly alter the basal levels of *LMP1* promoter activity. It was also observed that pEFBOSneo did not affect EBNA2-mediated trans-activation of the *LMP1* promoter.

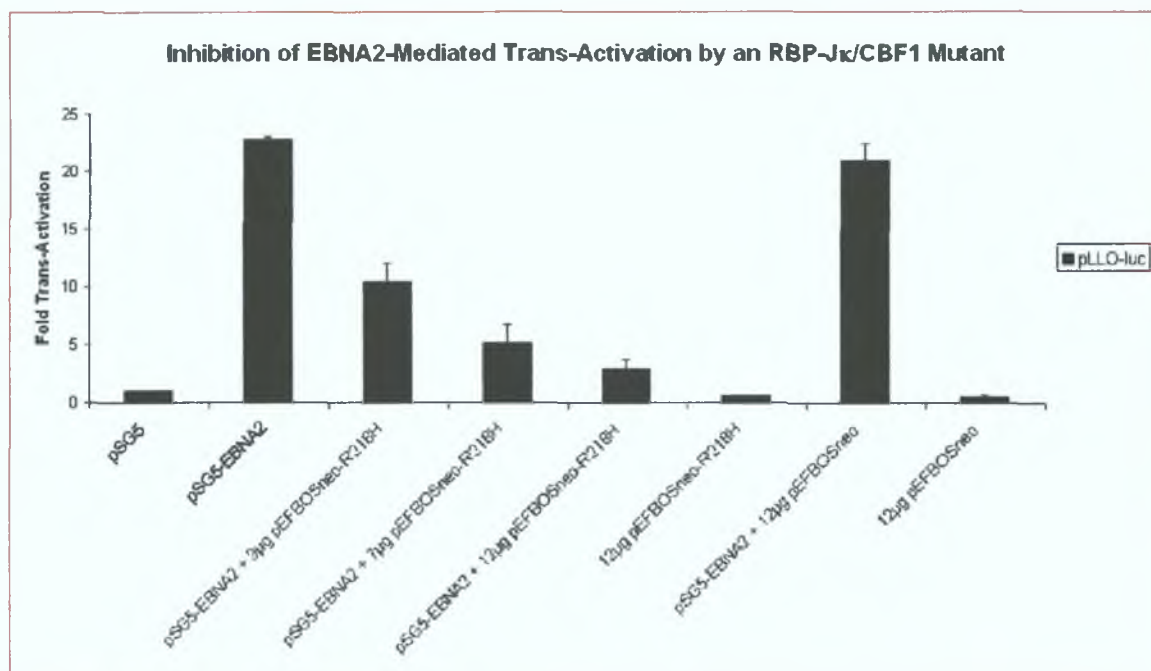


Figure 3.11: The RBP-J κ /CBF1 mutant blocks EBNA-2-mediated trans-activation of the *LMP1* promoter reporter construct, pLLO-luc. DG75 cells were co-transfected with a constant amount of pSG5-EBNA2 (7 μ g) and various amounts of the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H), along with 2 μ g of the *LMP1* promoter reporter construct pLLO-luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for co-transfection with the empty vector (pSG5) and the reporter. During control transfections, pSG5-EBNA2 was co-transfected with the empty vector for the RBP-J κ /CBF1 mutant, pEFBOSneo.

These results showed that EBNA2 trans-activated the *LMP1* promoter in DG75 cells, and that RBP-J κ /CBF1 is essential for this effect. Based on the evidence that the RBP-J κ /CBF1 mutant significantly inhibits EBNA2-mediated trans-activation of the *LMP1* promoter, in addition to the control reporter constructs pGa981-6 and the *bfl-1* promoter, it may be considered a useful tool for blocking EBNA2 mediated activity.

3.2.5 The cellular functional equivalent of EBNA2, Notch1IC, does not trans-activate the *bfl-1* promoter

Although no cellular homologue of the EBNA2 protein has been identified, the intracellular domain of the Notch receptor (Notch1IC) is considered to be a cellular equivalent from a mechanistic and functional point of view (Hsieh *et al.*, 1996). There is no obvious sequence homology between EBNA2 and Notch1IC, however both proteins interact with RBP-J κ /CBF1 to modulate gene expression. Both EBNA2 and

NotchIC interact with similar regions of RBP-J κ /CBF1 and displace repressor proteins by their trans-activation domains (Zimber-Strobl and Strobl, 2001). The set of promoters, which is regulated by EBNA2 and NotchIC is overlapping but not identical (reviewed in Chapter 1). In order to understand to what extent EBNA2 is a functional equivalent of NotchIC, it would prove interesting to identify further target genes for NotchIC and EBNA2 in B cells. This study set out to investigate if the *bfl-1* gene is a target of NotchIC.

In a preliminary experiment to demonstrate NotchIC-mediated promoter activation via RBP-J κ /CBF1, transient transfections using DG75 cells with increasing amounts of a mouse Notch1IC expression vector (pSG5-HA-mNotch1IC (1751-2294), Hsieh *et al.*, 1996) and the luciferase reporter construct pGa981-6 were performed. Notch1IC used in these experiments consists of amino acids 1751-2294 of the Notch receptor (Figure 3.15). This region encompasses the sequences between the trans-membrane domain and the PEST motif at the C-terminus, and includes 6 tandem cdc10/ankyrin repeats and 2 putative nuclear localisation sequences (NLS). Similarly to EBNA2, it can be seen that mNotch1IC trans-activated pGa981-6 in a dose-dependent manner (Figure 3.12). It was noted that the maximum trans-activation for EBNA2 (approximately 4,500-fold using 11 μ g of effector plasmid, Figure 3.4) was greater than the maximum trans-activation for mNotch1IC (approximately 2,300-fold using 11 μ g of effector plasmid). This observation of greater trans-activation efficiency for EBNA2 when compared with mNotch1IC is in keeping with the findings of others (Hsieh *et al.*, 1996).

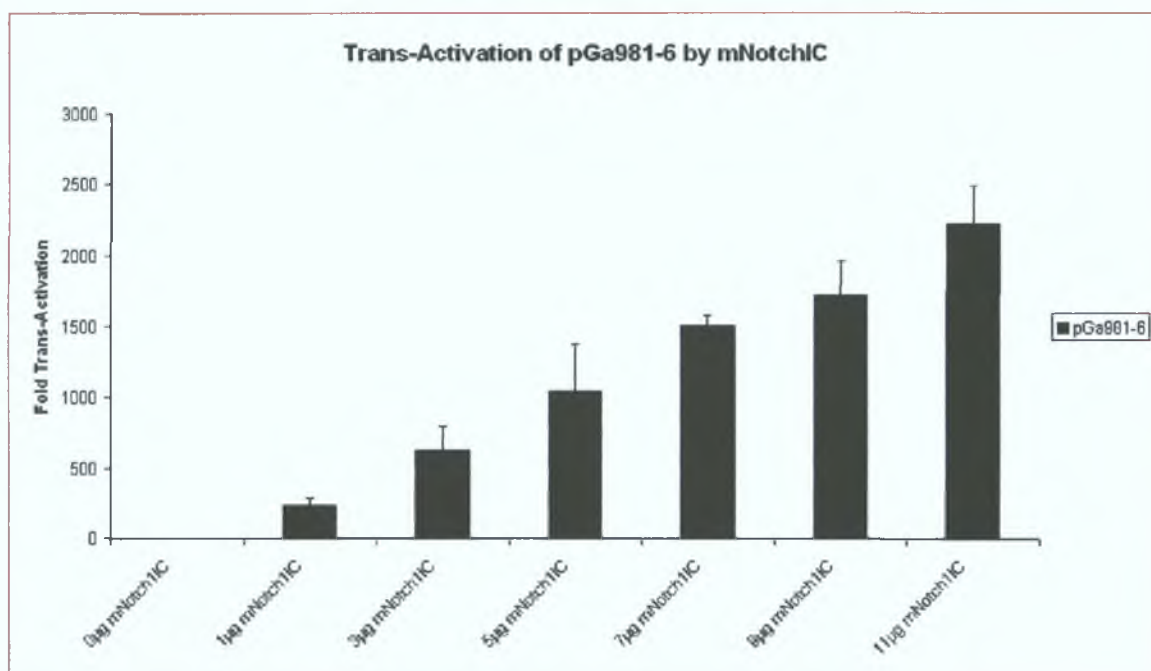


Figure 3.12: mNotch1IC trans-activate the luciferase reporter construct pGa981-6. DG75 cells were co-transfected with increasing amounts pSG5-HA-mNotch1IC (1751-2294) and 2 µg of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection. Luciferase values were normalised as described earlier and expressed as activation (*n*-fold) relative to the corresponding value obtained for co-transfection with the empty vector (pSG5) and pGa981-6.

The role of RBP-Jκ/CBF1 in mediating mNotch1IC-mediated responsiveness was further investigated using the expression vector for the non-DNA binding RBP-Jκ/CBF1 mutant (pEFBOSneo-R218H). To investigate if pEFBOSneo-R218H could inhibit trans-activation of the luciferase reporter construct pGa981-6 by Notch1IC, co-transfections were performed in DG75 cells with increasing amounts of pEFBOSneo-R218H and constant quantity of mNotch1IC. It can be seen from this experiment that expression of the mutant RBP-Jκ/CBF1 (pEFBOSneo-R218H) efficiently inhibited mNotch1IC-mediated trans-activation of pGa981-6 (Figure 3.13). This effect was dose-dependent, with maximum inhibition of approximately 66%, when 12 µg of pEFBOSneo-R218H was used. pEFBOSneo-R218H or the corresponding empty vector (pEFBOSneo) did not significantly alter the basal levels of promoter activity. It was also observed that pEFBOSneo did not affect mNotch1IC-mediated trans-activation of the luciferase reporter construct pGa981-6.

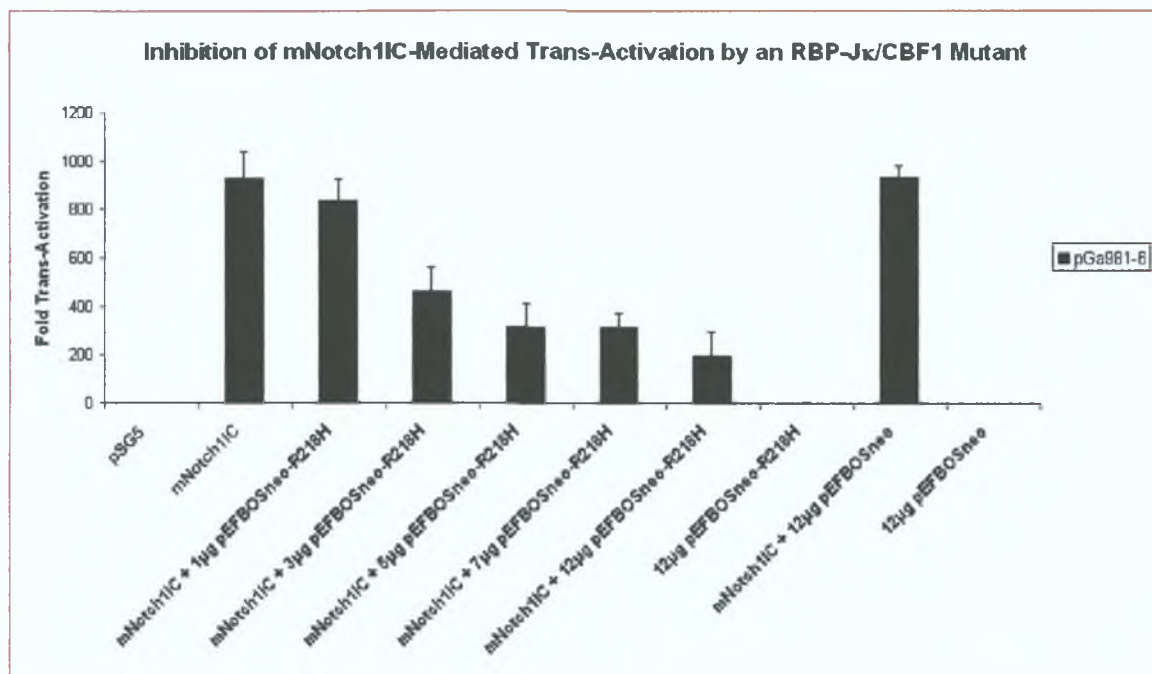


Figure 3.13: An RBP-J κ /CBF1 mutant inhibits mNotch1IC-mediated trans-activation of the luciferase reporter construct pGa981-6. DG75 cells were co-transfected with a constant amount of mNotch1IC (5 μ g) and increasing amounts of the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H), along with 2 μ g of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for co-transfection with the empty vector (pSG5) and the reporter. In a control experiment, mNotch1IC was co-transfected with the empty vector for the RBP-J κ /CBF1 mutant, pEFBOSneo.

These results demonstrated Notch1IC-mediated activation via RBP-J κ /CBF1 and showed that expression of the RBP-J κ /CBF1 mutant, pEFBOSneo-R218H, effectively blocks mNotch1IC-mediated responsiveness on the luciferase reporter construct pGa981-6 in DG75 cells.

It was demonstrated in Section 3.2.2 that EBNA2 trans-activated the *bfl-1* promoter in DG75 cells. Because both EBNA2 and mNotch1IC trans-activated the RBP-J κ /CBF1 regulated promoter pGa981-6 in DG75 cells, and this activation was effectively inhibited by an RBP-J κ /CBF1 mutant, it would prove interesting to determine whether mNotch1IC could trans-activate the *bfl-1* promoter in this cell context. To this end, transient co-transfections were carried out with DG75 cell using various amounts of the expression vector for mNotch1IC [pSG5-HA-mNotch1IC (1751-2294)] together with the *bfl-1* promoter reporter construct -1374/+81-Luc (Figure 3.14), and the resulting data compared with that obtained in the EBNA2 co-transfections. EBNA2 was seen to trans-activate the *bfl-1* promoter reporter construct in a dose-dependent manner.

However, the *bfl-1* reporter construct was not found to be mNotch1IC-responsive, even when up to 11 μ g of effector plasmid was used during co-transfections.

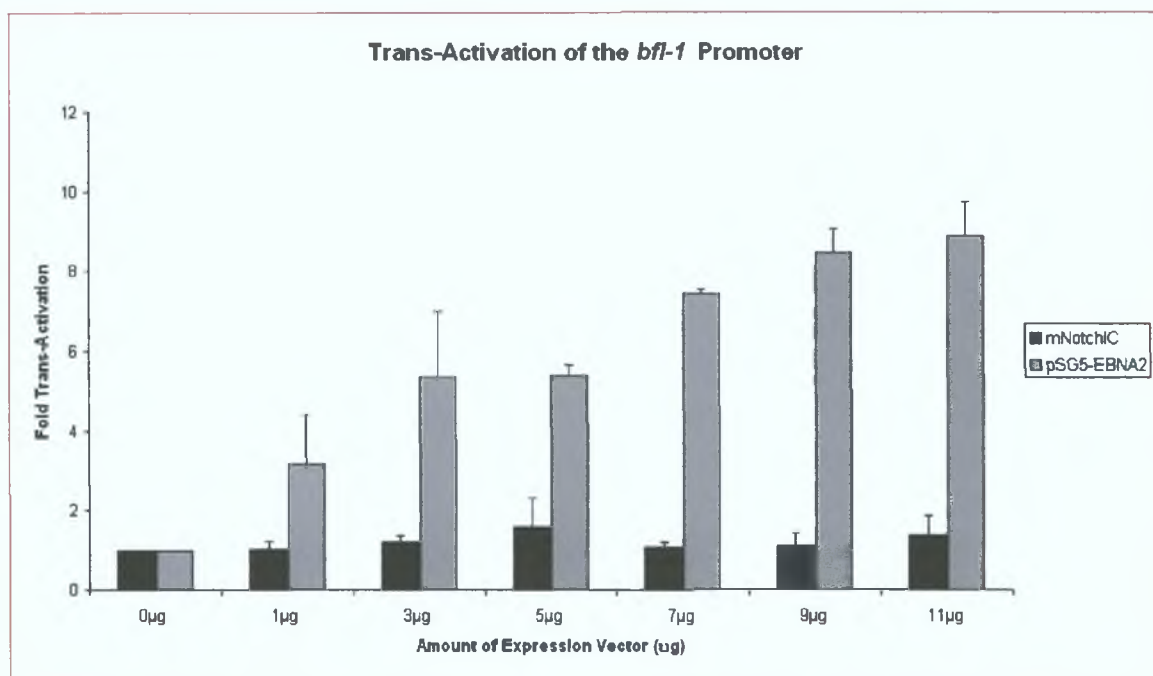


Figure 3.14: Unlike EBNA2, mNotch1IC does not trans-activate the *bfl-1* promoter reporter construct -1374/+81-Luc. DG75 cells were co-transfected with increasing amounts of pSG5-EBNA2 (grey) or mNotch1IC (black) along with 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained for transfection with the empty vector (pSG5).

This set of data indicated that, like EBNA2, mNotch1IC trans-activates the RBP-J κ /CBF1-regulated control promoter pGa981-6 and that RBP-J κ /CBF1 is essential to this effect. However, unlike EBNA2, mNotch1IC does not trans-activate the *bfl-1* promoter reporter construct -1374/+81-Luc in DG75 cells.

3.2.6 Failure of Notch1IC to trans-activate the *bfl-1* promoter is not due to a weak trans-activation domain.

It has been reported that the Notch1IC trans-activation domain is weaker than that of EBNA2 (Hsieh *et al.*, 1996). Further experiments in the EBV-negative BL-derived cell line DG75 were conducted to determine whether the inability of mNotch1IC to trans-activate the *bfl-1* promoter was due to differences in the strength of the Notch1IC trans-activation domain (TAD) relative to that of EBNA2. For this purpose, co-transfections

were carried out using a vector expressing a chimeric protein in which the transcriptional activation domain and nuclear localisation sequence of the EBNA2 protein (E2TANLS) were fused to the carboxy terminus of mNotch1IC (mNotch1IC:E2TANLS, Hsieh *et al.*, 1996). The E2TANLS domain lies outside the RBP-J κ /CBF1 interaction domain of EBNA2, which is located between amino acids 252 and 425. Therefore, the chimeric protein mNotch1IC:E2TANLS binds RBP-J κ /CBF1 via mNotch1IC and contains the additional TAD and NLS of EBNA2. Schematic representations of EBNA2, Notch1IC and Notch1IC:E2TANLS are shown in Figure 3.15.

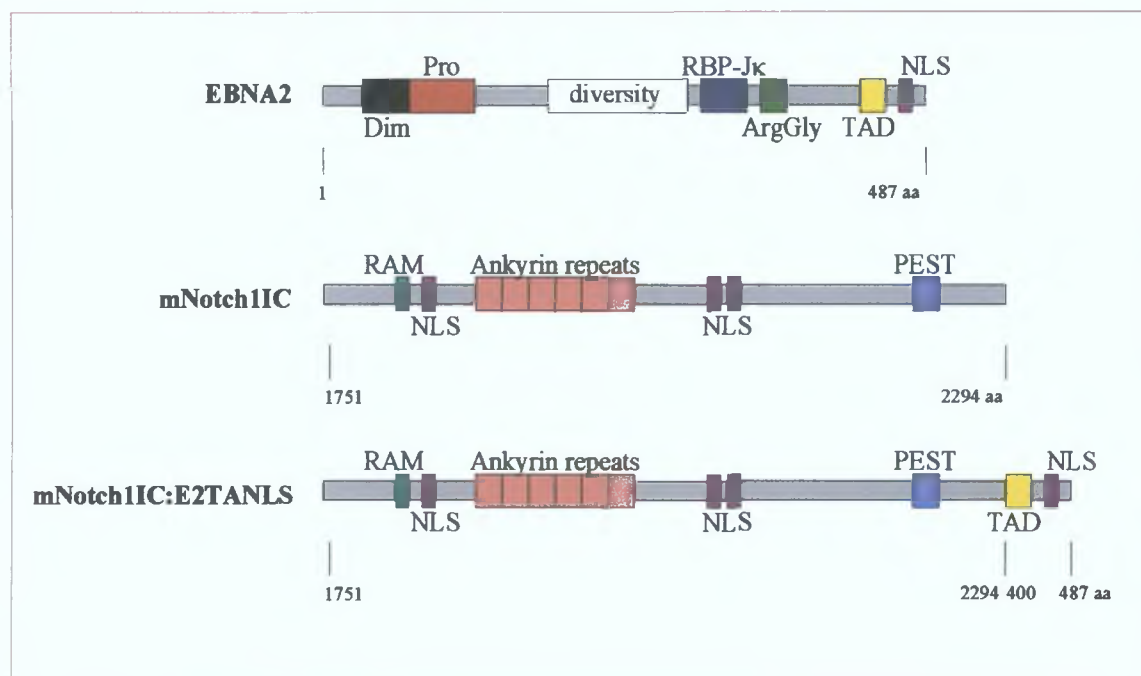


Figure 3.15: Schematic representation of EBNA2, mNotch1IC and mNotch1IC:E2TANLS. The upper panel shows the structure of the EBNA2 protein. EBNA2 consists of a negatively charged region at the amino-terminus, which is likely to play a role in homo-dimerization (Dim), a polyproline region (Pro) consisting of 10-40 consecutive prolines depending on the virus strain, a diversity region, a domain responsible for interaction with RBP-J κ /CBF1 (RBP-J κ), an arginine-glycine rich stretch (ArgGly) and a negatively charged region carboxy terminus, which harbors a trans-activation domain (TAD) and nuclear localization signal (NLS). The middle panel is a schematic representation of mNotch1IC from amino acids 1751 to 2294. This region encompasses the sequences between the trans-membrane domain and the PEST motif at the C-terminus, and includes the RAM domain, 6 ankyrin repeats and 3 putative nuclear localisation sequences (NLS). The structure of mNotch1IC:E2TANLS is shown in the lower panel. This is a fusion protein in which the NLS and TA of the EBNA2 protein (amino acids 400 to 487) are fused to the carboxy terminus of mNotch1IC.

Co-transfections using the expression vector for mNotch1IC:E2TANLS (pSG5-HA-mNotch1IC (1751-2294):E2TANLS) and the luciferase reporter construct pGa981-6 are shown in Figure 3.16. Similarly to co-transfections using EBNA2 (Figure 3.4) and mNotch1IC (Figure 3.12) together with pGa981-6, it was found that mNotch1IC:E2TANLS trans-activated pGa981-6 in a dose-dependent manner. A maximum trans-activation of approximately 9,000-fold was observed when 11 µg of expression vector was used. It was noted that the maximum trans-activation for mNotch1IC:E2TANLS was greater than the maximum trans-activation for both EBNA2 (approximately 4,500-fold using 11 µg of effector plasmid) and mNotch1IC (approximately 2,300-fold using 11 µg of effector plasmid). This observation is in keeping with the findings of others (Hsieh *et al.*, 1996).

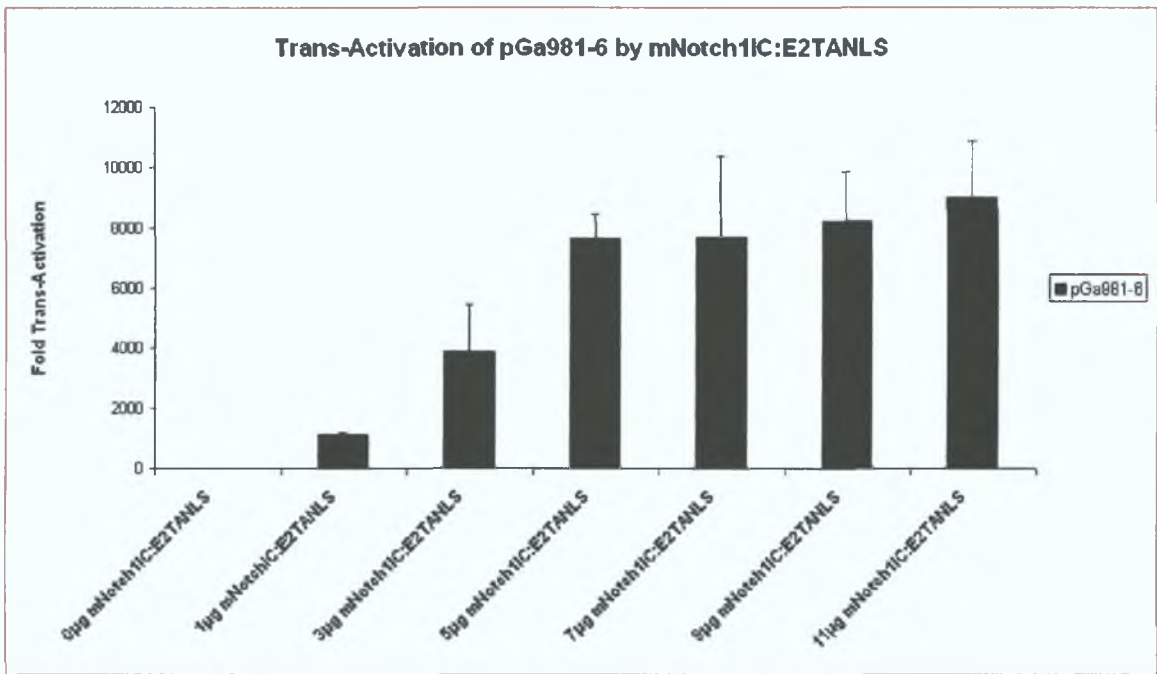


Figure 3.16: A fusion protein between mNotch1IC and the trans-activation domain and nuclear localisation sequence of EBNA2 trans-activates the luciferase reporter construct pGa981-6. Co-transfections were performed using DG75 cells with increasing amounts of an expression vector for a fusion protein between mNotch1IC and the trans-activation domain and nuclear localisation sequence of EBNA2 (mNotch1IC:E2TANLS) together with 2 µg of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (n-fold) relative to the corresponding value obtained for transfection with the empty vector (pSG5).

Once it was established that mNotch1IC:E2TANLS could trans-activate the control RBP-Jκ/CBF1-regulated promoter pGa981-6, further experiments were conducted in order to determine whether the *bfl-1* promoter reporter construct -1374/+81-Luc was

responsive to this fusion protein. It was observed during earlier co-transfections that 4 μ g of EBNA2 expression vector (from Figure 3.4), 11 μ g of mNotch1IC (from Figure 3.12) and 2 μ g of mNotch1IC:E2TANLS (Figure 3.16) trans-activated the control reporter construct pGa981-6 to a similar degree (approximately 2,300-fold). It was therefore reasoned that these quantities of expression vector should be used in an attempt to directly compare the ability of EBNA2, mNotch1IC and mNotch1IC:E2TANLS to trans-activate the *bfl-1* promoter. Firstly, co-transfections were performed in DG75 cells using these quantities of effector plasmid along with pGa981-6 and the resulting data is displayed in Figure 3.17A. These quantities of expression vector for EBNA2, mNotch1IC and mNotch1IC:E2TANLS were seen to result in a comparable rate of trans-activation. Subsequently, these quantities were used for co-transfections using the *bfl-1* promoter reporter construct -1374/+81-Luc (Figure 3.17B). As expected, EBNA2 trans-activated the *bfl-1* promoter. By contrast, neither mNotch1IC or mNotch1IC:E2TANLS were seen to trans-activate the *bfl-1* promoter.

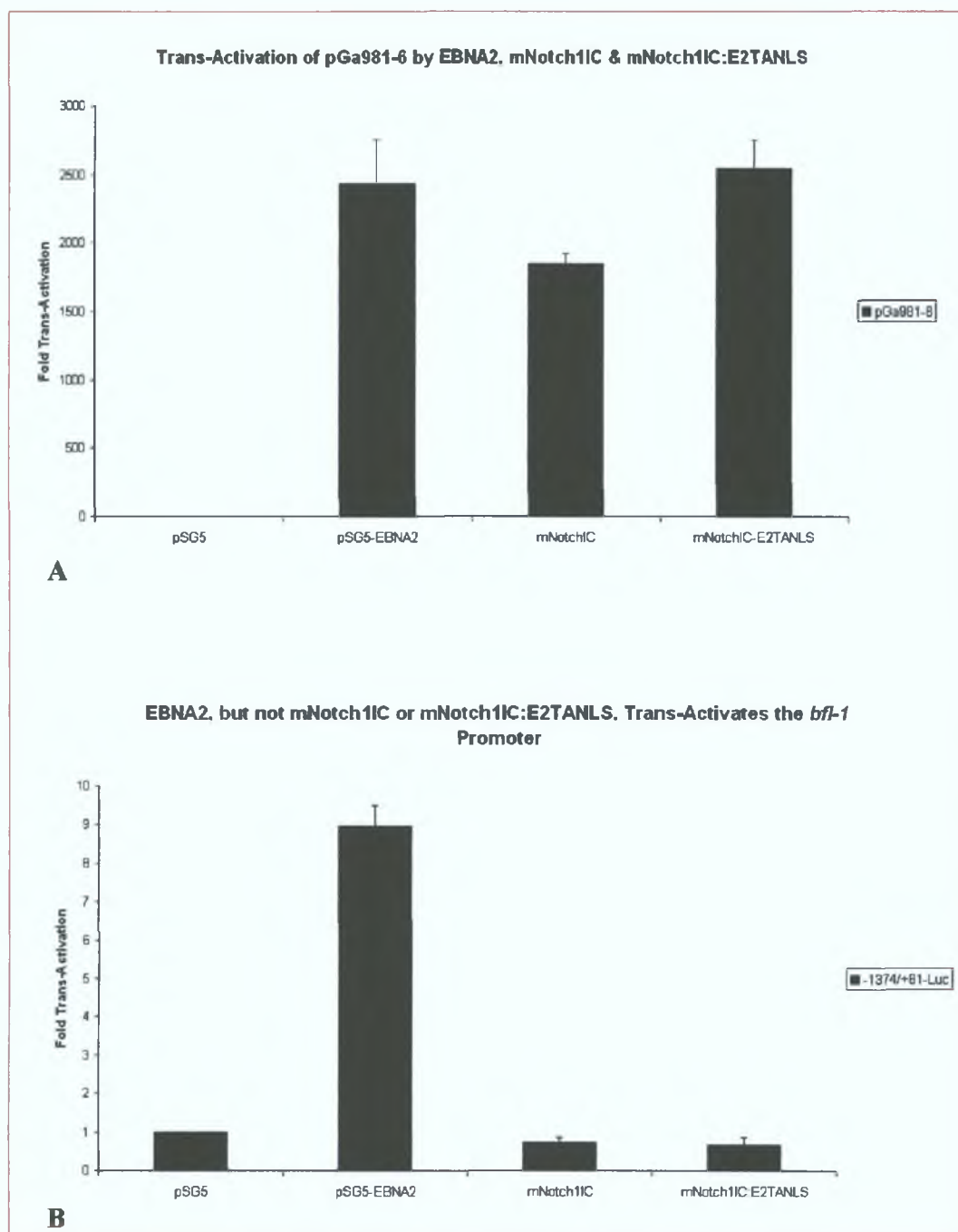


Figure 3.17: Trans-activation of the luciferase reporter constructs pGa981-6 and -1374/+81-Luc by EBNA2, mNotch1IC and mNotch1IC:E2TANLS. Transient co-transfections were performed using DG75 cells with expression vectors for EBNA2 (pSG5-EBNA2, 4 μ g), mNotch1IC (pSG5-HA-mNotch1IC (1751-2294), 11 μ g) and mNotch1IC:E2TANLS (pSG5-HA-mNotch1IC (1751-2294):E2TANLS, 2 μ g) together with 2 μ g of the RBP-J κ /CBF1-regulated luciferase reporter construct pGa981-6 (A) or 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc (B). Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) over the corresponding value obtained upon co-transfection with the empty vector (pSG5).

It was demonstrated in Section 3.2.4 that EBNA2 trans-activated the *LMP1* promoter and that this activation was effectively inhibited by expression of an RBP-J κ /CBF1 mutant. To compare EBNA2 activity with that of mNotch1IC and

mNotch1IC:E2TANLS with regard to the *LMP1* promoter, co-transfections were performed using DG75 cells and expression vectors for EBNA2, mNotch1IC and mNotch1IC:E2TANLS along with the *LMP1* promoter reporter construct pLLO-luc (Figure 3.18). It was found, as before that EBNA2 trans-activated the *LMP1* promoter approximately 22-fold. The *LMP1* promoter construct not found to be responsive to mNotch1IC. This observation is in keeping with others (Zimber-Strobl and Strobl, 2001). It can also be seen from Figure 3.18 that mNotch1IC:E2TANLS did not trans-activate the *LMP1* promoter reporter construct pLLO-luc in DG75 cells.

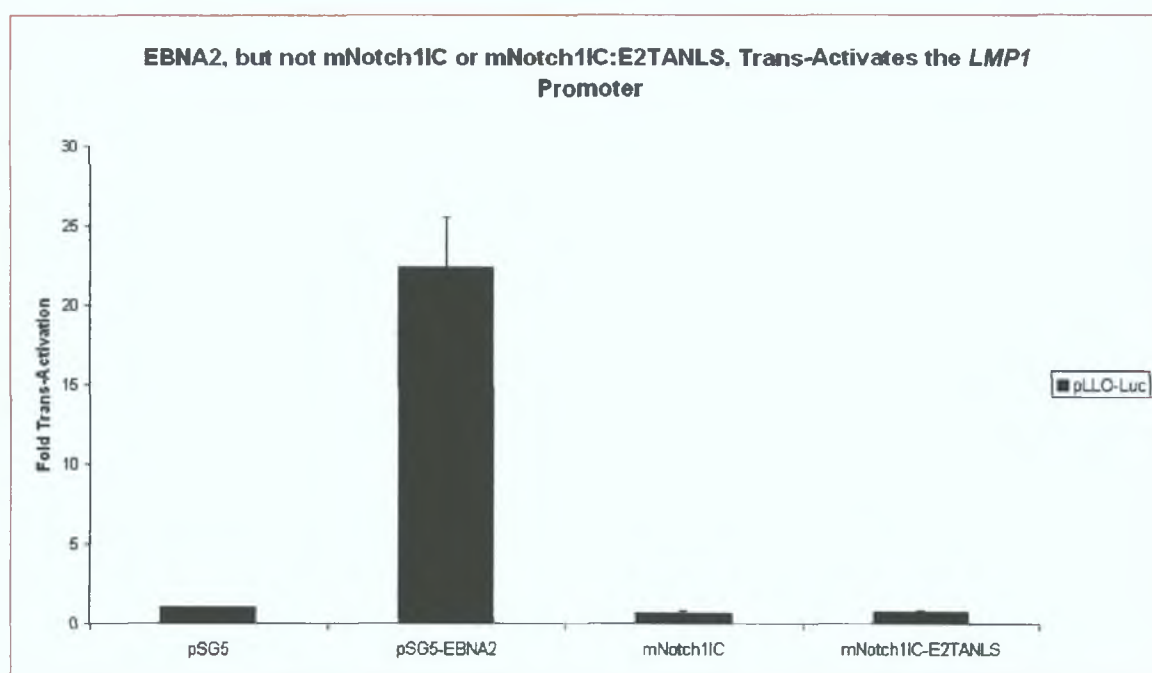


Figure 3.18: EBNA2, but not mNotch1IC or mNotch1IC:E2TANLS, trans-activates the *LMP1* promoter. DG75 cells were co-transfected with expression vectors for EBNA2 (pSG5-EBNA2, 4 µg), mNotch1IC (pSG5-HA-mNotch1IC (1751-2294), 11 µg) and mNotch1IC:E2TANLS (pSG5-HA-mNotch1IC (1751-2294):E2TANLS, 2 µg) together with 2 µg of the *LMP1* promoter reporter construct pLLO-luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) over the corresponding value obtained upon co-transfection with the empty vector (pSG5).

This data demonstrated that even when the transcriptional activation domain and nuclear localisation sequence from EBNA2 were fused to the mNotch1IC protein, it was not sufficient to activate either the *bfl-1* promoter or the *LMP1* promoter in the EBV-negative BL-derived cell line DG75. Therefore, failure of mNotch1IC to trans-activate these promoters is not merely due to a weaker trans-activation domain than that of EBNA2. It is possible that binding of additional proteins (e.g. members of the Spi

family), as well as RBP-J κ /CBF1, to EBNA2 is required for EBNA2-mediated promoter activation.

3.2.7 A strong trans-activation domain alone is not sufficient for trans-activation of the *bfl-1* promoter

To further evaluate the role of trans-activation domains in *bfl-1* promoter activation, co-transfections were performed using a construct containing the acidic activation domain of the herpes simplex virus VP16 transcriptional activator. Because RBP-J κ /CBF1 is a transcriptional repressor that tethers a repression complex to the promoter, it was tested whether providing RBP-J κ /CBF1 with a trans-activation domain would be sufficient to counter-balance repression. For this purpose, the sequence coding for the acidic activation domain of the herpes simplex virus VP16 transcriptional activator was fused to the C-terminus of the RBP3 coding sequence (RBP3 being one of the known RBP-J κ /CBF1 isoforms, Brou *et al.* 1994). This protein, called RBP-VP16 (Figure 3.19) has been shown by EMSA to bind to DNA as efficiently as RBP3 (Waltzer *et al.*, 1995).

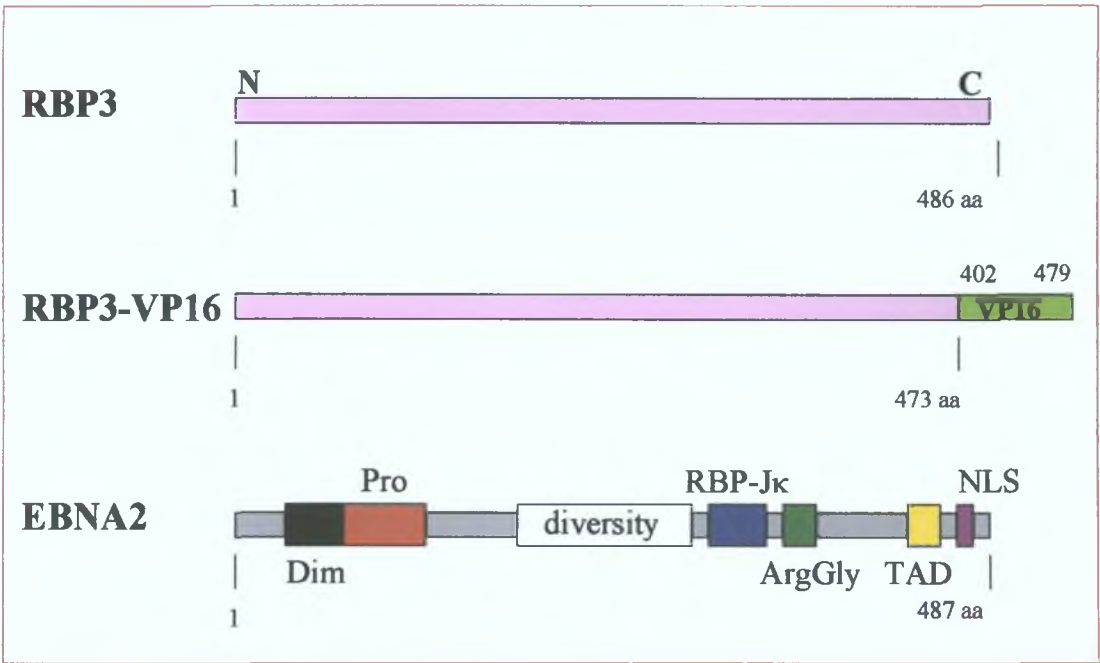


Figure 3.19: Schematic representation of the proteins used during co-transfections. The upper panel represents the RBP3 protein from amino acids 1 to 486. RBP3 is one of the known RBP-J κ /CBF1 isoforms. The middle panel represents a fusion protein between the herpes simplex virus VP16 trans-activation domain and RBP3. The EBNA2 protein is illustrated in the lower panel.

Firstly, it was evaluated if this fusion protein could activate transcription of the luciferase reporter construct pGa981-6 in DG75 cells. Upon co-transfection of the expression vector for the RBP3 protein alone with the reporter construct, a level of luciferase activity similar to that obtained upon co-transfection with the empty vector (pSG5FLAG) was observed (Figure 3.20A). This result was in keeping with a role in promoter repression for RBP3. However, when the RBP3 protein was fused to the VP16 trans-activation domain, a significant increase in luciferase activity was detected (approximately 1,700-fold (Figure 3.20A). This result shows that RBP-J κ /CBF1 provided with an activation domain functions as an transcriptional activator, indicating that the VP16 activation domain added *in cis* to RBP-J κ /CBF1 counter-balanced RBP-J κ /CBF1-mediated repression. The expression vector for EBNA2 was also co-transfected with the RBP-J κ /CBF1-regulated reporter construct to act as a positive control for luciferase activity.

In order to determine whether the *bfl-1* promoter was responsive to the RBP3-VP16 fusion protein, transfections were also performed using the expression vector for the fusion protein together with the *bfl-1* promoter reporter construct -1374/+81-Luc. The promoter was not found to be RBP3-VP16 responsive (Figure 3.20B).

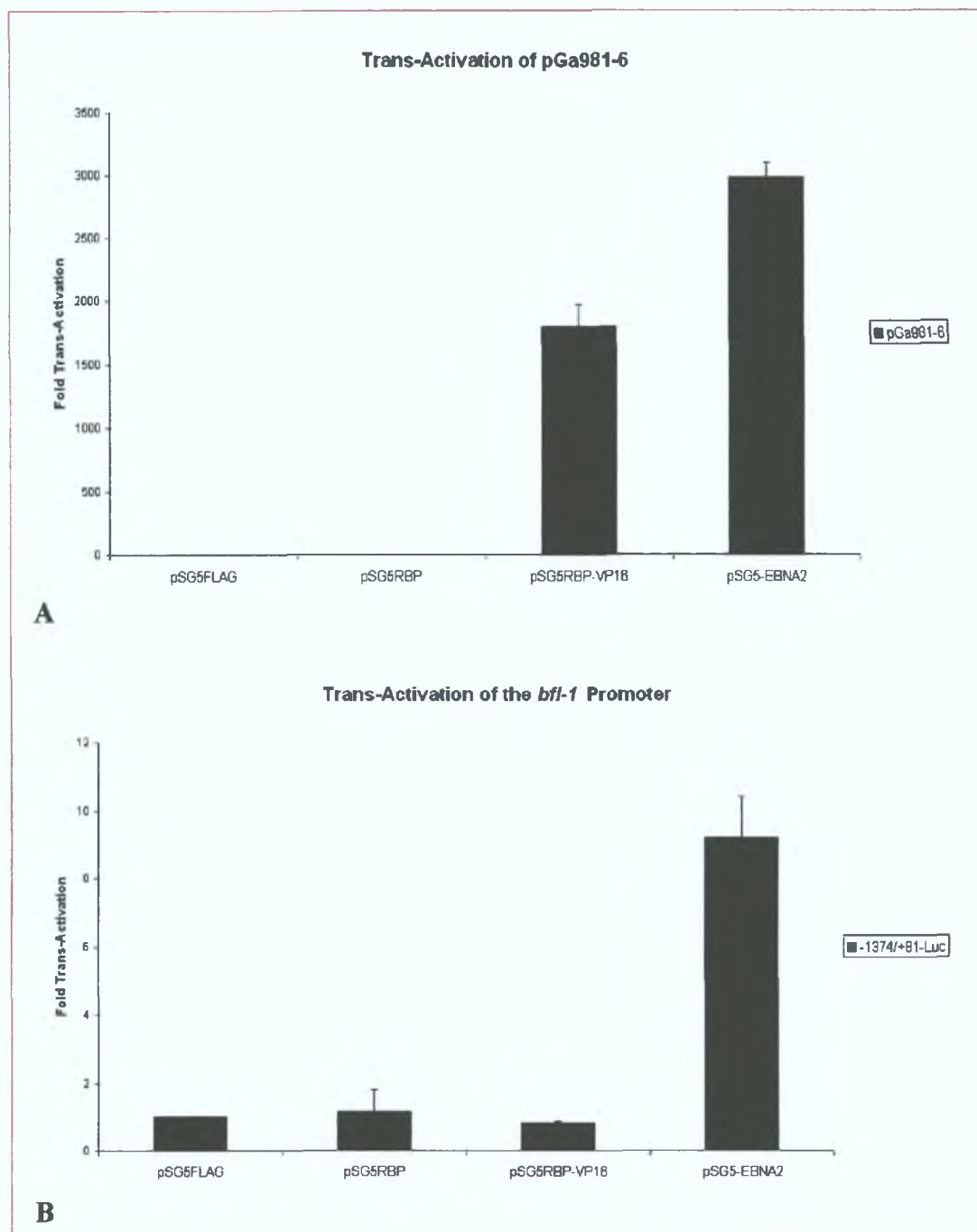


Figure 3.20: An RBP-VP16 fusion protein trans-activates the RBP-J κ /CBF1-regulated reporter construct pGa981-6, but not the *bfl-1* promoter. DG75 cells were transiently co-transfected with 2 μ g of either the luciferase reporter construct pGa981-6 (A) or the *bfl-1* promoter reporter construct -1374/+81-Luc (B) together with 7 μ g of the expression vectors indicated underneath the graphs. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the corresponding value obtained upon co-transfection with the empty vector (pSG5FLAG) along with the reporter construct.

These results suggested that providing RBP-J κ /CBF1 with a strong trans-activation domain without concomitant masking of the RBP-J κ /CBF1 repression domain, is sufficient to counter-balance RBP-J κ /CBF1-mediated repression of the pGa981-6

reporter construct. However, recruitment of a strong trans-activation domain alone is not sufficient for trans-activation of the *bfl-1* promoter in the EBV-negative BL-derived cell line DG75. It is possible that EBNA2 trans-activates transcription of the *bfl-1* promoter by (i) providing an activation domain, (ii) interfering with the function of a co-repressor recruited by RBP-J κ /CBF1 and (iii) binding to co-activator proteins.

3.2.8 *bfl-1* promoter sequence analysis

It has previously been shown in our laboratory using *bfl-1* promoter deletion reporter constructs, that the sequence from position -367 to -129 is essential in mediating EBNA2-responsiveness on the *bfl-1* promoter (Pegman *et al.*, submitted December 2004). Analysis of this region of the promoter using transcription element search software and the Alibaba transcription factor prediction database revealed a consensus binding sequence for RBP-J κ /CBF1 in the reverse orientation at position -243 to -249 relative to the transcription start site. This sequence has been found in all EBNA2 responsive promoters characterised to date (Tun *et al.*, 1994). Further analysis revealed additional 5'-GGAA-3' tracts, the core sequence motif known to be central in DNA binding of the Ets family of transcription factors (Karim, 1990; Gutman and Wasylyk, 1991). This was identified in four places between -367 and -129 on the promoter. The locations of these possible transcription factor binding sites are shown in Figure 3.23. The contribution of these sites in facilitating EBNA2-mediated trans-activation of the *bfl-1* promoter was then investigated using site directed mutagenesis to knock out the relevant transcription factor binding sites, and thus assess their potential contribution to EBNA2-responsiveness on the promoter. Mutation of any of the transcription factor binding sites resulted in a dramatic reduction in EBNA2-mediated activation of the *bfl-1* promoter (Pegman *et al.*, submitted December 2004).

To determine whether sequences from the *bfl-1* promoter could confer EBNA2-responsiveness on a heterologous minimal promoter, portions of the -367 to -40 region of the sequence were sub-cloned upstream of the minimal β -globin promoter in the luciferase reporter construct pGa50-7. The sub-cloning strategy is summarised in Figure 3.21. Firstly 3 different regions of the *bfl-1* promoter were amplified using the construct -1374/+81-Luc as template DNA. This construct contains the longest available *bfl-1* promoter sequence from position -1374 to +81 sub-cloned upstream of

the luciferase gene in promoterless vector pGL2-Basic (D'Souza *et al.*, 2004). This DNA was characterised by restriction digestion prior to PCR and the digested fragments are shown in Figure 3.22.

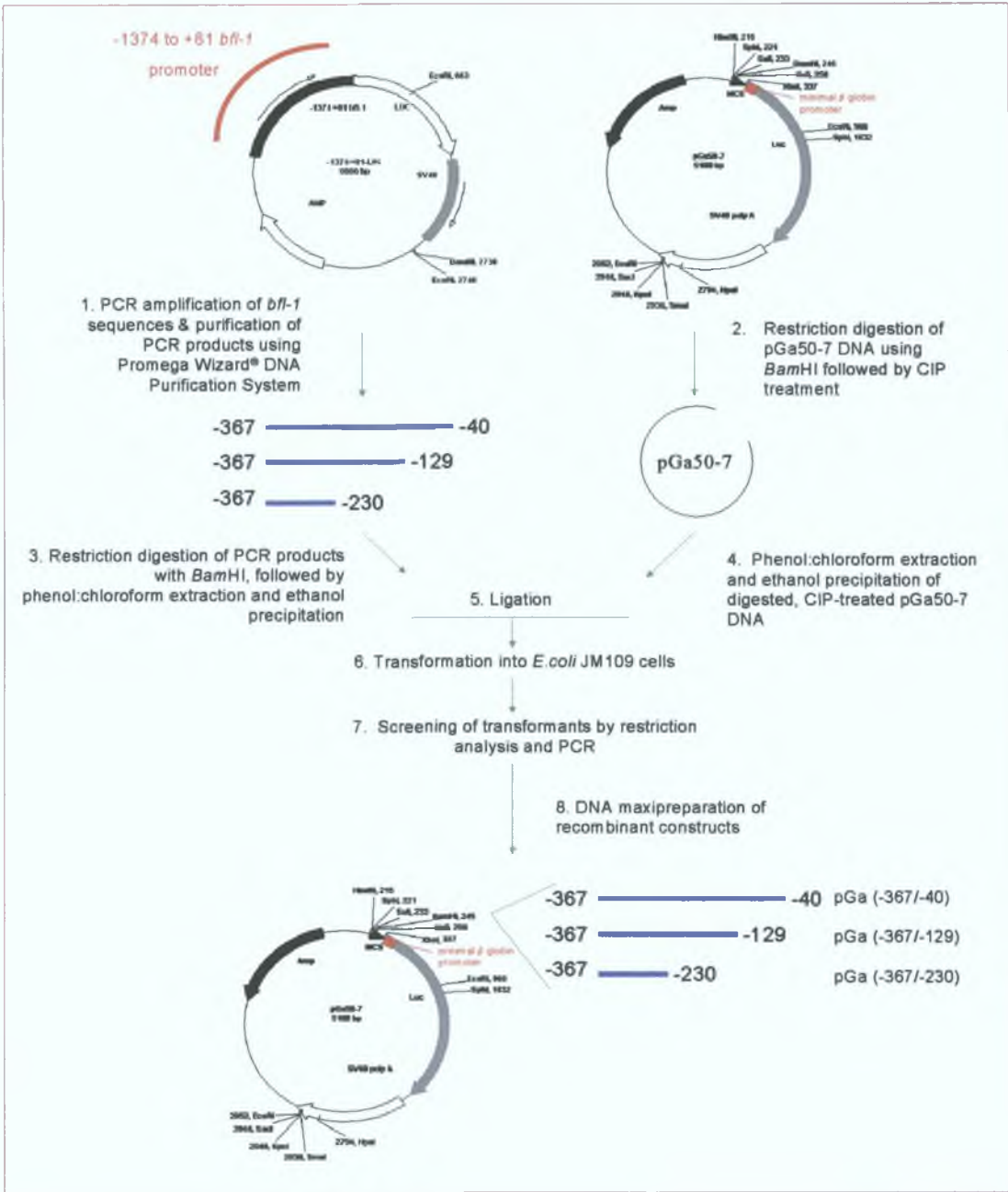


Figure 3.21: Generation of luciferase reporter constructs by sub-cloning portions of the *bfl-1* promoter into pGa50-7. Flow diagram detailing the steps involved in generating the pGa50-7 luciferase reporter constructs. Briefly, portions of the *bfl-1* promoter were amplified by PCR, digested with *Bam*HI and sub-cloned into the *Bam*HI site upstream of the β -globin minimal promoter in the pGa50-7 vector. Competent *E. coli* JM109 cells were transformed with the recombinant plasmid and a number of independently transformed colonies were selected for growth of small-scale cultures. Plasmid DNA isolated from each culture was then analysed by digestion with restriction enzymes and PCR. Large-scale DNA preparations of the recombinant plasmids were performed using the Qiagen® Midi Kit and the pGa50-7 luciferase reporter constructs were used in co-transfections with DG75 cells.

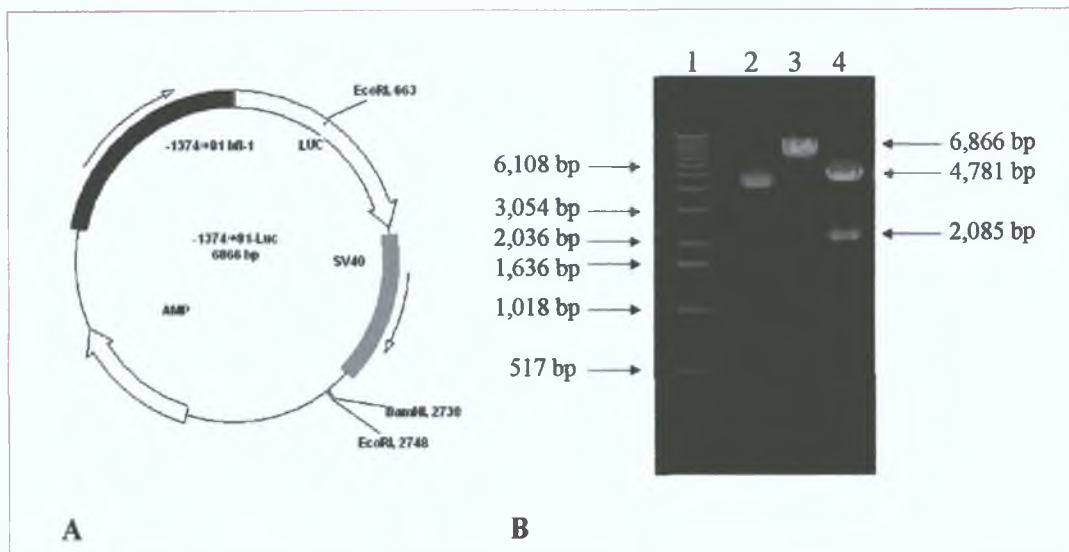


Figure 3.22: Restriction analysis of -1374/+81-Luc. (A) Schematic circular map of -1374/+81-Luc. Indicated on the map are the *Bam*HI and *Eco*RI sites targeted during restriction digestion. (B) The 6,866 bp -1374/+81-Luc plasmid was characterised prior to PCR by restriction digestion using *Bam*HI and *Eco*RI. A 1 Kb DNA ladder (Invitrogen) is shown in lane 1. Undigested DNA can be seen in lane 2. DNA linearised by restriction digestion with *Bam*HI resulted in a fragment at 6,866 bp as expected (lane 3). Restriction digestion with *Eco*RI yields two fragments of 4,781 bp and 2,085 bp (lane 4). Electrophoresis was carried out at 100 V for 1 hour using a 1 % agarose-1X TAE gel.

bfl-1 promoter sequences were amplified by PCR with a forward oligonucleotide primer from the -367 region of the *bfl-1* promoter and one of three reverse primers from various regions on the promoter (-40, -129 and -230). The locations of these primers relative to the full-length *bfl-1* promoter are indicated in Figure 3.23. All primers included a *Bam*HI restriction site (GGATCC) and a digestion clamp (CGC) at its 5' end. Details of primer sequences are shown in Table 3.1.

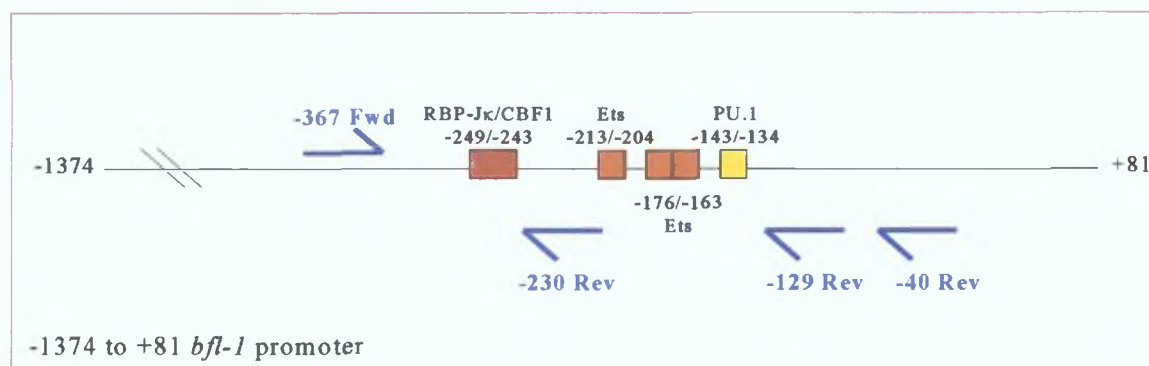


Figure 3.23: Schematic representation of the *bfl-1* promoter. The full-length *bfl-1* promoter from position -1374 to +81 relative to the transcription start site is illustrated. The locations of the putative RBP-Jκ/CBF1 (red), Ets (orange) and PU.1 (yellow) transcription factor binding sites are indicated. The positions of the primers used during PCR are also shown.

Table 3.1: Primers used for PCR amplification of *bfl-1* promoter sequences

Target	Oligonucleotide Primer Sequence		Product Size
-367/-40 promoter	<i>bfl-1</i>	(Forward) 5'-CGCGGATCCGGTGTATACCTATGAATG-3'	351 bp
		(Reverse) 5'-CGCGGATCCTGATACATGGAGGCTGGT-3'	
-367/-129 promoter	<i>bfl-1</i>	(Forward) 5'-CGCGGATCCGGTGTATACCTATGAATG-3'	240 bp
		(Reverse) 5'-CGCGGATCCAAAATGTGAAAGAGGAAA-3'	
-367/-230 promoter	<i>bfl-1</i>	(Forward) 5'-CGCGGATCCGGTGTATACCTATGAATG-3'	151 bp
		(Reverse) 5'-CGCGGATCCGAAATTAGAATCCTTGTA-3'	

Following PCR, the products were analysed by agarose gel electrophoresis to check for migration of fragments at the expected size (Figure 3.24). The PCR products were subsequently purified using the Promega Wizard® DNA purification system, digested with *Bam*HI and purified again by phenol:chloroform extraction followed by ethanol precipitation. The individual digested, purified PCR products were then sub-cloned into the *Bam*HI site of pGa50-7.

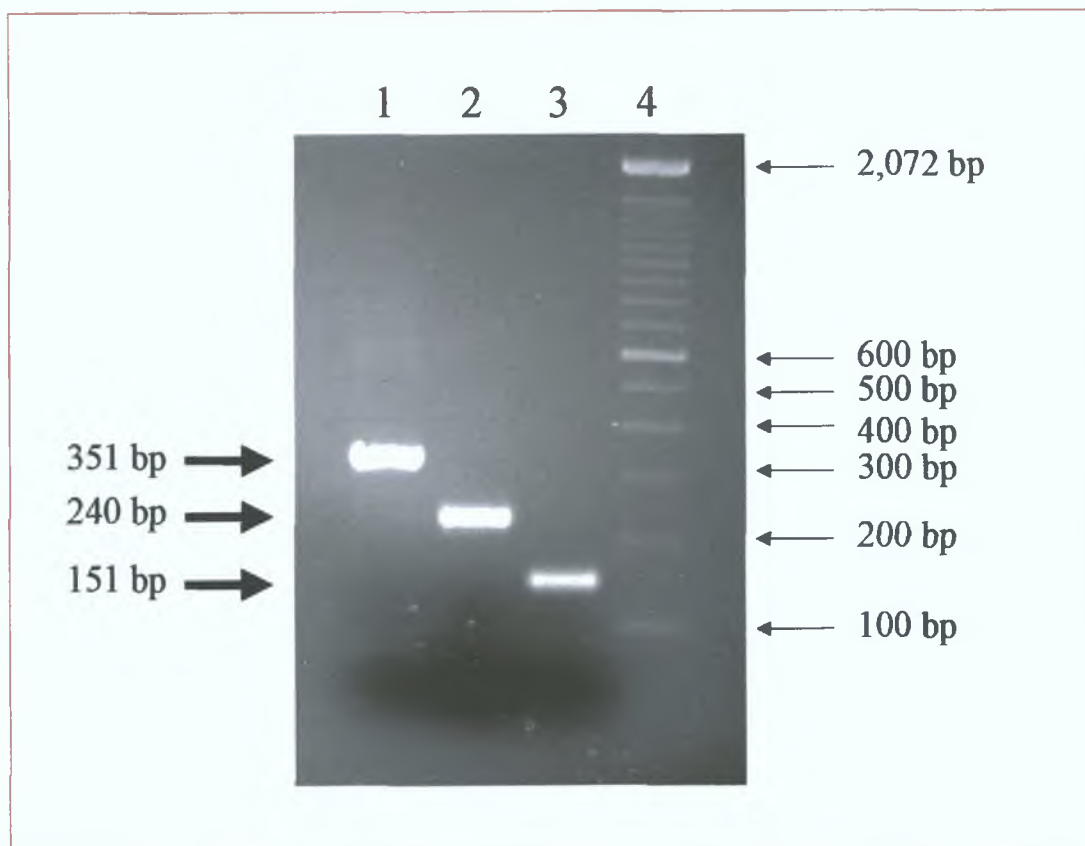


Figure 3.24: Agarose gel electrophoresis of PCR products. PCR products amplified from the expression vector for the full-length *bfl-1* promoter sequence (-1374/+81-Luc) were analysed by agarose gel electrophoresis on a 2 % agarose-1 X TAE gel and electrophoresis was carried out at 100 V for 1 hour in 1 X TAE. Amplification of the -367 to -40 region of the promoter resulted in a band at 351 bp (lane 1), amplification of the -367 to -129 region resulted in a band at 240 bp (lane 2) and amplification of the -367 to -230 region of the sequence resulted in a band at 151 bp (lane 3). A 100 bp DNA ladder (Invitrogen) is shown in lane 4.

A circular map of pGa50-7 is shown in Figure 3.25A. The plasmid was firstly characterised by restriction digestion (Figure 3.25B). Prior to ligation, the plasmid was linearised by digestion with *Bam*HI, followed by dephosphorylation using calf intestinal phosphatase (CIP) to prevent recircularisation of the linearised DNA. The linearised, CIP-treated DNA was then purified by phenol:chloroform extraction followed by ethanol precipitation. Ligations of vector and insert DNA were carried out overnight at 16°C.

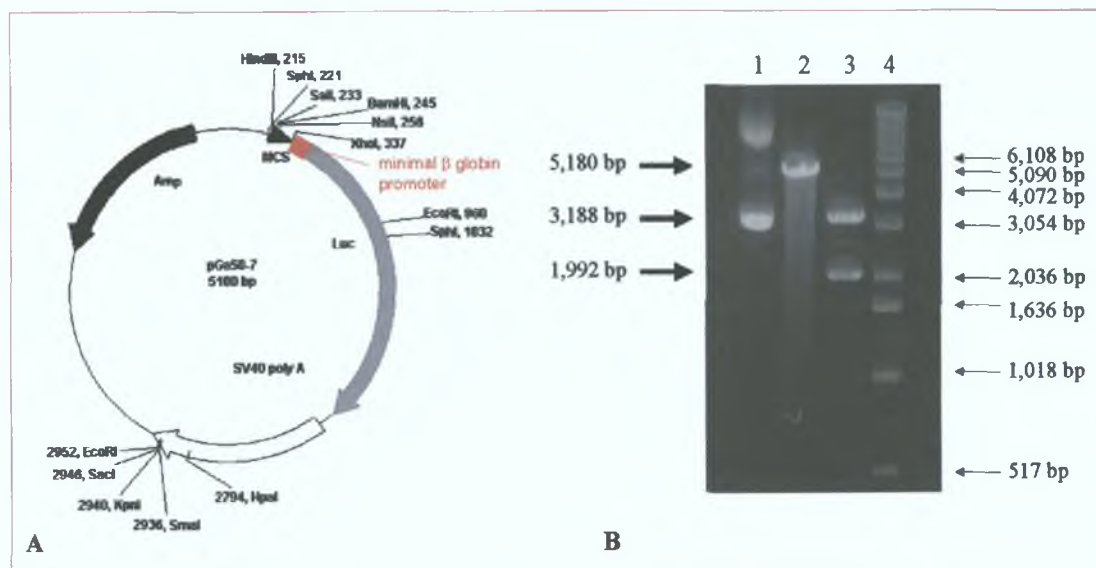


Figure 3.25: Restriction analysis of pGa50-7. (A) A schematic circular map of the pGa50-7 vector. The *Bam*HI site (into which insert DNA was sub-cloned) can be seen at position 245 on the plasmid. (B) The 5,180 bp pGa50-7 plasmid was characterised by restriction digestion. Digested samples were analysed by agarose gel electrophoresis on a 1 % agarose-1 X TAE gel and electrophoresis was carried out at 100 V for 1 hour in 1 X TAE. Uncut plasmid DNA is shown in lane 1. The plasmid was linearised by digestion with *Bam*HI yielding a band at 5,180 bp (lane 2). Digestion with *Eco*R1 resulted in two fragments at 3,208 bp and 1,972 bp as expected. A 1 Kb DNA ladder (Invitrogen) is shown in lane 4.

Following the ligation reaction, competent *E.coli* JM109 cells were transformed with the recombinant plasmid DNA. Single colonies were used to inoculate cultures from which DNA mini-preparations were generated. Restriction analysis was performed on the DNA to detect the presence of insert DNA. Because both PCR product and the pGa50-7 plasmid were digested with the *Bam*HI restriction endonuclease, it was possible for the insert DNA to ligate into the vector in either the forward or reverse orientation. To determine whether insert DNA had ligated in the forward orientation, a PCR based strategy was employed using the -367 forward primer and a reverse primer from the luciferase region of the pGa50-7 vector (Table 2.3). A PCR product would only be generated if the insert DNA had ligated in the forward orientation. To confirm correct insertion, further PCR was carried out using the individual reverse primers (i.e. -40 Rev, -129 Rev and -230 Rev) and a forward primer from the ampicillin resistance region of the pGa50-7 vector. Once it was established that the *bfl-1* sequences had ligated into pGa50-7 in the forward orientation, DNA maxi-preparations were carried out using the Qiagen® Midi Kit and restriction analysis of the resulting DNA is shown in Figure 3.26.

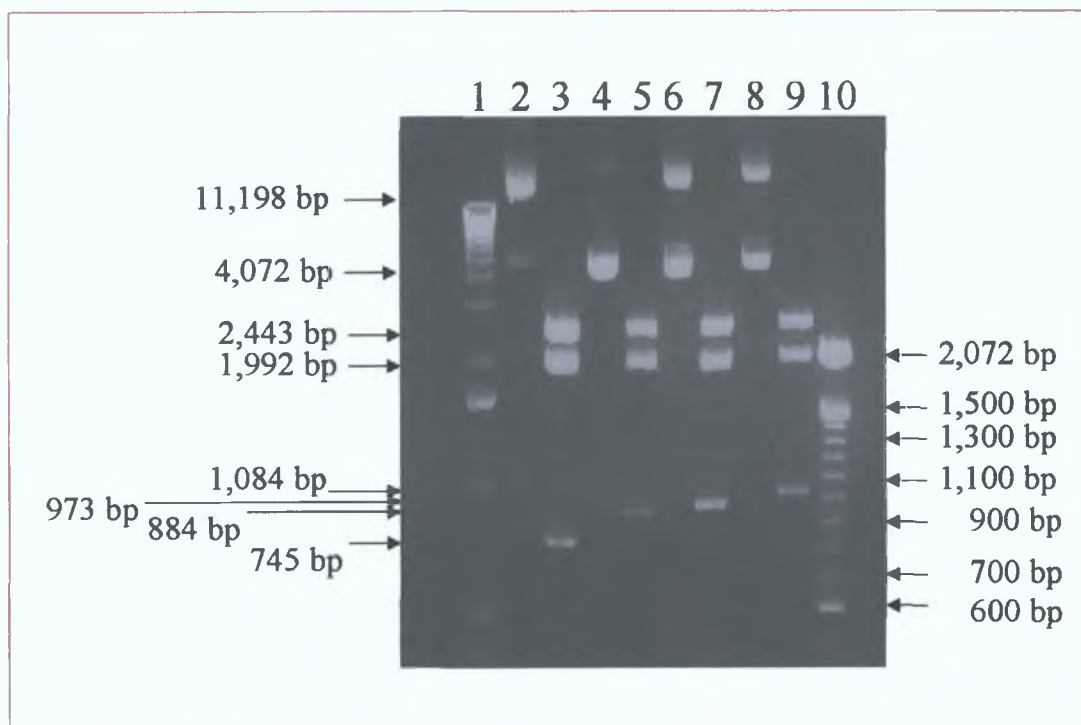


Figure 3.26: Restriction analysis of recombinant plasmid DNA. Bacterial colonies transformed with recombinant plasmid were analysed by digestion with restriction enzymes followed by agarose gel electrophoresis using a 1.2 % agarose-1X TAE gel. A 1 Kb DNA ladder (Invitrogen) is shown in lane 1. Undigested pGa50-7 DNA can be seen in lane 2. pGa50-7 digested with *EcoRI* and *HindIII* yielded three fragments at 2,443 bp, 1,992 bp and 745 bp as expected. The pGa (-367/-230) plasmid created by ligating the 139 bp digested PCR product from -1374/+81-Luc into pGa50-7 is shown undigested in lane 4. When this construct was digested with *EcoRI* and *HindIII*, three fragments at 2,433 bp, 1,992 bp (as for the empty vector) and at 884 bp were found (lane 5). This increase in fragment size from 745 bp to 884 bp indicates the presence of insert DNA. The pGa (-367/-129) plasmid created by ligating the 228 bp digested PCR product from -1374/+81-Luc into pGa50-7 is shown undigested in lane 6. Digestion of this construct with *EcoRI* and *HindIII* yielded fragments at 2,433, 1,992 bp and 973 bp. The pGa (-367/-40) construct created by ligating the digested 339 bp PCR product from -1374/+81-Luc into pGa50-7 is shown undigested in lane 8. Digestion of this construct with the same restriction endonucleases yielded fragments at 2,433 bp, 1,992 bp and 1,084 bp. A 100 bp DNA ladder (Invitrogen) is shown in lane 10.

Transient co-transfections of the pGa50-7 luciferase reporter constructs (Figure 3.27A) and the expression vector for EBNA2 (pSG5-EBNA2) were performed using DG75 cells (Figure 3.27B). As expected EBNA2 expression trans-activated the full-length *bfl-1* promoter (approximately 11-fold). However, none of the pGa50-7 luciferase reporter constructs were found to be EBNA2-responsive.

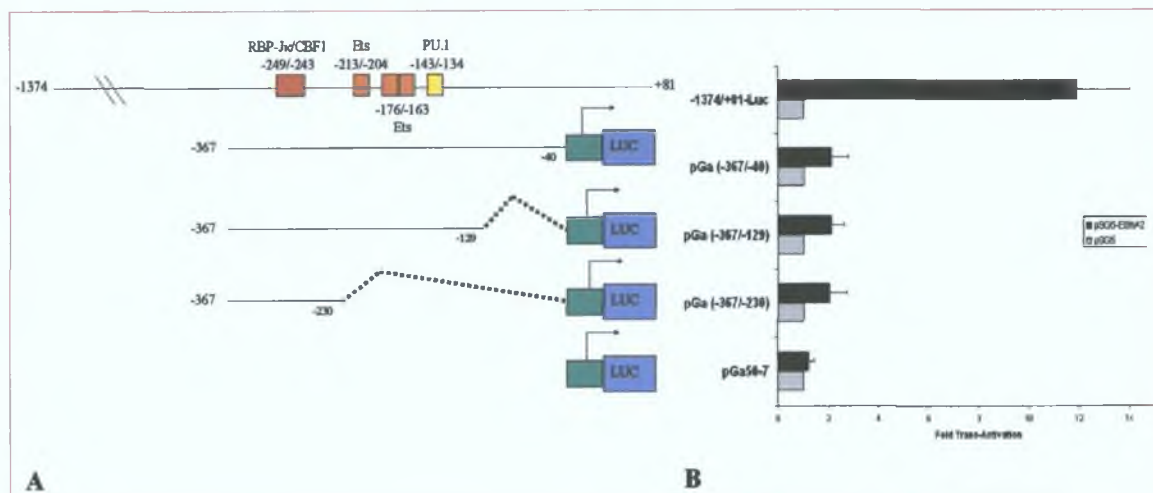


Figure 3.27: The recombinant luciferase reporter constructs are not EBNA2-responsive. (A) Schematic representation of the recombinant luciferase reporter constructs generated after sub-cloning portions of the *bfl-1* promoter upstream of the β -globin minimal promoter (green box with bent arrow) in pGa50-7. The name of each reporter construct includes the relevant 5' and 3' ends of the *bfl-1* sequence. The upper panel shows the full-length *bfl-1* promoter. Putative RBP-J κ /CBF1 (red), Ets (orange) and PU.1 transcription factor binding sites are indicated. The constructs pGa (-367/-40), pGa (-367/-129), pGa (-367/-230) and pGa50-7 are also shown. (B) DG75 cells were co-transfected with 7 μ g of either pSG5 or pSG5-EBNA2 along with 2 μ g of reporter construct indicated to the left of the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (n-fold) relative to the corresponding value obtained upon co-transfection with the empty vector (pSG5).

These results indicated that the -367 to -40 region of the *bfl-1* promoter does not function as an EBNA2-dependent transcriptional enhancer in the EBV-negative BL-derived cell line DG75.

3.2.9 Identification of an EBNA2 mutant demonstrating dominant-negative activity

This study set out to investigate the ability of a set of established EBNA2 mutants to impair wild type EBNA2-mediated promoter activation, with a view to identifying an EBNA2 mutant that demonstrated dominant-negative activity. The application of such an inhibitor molecule would prove useful in further investigating the function of EBNA2, especially in EBV-infected cells, and would possibly have useful therapeutic applications. The first mutant investigated (EBNA2-WW323SR, Figure 3.28A) was identical to wild type EBV B95.8 EBNA2 except that two tryptophan residues at positions 323 and 324 were mutated to serine and arginine respectively (Ling *et al.*,

1993). Therefore this form of EBNA2 is unable to bind RBP-J κ /CBF1. The ability of EBNA2-WW323SR to activate the RBP-J κ /CBF1-regulated control promoter pGa981-6 was tested. It was demonstrated that wild type EBNA2 trans-activated this promoter efficiently (approximately 2,700-fold when 5 μ g of expression vector was used, Figure 3.28B). However, upon co-transfection of 7 μ g of EBNA2-WW323SR with the luciferase reporter construct, no activation was observed. Since EBNA2-WW323SR did not trans-activate pGa981-6, it was postulated that it might be able to inhibit wild type EBNA2 function. To test this hypothesis co-transfections were performed using a constant amount of expression vector for wild type EBNA2 (pSG5-EBNA2) and various amounts of EBNA2-WW323SR along with the pGa981-6. EBNA2-WW323SR was not seen to inhibit wild type EBNA2-mediated activation of this reporter construct (Figure 3.28B).

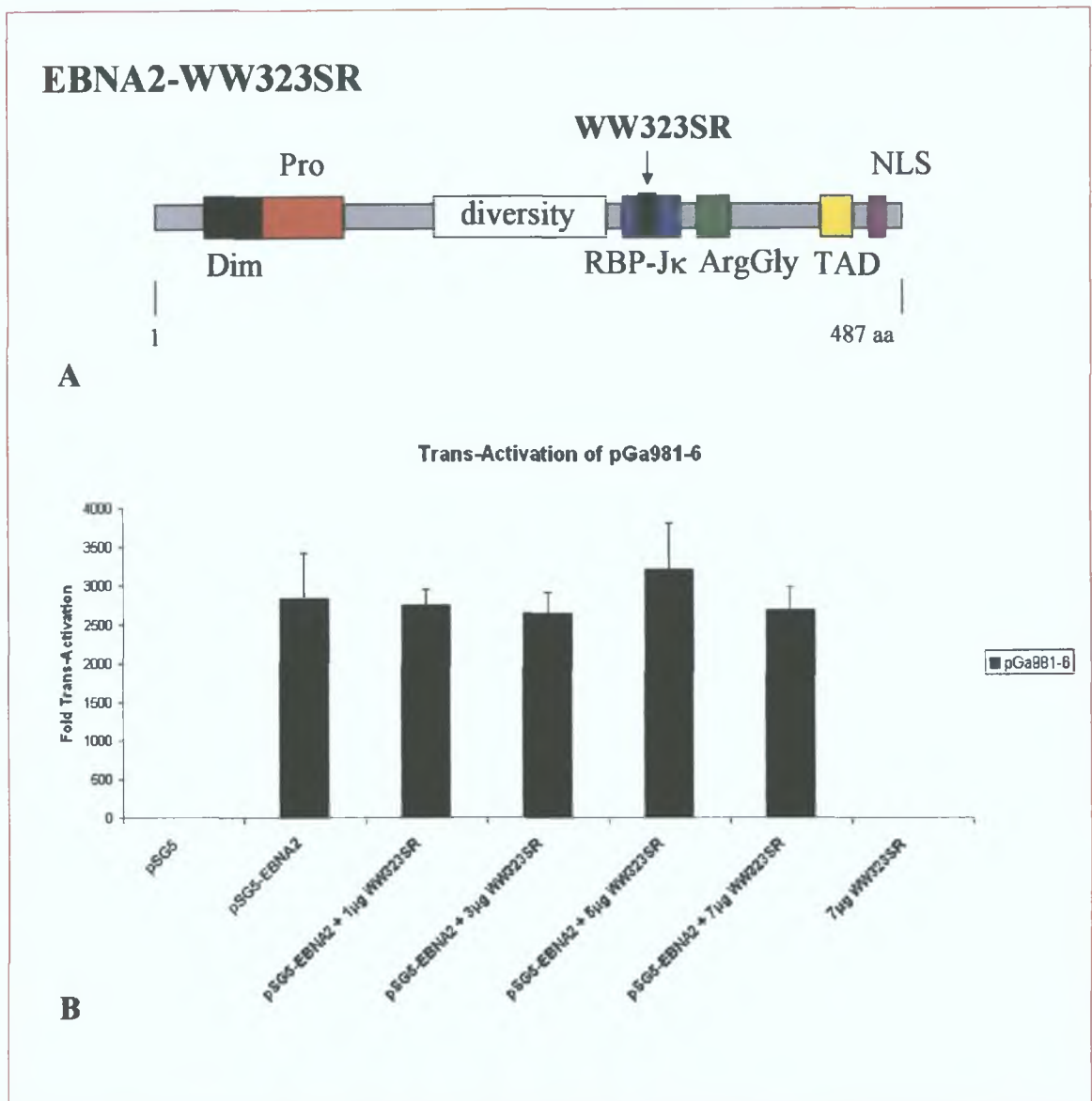


Figure 3.28: The EBNA2 mutant EBNA2-WW323SR does not inhibit EBNA2-mediated trans-activation of the luciferase reporter construct pGa981-6. (A) A schematic representation of the EBNA2 mutant EBNA2-WW323SR, which is identical to wild type EBNA2 except that two tryptophan residues at positions 323 and 324 have been replaced with serine and arginine respectively (indicated by a black rectangle). Therefore, this mutant form of EBNA2 is unable to bind RBP-Jκ/CBF1. (B) DG75 cells were co-transfected with a constant quantity of EBNA2 (5 μg) and/or various quantities of the expression vector for EBNA2-WW323SR together with 2 μg of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (n-fold) relative to the value obtained upon co-transfection with the empty vector (pSG5).

A second EBNA2 mutant was investigated for its ability to inhibit wild type EBNA2 function. This mutant (EBNA2 Mut (321-323), Figure 3.29A) contains deletions of the two tryptophan residues at position 322 and 323 (Waltzer *et al.*, 1995). As this deletion is located in the RBP-Jκ/CBF1 interaction domain, this mutant form of EBNA2 is unable to bind RBP-Jκ/CBF1. The ability of EBNA2 Mut (321-323) to activate the

RBP-J κ /CBF1-regulated control promoter pGa981-6 was tested. It was found that wild type EBNA2 trans-activated this promoter efficiently (approximately 2,000-fold when 5 μ g of expression vector was used, Figure 3.29B). However, upon co-transfection using 7 μ g of EBNA2 Mut (321-323) with the pGa981-6, no activation was observed. Even though EBNA2 Mut (321-323) was unable to trans-activate the luciferase reporter construct, it was not seen to inhibit wild type EBNA2 activation of this promoter, even when up to 7 μ g of the mutant was used in co-transfections (Figure 3.29B).

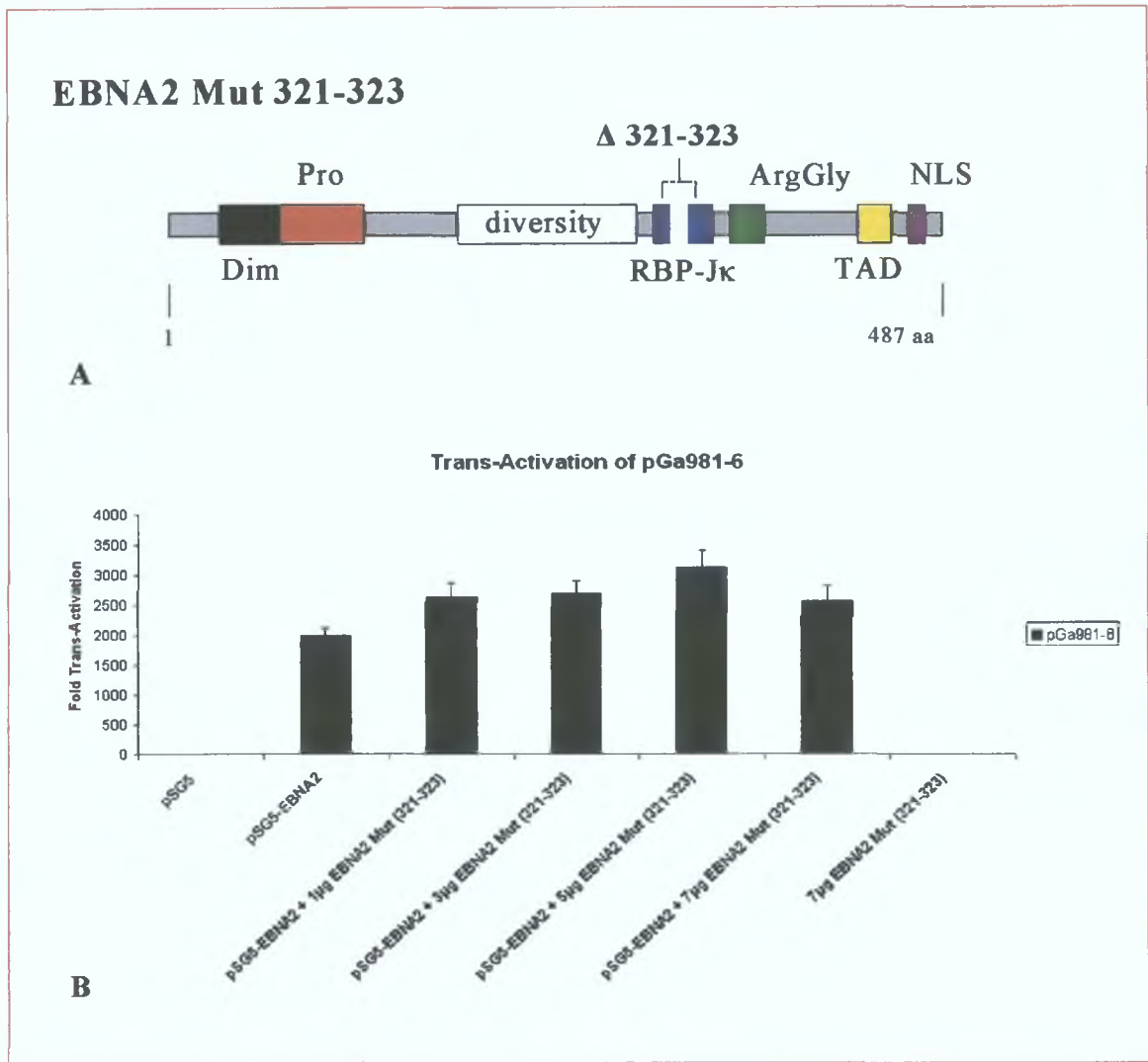


Figure 3.29: The EBNA2 mutant EBNA2 Mut (321-323) does not inhibit EBNA2-mediated trans-activation of the luciferase reporter construct pGa981-6. (A) A schematic representation of EBNA2 Mut (321-323), which is identical to wild type EBNA2 except that it contains an in-frame deletion from amino acids 321-323 (indicated by the orange rectangle) and therefore, cannot bind to RBP-J κ /CBF1. (B) DG75 cells were co-transfected with a constant quantity of EBNA2 (5 μ g) and/or various quantities EBNA2 Mut (321-323) together with 2 μ g of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the value obtained upon co-transfection with the empty vector (pSG5).

A third EBNA2 mutant was investigated for dominant-negative activity over wild type EBNA2. This mutant (EBNA2 Mut (447-472), Figure 3.30A) is identical to wild type EBNA2 except that it bears a trans-activation domain deletion from amino acids 447-472. When this mutant was co-transfected with the luciferase reporter construct pGa981-6 in DG75 cells, no activation was observed (Figure 3.30B). This contrasted with the result obtained when wild type EBNA2 was co-transfected with the reporter construct, where approximately 2,300-fold trans-activation was seen. When increasing amounts of EBNA2 Mut (447-472) were co-transfected with a constant quantity of wild type EBNA2 along with the reporter construct, a dose-dependent inhibition was observed. A maximum inhibition of approximately 66 % resulted when 12 μ g of the mutant were co-transfected along with EBNA2.

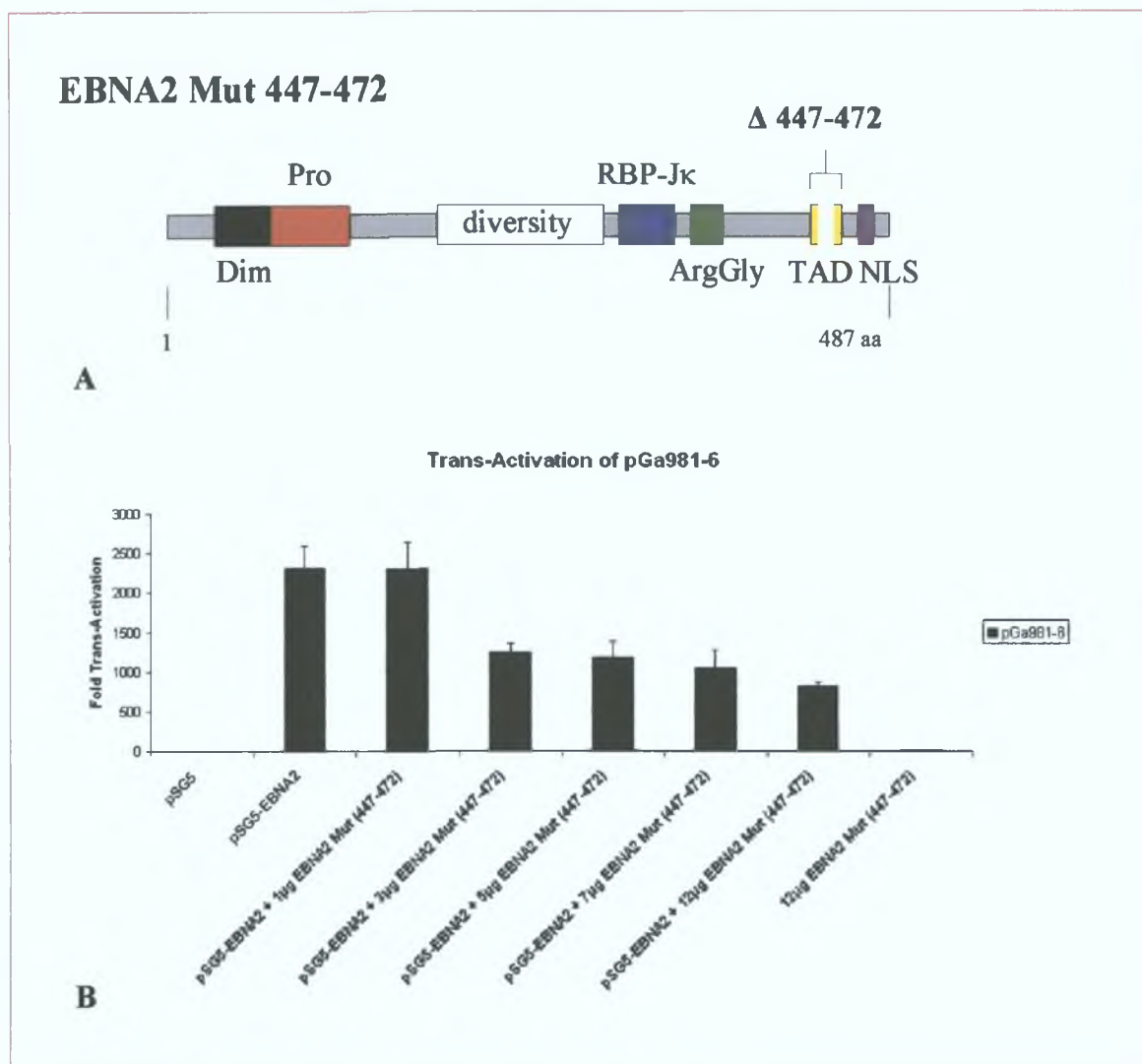


Figure 3.30: The EBNA2 mutant EBNA2 Mut (447-472) inhibits EBNA2-mediated trans-activation of the luciferase reporter construct pGa981-6. (A) A schematic representation of EBNA2 Mut (447-472), which is identical to wild type EBNA2 except that it contains a trans-activation domain deletion from amino acids 447 to 472 (indicated by orange rectangle). (B) Co-transfections were performed using DG75 cells with a constant quantity of EBNA2 (5 μ g) and/or various quantities EBNA2 Mut (447-472) together with 2 μ g of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the value obtained upon co-transfection with the empty vector (pSG5).

The final EBNA2 mutant investigated for dominant-negative activity also contained a deletion in the trans-activation domain (EBNA2 Mut (437-477), Figure 3.31A). In the case of EBNA2 Mut (437-477), an in-frame deletion is present from amino acids 437 to 477 (Waltzer *et al.*, 1995). This mutant was unable to trans-activate the reporter construct (Figure 3.31B). This contrasted with the result obtained when wild type EBNA2 was co-transfected with the reporter construct, where approximately 2,250-fold trans-activation was observed. When increasing amounts of EBNA2 Mut (437-477) were co-transfected with a constant quantity of wild type EBNA2 along with the

reporter construct, a dose-dependent inhibition was observed. A maximum inhibition of approximately 90 % resulted when 12 μ g of the mutant were co-transfected along with EBNA2. Of all the EBNA2 mutants tested, EBNA2 Mut (437-477) was the most efficient dominant inhibitor of wild type EBNA2-mediated activation of the RBP-J κ /CBF1-regulated control promoter pGa981-6 in DG75 cells.

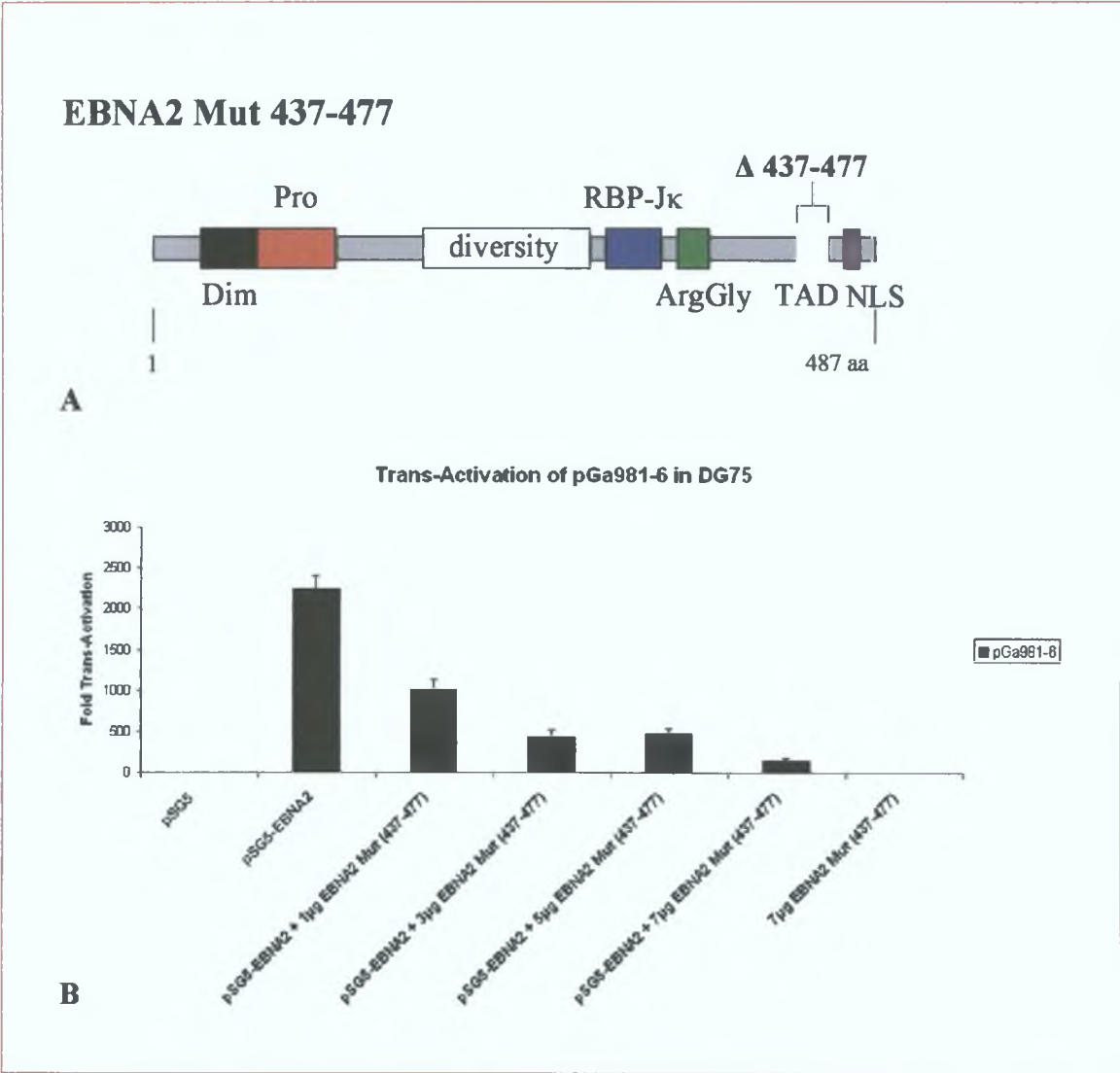


Figure 3.31: The EBNA2 mutant EBNA2 Mut (437-477) inhibits EBNA2-mediated trans-activation of the luciferase reporter construct pGa981-6. (A) A schematic representation of EBNA2 Mut (447-472), which is identical to wild type EBNA2 except that it contains an in-frame deletion in the trans-activation domain from amino acids 437 to 477. (B) Co-transfections were performed using DG75 cells with a constant quantity of EBNA2 (5 μ g) and/or various quantities EBNA2 Mut (437-477) together with 2 μ g of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the value obtained upon co-transfection with the empty vector (pSG5).

To strengthen the observation that EBNA2 Mut (437-477) was an effective inhibitor of wild type EBNA2 activity, its ability to inhibit EBNA2-mediated trans-activation of both the cellular *bfl-1* promoter and the viral *LMP1* promoter was investigated. To this end, co-transfections were conducted using equal quantities of the expression vectors for EBNA2 and EBNA2 Mut (437-477) together with the *bfl-1* promoter reporter construct -1374/+81-Luc (Figure 3.32A) or the *LMP1* promoter reporter construct pLLO-luc (Figure 3.32B). It was observed that EBNA2 Mut (437-477) dramatically inhibited EBNA2-mediated trans-activation of both the *bfl-1* and *LMP1* promoters when co-expressed with wild type EBNA2.

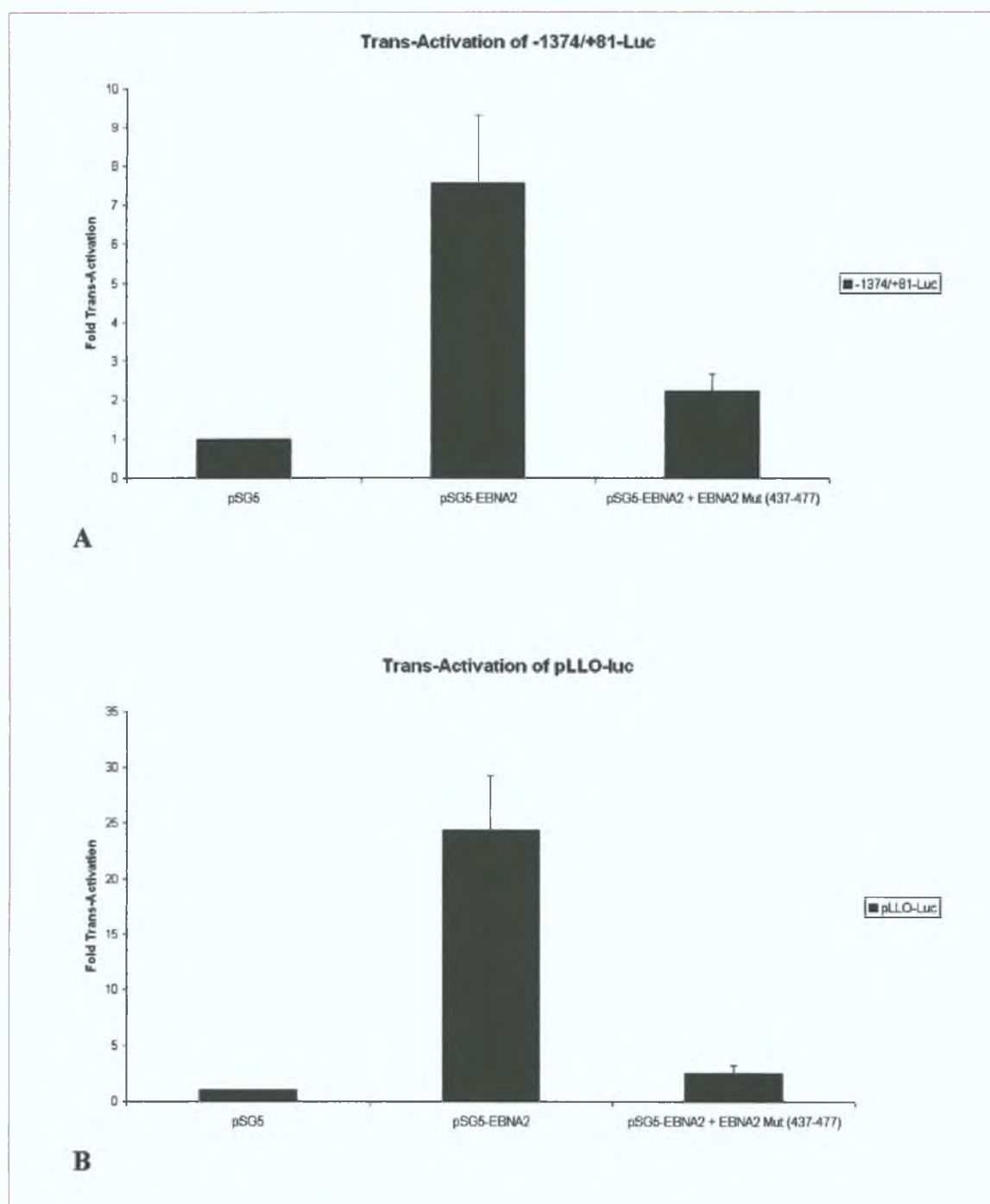


Figure 3.32: The EBNA2 mutant EBNA2 Mut (437-477) inhibits EBNA2-mediated trans-activation of the *bfl-1* promoter and the *LMP1* promoter. Co-transfections were performed using DG75 cells with equal amounts (7 μ g) of EBNA2 and EBNA2 Mut (437-477) together with 2 μ g of either the *bfl-1* promoter reporter construct -1374/+81-Luc (A) or the *LMP1* promoter reporter construct pLLO-luc (B). Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as activation (*n*-fold) relative to the value obtained upon co-transfection with the empty vector (pSG5).

Taken together, this set of data demonstrated that an EBNA2 mutant with a deletion in the trans-activation domain [(EBNA2 Mut (437-477))], efficiently impaired EBNA2-mediated activation of the luciferase reporter construct pGa981-6, the *bfl-1* promoter and the *LMP1* promoter in the EBV-negative BL-derived cell line DG75. This mutant

form of EBNA2 would prove a useful tool in elucidating the role of EBNA2 in *bfl-1* regulation in EBV-infected cells.

3.3 DISCUSSION

It has already been shown in our laboratory that expression of LMP1 in EBV-negative BL-derived cell lines coincides with a dramatic increase in the level of *bfl-1* mRNA (D'Souza *et al.*, 2000), and that LMP1 trans-activates the *bfl-1* promoter in this cell context (D'Souza *et al.*, 2004). Additionally, an important role for the transcription factor NF- κ B was implied in the LMP1-mediated regulation of the *bfl-1* promoter in this study. Firstly, co-expression of a super-repressor I κ B α DN mutant inhibited LMP1-mediated trans-activation of the *bfl-1* promoter. Secondly, treatment with PMA, a well-known activator of NF- κ B in many cell types, stimulated *bfl-1* promoter activity (D'Souza *et al.*, 2004). The implication of a role for NF- κ B is in keeping with other studies, where increases in *bfl-1* mRNA levels have been demonstrated in response to agents such as LPS, TNF α or etoposide, all of which are known to lead to activation of NF- κ B (Hu *et al.*, 1998; Wang *et al.*, 1999; Zong *et al.*, 1999). In addition, mitogen stimulation of primary B cells was shown to induce expression of mouse A1 (the murine *bfl-1* homologue) in a c-Rel-dependent fashion (Grumont *et al.*, 1999). Elsewhere, ectopic expression of the NF- κ B subunits c-Rel and p65 (but not p50) was also shown to independently up-regulate *bfl-1* mRNA levels in the epithelial cell line HeLa (Zong *et al.*, 1999). In the same study, it was shown that c-Rel trans-activated the *bfl-1* promoter through its interaction with a consensus NF- κ B binding site at position -833 to -823 relative to the transcription start site of the *bfl-1* gene. However, experiments performed in our laboratory demonstrated that removal of this binding site did not affect the level of LMP1-mediated *bfl-1* promoter activity in EBV-negative BL-derived cell lines (D'Souza *et al.*, 2004). Subsequently it was found that a 210 bp *bfl-1* promoter fragment (-129 to +81) mediated NF- κ B-dependent trans-activation by both LMP1 and PMA in this cell context. Furthermore, it was demonstrated that an NF- κ B-like binding site at position -52 to -43 relative to the transcription start site of the *bfl-1* gene, is essential for LMP1-mediated trans-activation of the *bfl-1* promoter (D'Souza *et al.*, 2004).

Regulation of NF- κ B-dependent transcription displays several levels of complexity. A number of NF- κ B sites have been found in association with various cellular and viral genes. Although these sites are recognised by NF- κ B, they exhibit considerable DNA sequence variation (Perkins *et al.*, 1992). In addition, the sub-unit composition of NF- κ B can greatly influence the ability of this transcription factor to bind a particular DNA sequence and the extent of promoter activation (Perkins *et al.*, 1992). Specific combinations of Rel proteins can therefore distinguish between variant NF- κ B sites. In the current study, it was demonstrated that homo-dimers of p65 directly trans-activated the 210 bp *bfl-1* promoter fragment (-129 to +81 relative to the transcription start site) and that the NF- κ B-like binding site at -52 to -43 was essential both for this effect, and for conferring p65-responsiveness on a heterologous minimal promoter. Classic NF- κ B (p65/p50) binds the sequence 5'-GGGRNNYYCC-3', whereas the p65/c-Rel dimer binds to the sequence 5'-HGGARNYYCC-3' [H indicates A, C or T; R is purine (A or G); and Y is pyrimidine (T or C)] (Baldwin, 1996). The NF- κ B site at position -52 to -43 on the *bfl-1* promoter differs from the p65/p50 and p65/c-Rel consensus binding sites at nucleotide positions 3 and 2 respectively. NF- κ B-like sites that diverge from the consensus sequence have been identified in the promoters of several apoptosis-related genes, such as *bcl-xL*, *c-IAP1*, *c-IAP2* and mouse *A1*, as well as cytokine genes such as *IL-8*, and the functionality of these sites in the promoter activation has been demonstrated in several of these cases (Chen *et al.*, 1999a; Grumont *et al.*, 1999; Hong *et al.*, 2000; Okamoto *et al.*, 1994). Significantly, the -52 to -43 site on the *bfl-1* promoter is 100 % complimentary to a CD28-responsive κ B site in the *IL-2* promoter (Civil *et al.*, 1996; Civil *et al.*, 1999; Verweij *et al.*, 1991) and both c-Rel and p65 have been shown to play roles in mediating this process (Ghosh *et al.*, 1993).

The previously identified NF- κ B binding site at position -833 to -823 relative to the transcription start site of the *bfl-1* gene is regulated by c-Rel in the HeLa epithelial cell line (Zong *et al.*, 1999). It has already been shown in our laboratory that, in the context of EBV-negative BL-derived cells, that removal of this -833 to -823 site by deletion or mutation did not affect LMP1-associated activation of the *bfl-1* promoter (D'Souza *et al.*, 2004). In the current study, p65 trans-activated a 210 bp *bfl-1* promoter fragment (-129 to +81) that did not contain the -833 to -823 site, indicating that this previously identified NF- κ B binding site is not essential for p65-mediated trans-activation of the *bfl-1* promoter in DG75 cells. Although LMP1 activates different subsets of NF- κ B subunit proteins in a cell-type dependent manner (Chien and Hammaraskjold, 2000;

Herrero *et al.*, 1995; Paine *et al.*, 1995), c-Rel is known to be LMP1-responsive in the context of the B cell, including in DG75 cells (Chien and Hammarskjold, 2000; Liljeholm *et al.*, 1998). Because the -833 to -823 site was not essential for mediating trans-activation of the *bfl-1* promoter by p65, and the fact that c-Rel failed to activate luciferase expression via the -52 to -43 site, this may indicate that p65, and not c-Rel, is the predominant subunit involved in regulating the *bfl-1* promoter in EBV-negative BL cells in response to LMP1. The failure of LMP1 to trans-activate via the -833 to -823 site might therefore be due to its low affinity for p65. It is also possible, however, that access to this site may be obstructed in EBV-negative BL cells by lymphocyte-specific nuclear proteins binding in its vicinity.

It has also recently been demonstrated in our laboratory that induction of EBNA2 in the cell line DG75-tTA-EBNA2 (Floettmann *et al.*, 1996) coincides with an increase in the level of *bfl-1* mRNA (Pegman *et al.*, submitted December, 2004), indicating for the first time that a second EBV protein modulates expression of the *bfl-1* gene in a system where LMP1 is not present. Furthermore, EBNA2 trans-activates the *bfl-1* promoter in EBV-negative BL-derived cell lines. The DNA binding protein RBP-J κ /CBF1 was found to be essential for this effect, as over-expression of an EBNA2 mutant (EBNA2-WW323SR), that cannot bind RBP-J κ /CBF1, did not lead to trans-activation of the *bfl-1* promoter (Pegman *et al.*, submitted December, 2004). The observation that RBP-J κ /CBF1 is required for EBNA2-mediated trans-activation was strengthened in the current study, when it was shown that co-expression of an RBP-J κ /CBF1 replacement mutant (pEFBOSneo-R218H, Chung *et al.*, 1994), that is unable to bind DNA, inhibited EBNA2-responsiveness of the *bfl-1* promoter. The RBP-J κ /CBF1 mutant could compete with endogenous wild type RBP-J κ /CBF1 for binding to EBNA2. This mutant has been shown by EMSA to reduce the DNA binding activity of RBP-J κ /CBF1 to 2 % (Chung *et al.*, 1994) and block both EBNA2- and NotchIC-mediated trans-activation of the *HES1* promoter in COS7 cells (Kato *et al.*, 1997; Sakai *et al.*, 1998). In addition, co-expression of the non DNA-binding RBP-J κ /CBF1 mutant blocked EBNA2-mediated transcriptional activation of the *LMP1* promoter in the current study, demonstrating that RBP-J κ /CBF1 is also essential for EBNA2-mediated activation of this viral promoter. The observation that RBP-J κ /CBF1 is required for *LMP1* promoter activity is in keeping with the findings of others, who have demonstrated that mutation of the RBP-J κ /CBF1 binding site in the *LMP1* promoter dramatically decreases EBNA2-responsiveness (Laux *et al.*, 1994b; Johannsen *et al.*, 1995).

Because *bfl-1* is a known NF- κ B-responsive gene, and recent evidence has demonstrated cell-context specific interplay between the NF- κ B and Notch signalling pathways (Guan *et al.*, 1996; Bash *et al.*, 1999; Wang *et al.*, 2001 Oakley *et al.*, 2003), it was therefore necessary to monitor the level of activated NF- κ B in DG75 cells when EBNA2 was expressed. EBNA2 was not seen to affect the level of activated NF- κ B in the context of the EBV-negative BL-derived cell line DG75. Furthermore, over-expression of an I κ B α super-repressor did not affect the ability of EBNA2 to trans-activate the *bfl-1* gene. By contrast, it was shown in the current study that LMP1 expression resulted in the activation of the NF- κ B transcription factor, and it has already been shown in our laboratory that over-expression of the I κ B α super-repressor inhibits LMP1-mediated trans-activation of *bfl-1* (D'Souza *et al.*, 2004). These findings suggest that, unlike LMP1, EBNA2-mediated trans-activation of the *bfl-1* promoter does not lead to the activation of NF- κ B.

Because both EBNA2 and NotchIC trans-activate genes by interacting with the DNA binding transcription factor RBP-J κ /CBF1, they are often regarded as functional homologues. Although there is no obvious sequence homology between these two proteins, they interact with similar regions of RBP-J κ /CBF1 and replace repressor proteins with their trans-activation domains. Moreover, there are parallels between EBNA2 and NotchIC in the ways in which transcriptional activation through RBP-J κ /CBF1 is modified. Both EBNA2 and NotchIC interact with co-activating proteins to modulate gene expression. These co-activating proteins include members of the HAT family of proteins (Zimber-Strobl and Strobl, 2001), which play a role in chromatin opening and initiation of transcription. Gene regulation by both proteins is regulated, in part, by loss of RBP-J κ /CBF1 binding as a result of antagonistic proteins that prevent either EBNA2 or NotchIC binding to RBP-J κ /CBF1 (Zimber-Strobl and Strobl, 2001). Since both EBNA2 and NotchIC share common mechanisms for regulating target gene expression, it is not surprising that they also overlap in the genes that they regulate. Both proteins have been found to modulate the same RBP-J κ /CBF1-regulated genes. Common gene targets include the EBV LMP2A (Strobl *et al.*, 2000) and the cellular genes BATF (Johansen *et al.*, 2003), CD21 (Strobl *et al.*, 2000), Hes1 (Sakai *et al.*, 1998; Kuroda *et al.*, 1999) and the Ig enhancer (Strobl *et al.*, 2000; Morimura *et al.*, 2001). However, although the set of promoters, which is regulated by EBNA2 and NotchIC is overlapping, it is not identical. Only EBNA2, but not NotchIC, can induce LMP1, CD23 and c-Myc expression (Strobl *et al.*, 2000). The results presented here

also demonstrate that the *LMP1* promoter is not NotchIC-responsive, and extend the list of genes that are responsive to EBNA2, but not to NotchIC, to include the anti-apoptotic cellular gene *bfl-1*.

Although the NotchIC trans-activation domain is considered weaker than that of EBNA2 (Hsieh *et al.*, 1996), this was not found to be the reason for the inability of NotchIC to trans-activate the *bfl-1* promoter. Even when the trans-activation domain and nuclear localisation sequence of EBNA2 were fused to the NotchIC protein, it was not sufficient to drive transcription from the *bfl-1* promoter in the EBV-negative BL-derived cell line DG75. It was shown that the presence of a strong trans-activation alone is not sufficient for transcriptional activation of the *bfl-1* gene. Because RBP-J κ /CBF1 is a transcriptional repressor that tethers a co-repressor complex to promoters with cognate J κ binding sites, it was tested whether providing RBP-J κ /CBF1 with the strong VP16 trans-activation domain would be sufficient to counter-balance repression. This system mimics the recruitment of an activation domain by RBP-J κ /CBF1 without concomitant masking of the RBP-J κ repression domain. Although this fusion protein was seen to trans-activate the control promoter, it did not trans-activate the *bfl-1* promoter, in contrast to EBNA2. These observations suggest that the mechanism by which EBNA2 trans-activates the *bfl-1* promoter involves more than simply providing a trans-activation domain. It is likely that EBNA2 mediates trans-activation of *bfl-1* by interfering with the function of a co-repressor in addition to the recruitment of a trans-activation domain. This mechanism of EBNA2-mediated promoter activation is in keeping with the findings of others (Hsieh *et al.*, 1995; Waltzer *et al.*, 1995). Another possible reason for the inability of the RBP3-VP16 fusion protein to trans-activate the *bfl-1* promoter, could be that the RBP-J κ /CBF1 isoform (RBP3) present in the fusion protein does not bind to the RBP-J κ /CBF1 binding site in the *bfl-1* promoter with high affinity. Although the RBP-J κ /CBF1 consensus binding motif is present in the *bfl-1* promoter, it has been reported that nucleotides on both sides of the core sequence may contribute to maximal DNA binding (Chung *et al.*, 1994).

Because NotchIC is also thought to mediate promoter trans-activation by interfering with RBP-J κ /CBF1-mediated repression and providing a trans-activation (Hayward, 2004), and yet still cannot trans-activate the *bfl-1* promoter, it is likely that other proteins play a role in EBNA2-mediated activation this promoter. Additional factors have been demonstrated to play a role in EBNA2-mediated trans-activation of other

EBNA2-responsive promoters that are not NotchIC-responsive. For example, although the RBP-J κ /CBF1 binding site is essential for EBNA2-responsiveness on the *LMP1* promoter, it is not sufficient for trans-activation. Other binding sites have been identified in the EBNA2 responsive element (ERE) of the *LMP1* promoter. One such binding site was identified as Spi-1, also known as PU.1, a member of the Ets family of transcription factors, which plays a pivotal role in regulating transcription from the *LMP1* promoter (Johannsen *et al.*, 1995; Laux *et al.*, 1994b). It has also been shown that a POU domain protein is involved in the EBNA2-mediated trans-activation of *LMP1* (Sjoblom *et al.*, 1995). 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). Interestingly, transcription element search software has revealed four possible Ets binding sites, as well as a possible RBP-J κ /CBF1 binding site, on the *bfl-1* promoter and mutation of any of these sites results in a dramatic decrease in EBNA2-mediated transcriptional activation of the *bfl-1* gene (Pegman *et al.*, submitted December, 2004). It is likely that EBNA2 acts as an adaptor molecule that has the potential to recruit a large number of interacting proteins in order to modulate gene expression. One explanation as to why NotchIC does not trans-activate the *bfl-1* promoter, therefore, may be that it is unable to interact with some of these proteins.

It has previously been shown in our laboratory using *bfl-1* promoter reporter constructs with 5' deletions, that the sequence from position -367 to -129 (which harbours the putative RBP-J κ /CBF1 and four Ets binding sites) is essential for mediating EBNA2-responsiveness on the *bfl-1* promoter (Pegman *et al.*, submitted December 2004). In order to define the limits of an EBNA2-responsive enhancer on the *bfl-1* promoter, portions of the sequence (between -367 and -40) were sub-cloned upstream of the minimal β -globin promoter in the luciferase reporter construct pGa50-7. However, none of the *bfl-1* promoter sequences, even those containing the putative RBP-J κ /CBF1 binding site and four putative Ets binding sites, were found to confer EBNA2-responsiveness on the reporter construct. It is possible that other elements downstream of the region of the promoter tested (between -40 and +81) are required for EBNA2-mediated activity. Another possibility for the inactivity of the reporter constructs generated, is that the interaction between the transcription factors/co-activating proteins bound to the *bfl-1* promoter sequences, and basal promoter associated proteins on the minimal promoter used in the study, did not cooperate for efficient enhancement of

transcription initiation. Further experiments would need to be carried out in order to determine whether other minimal promoters, such as the conalbumin or HSV thymidine kinase minimal promoters, would yield more successful results regarding EBNA2-mediated activity.

Another aim of study set out to identify an EBNA2 mutant that exhibited dominant-negative activity. The identification of such an inhibitory molecule would provide a useful tool for investigation of EBNA2 function in EBV-infected cells. In total, four EBNA2 mutants were tested in their ability to block wild type EBNA2 function. Two of these mutants, namely EBNA2-WW323SR and EBNA2 Mut (321-323), contained replacements/deletions in the region where EBNA2 interacts with RBP-J κ /CBF1. The other two EBNA2 mutants, EBNA2 Mut (447-472) and EBNA2 Mut (437-477) contained deletions within the trans-activation domain of the EBNA2 protein. Only the EBNA2 mutants with trans-activation domain deletions were found to inhibit EBNA2-mediated promoter activation.

An explanation for this may be provided by studies that suggest the EBNA2 amino-terminus is involved in self-association, and that self association may be important for transcriptional activation, and as a result cell growth transformation (Harada *et al.*, 2001). First, purified recombinant EBNA2 is 440 kDa, and EBNA2 from EBV-infected cells sediments in sucrose gradients at 13S and 34S, indicative of complexes that are much larger than monomeric EBNA2 (Grasser *et al.*, 1991; Tsui and Schubach, 1994). Second, EBNA2 residues 122 to 344 can interact with residues 1 to 428 in yeast two hybrid assays (Tsui and Schubach, 1994). Third, EBNA2 interacts with several sites in all EBNA2-responsive promoters and mediates transcriptional activation by recruiting multiple factors through its acid trans-activation domain. For example, the LMP1 promoter has two RBP-J κ /CBF1 binding sites, but also requires EBNA2 interaction with PU.1, while the Cp promoter has one RBP-J κ /CBF1 site and also requires CBF2 (Jin and Speck, 1992; Johannsen *et al.*, 1995; Laux *et al.*, 1994b). Thus, there appears to be a requirement for several EBNA2 molecules to be in close proximity to enable interactions among transcription factors that are necessary for appropriate promoter regulation. The transcriptional effects of EBNA2 are mediated by the acidic trans-activation domain of the EBNA2 protein. This region of EBNA2 is responsible for interaction with factors of the basal transcription machinery, and includes TFIIF, TATA box binding protein (TBP), associated binding factor TAF40, TFIIB, and the co-

activator p100 that interacts with TFIIE (Tong *et al.*, 1995a; Tong *et al.*, 1995b; Tong *et al.*, 1995c). The trans-activation domain also interacts with the histone acetyltransferases (HATs) p300/CBP and PCAF (Wang *et al.*, 2000), in order to facilitate chromatin opening.

Upon co-transfection with wild type EBNA2 and any of the EBNA2 mutants, it is likely that the EBNA2 molecules (wild type or mutant) self-associate. It is possible that EBNA2 complexes containing EBNA2 mutants with RBP-Jk/CBF1 binding site deletions could still bind DNA via an RBP-Jk/CBF1 binding site on a wild type EBNA2 molecule. Because all of the EBNA2 molecules in such a complex contain intact trans-activation domains, the EBNA2 complex could still have the ability to mediate transcriptional activation. However, in complexes where there are mutant EBNA2 molecules with trans-activation domain deletions, the complex may be unable to recruit all the co-activating molecules required for EBNA2-mediated promoter activation.

It was observed that one of the EBNA2 mutants with a trans-activation domain deletion, of amino acids 437 to 477 was more efficient at inhibiting wild type EBNA2-mediated trans-activation than the other trans-activation domain deletion mutant tested [EBNA2 Mut (447-471)]. In addition EBNA2 Mut (437-477) was shown to significantly decrease EBNA2-mediated trans-activation of (i) the control promoter, (ii) the viral LMP1 promoter and (iii) the cellular *bfl-1* promoter. The EBNA2 trans-activation domain has been mapped to amino acids 425 to 462 on the EBNA2 protein (Kieff, 1996). It is possible that EBNA2 Mut (437-477) was a more effective inhibitor due to the fact that a greater proportion of the protein was deleted in this particular mutant.

CHAPTER 4

Regulation of the Anti-Apoptotic Cellular *bfl-1* Gene in EBV-Infected Cell Lines

4.1 INTRODUCTION

The regulation of the anti-apoptotic cellular *bfl-1* gene has been studied extensively in our laboratory using the EBV-negative Burkitt lymphoma-derived cell line DG75. It has been demonstrated that EBV latent membrane protein 1 (LMP1) trans-activated the *bfl-1* promoter in this cell context by a mechanism that is dependent upon the transcription factor NF- κ B (D'Souza *et al.* 2004). It was shown in the previous chapter that NF- κ B homo-dimers composed of p65 subunits directly trans-activated the *bfl-1* promoter and that the NF- κ B-like binding site at -52 to -43 was essential for this effect, thus highlighting the role of NF- κ B pathway signalling components in LMP1-mediated *bfl-1* promoter activation. In a related study in our laboratory, an increase in *bfl-1* mRNA levels was observed upon EBNA2 induction using a tightly regulatable tetracycline-based system to express EBNA2 in the EBV-negative BL cell line DG75 (DG75-tTA-EBNA2) (Floettman *et al.*, 1996), demonstrating the ability of EBNA2 to activate transcription of the *bfl-1* gene independently of LMP1 (Pegman *et al.*, submitted December 2004). It is known that EBNA2 does not bind to DNA directly, but is tethered to responsive promoters by the DNA binding repressor protein RBP-J κ /CBF1 (Henkel *et al.*, 1994; Grossmann *et al.*, 1994; Waltzer *et al.*, 1994; Zimmer-Strobl *et al.*, 1994). This protein, which is a component of the Notch signalling pathway, was shown in the previous chapter to be essential for EBNA2-mediated activation of the *bfl-1* promoter in EBV-negative DG75 cells.

The regulation of the *bfl-1* gene in the EBV-negative BL-derived cell line DG75 investigated up to this point was determined by transfection of either the LMP1 or EBNA2 gene into DG75 cells. Because EBV-negative BL cells are immortalised by a mechanism not requiring expression of LMP1 or EBNA2, it is difficult to assess the contribution of these genes to cell survival of EBV-infected primary B cells. To overcome this obstacle, experiments were performed using EBV-infected cell lines, where both EBNA2 and LMP1 are expressed, and transcription of the *LMP1* gene is induced by EBNA2 (Abbot *et al.*, 1990; Fahraeus *et al.*, 1990; Laux *et al.*, 1994a; Wang *et al.*, 1990). Because both EBNA2 and LMP1 are present in these cell lines, and these EBV proteins regulate *bfl-1* expression through two different cellular signalling pathways, experiments were performed to elucidate the roles of EBNA2 and LMP1 in the regulation of the *bfl-1* gene in EBV-infected cells. The regulation of the *bfl-1* promoter in the cell lines Ag876 and IB4 was investigated. Ag876 is a latency type III

BL cell line, in which all EBV latent genes including LMP1 and EBNA2 are expressed. The IB4 cell line is an EBV-transformed lymphoblastoid cell line (LCL) generated by infection of normal human umbilical cord blood lymphocytes with the EBV B95.8 strain (King *et al.*, 1980).

4.2 RESULTS

4.2.1 LMP1-mediated *bfl-1* promoter activation in the cell lines Ag876 and IB4

Firstly the role of LMP1 in the trans-activation of the *bfl-1* promoter in Ag876 and IB4 cells was investigated. In a preliminary experiment, co-transfections were performed using the NF- κ B responsive reporter construct (3 x enh κ B luc). When this reporter construct was transfected into either Ag876 cells (Figure 4.1A) or IB4 cells (Figure 4.1B), a high level of luciferase activity was observed, relative to the level obtained upon transfection with the empty vector. This value was set at 100 %. A high level of activated NF- κ B was expected as LMP1, which is present in both these cell lines, is known to trigger signalling pathways that result in the activation of NF- κ B (Huen *et al.*, 1995). The NF- κ B-responsive reporter construct was also co-transfected into Ag876 and IB4 cells along with the expression vector for a mutant form of I κ B α (pEFCX-I κ B α DN), in which the serine residues at positions 32 and 36 have been replaced with alanines (Liljeholm *et al.*, 1998). When various quantities of this mutant form of I κ B α were co-transfected along with the NF- κ B-responsive reporter construct 3 x enh κ B luc, a dramatic decrease in luciferase activity was observed in both Ag876 cells (Figure 4.1A) and IB4 cells (Figure 4.1B). This decrease in activated NF- κ B levels was observed upon co-transfection with just 1 μ g of pEFCX-I κ B α DN, with a decrease in luciferase activity of approximately 80 % in Ag876 cells and approximately 70 % in IB4 cells. When larger quantities of the expression vector for the I κ B α mutant were used in transfection experiments, luciferase activity was almost completely abolished in both cell lines.

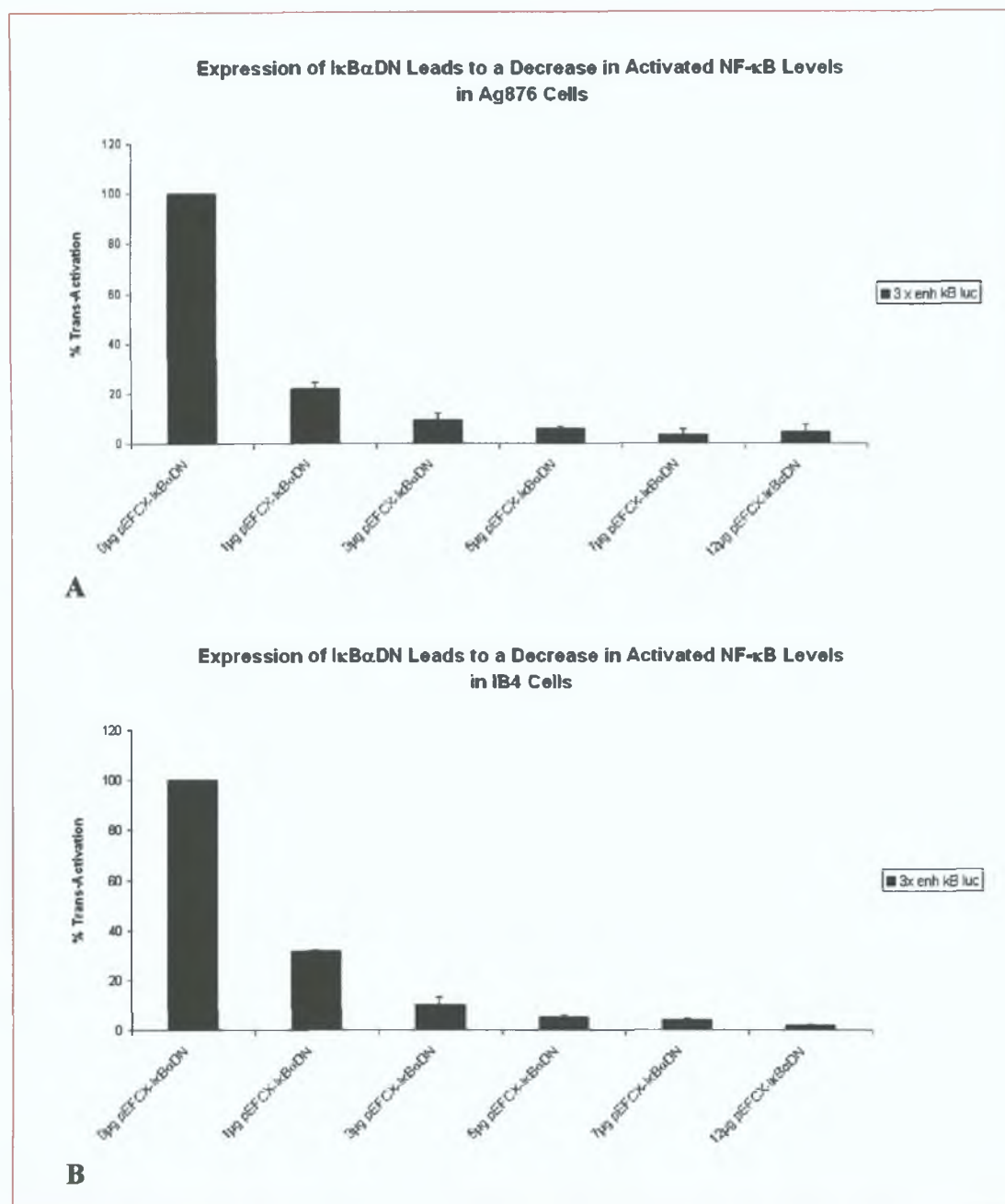


Figure 4.1: Over-expression of a dominant negative form of $\text{I}\kappa\text{B}\alpha$ leads to a decrease in activated NF- κB levels in the Ag876 and IB4 cell lines. Co-transfections were carried out in Ag876 cells (A) and IB4 cells (B) using various amounts of pEFCX-I $\kappa\text{B}\alpha\text{DN}$ and 2 μg of the NF- κB -responsive luciferase reporter construct 3 x enh κB luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the reporter and the empty vector (pEFCX). Luciferase activity obtained upon co-transfection with the reporter construct along with the empty vector was arbitrarily assigned a value of 100 %.

These results indicated that there is a high level of activated NF- κB in the EBV-infected cell lines Ag876 and IB4, most likely as a result of LMP1 signalling. Over-expression

of a dominant negative I κ B α mutant effectively decreases this level of activated NF- κ B in these cell contexts.

Once it was established that the dominant negative I κ B α mutant effectively blocked activation of NF- κ B in Ag876 and IB4 cells, co-transfections were performed to investigate if this mutant could block *bfl-1* promoter activation in these EBV-infected cell lines. To this end, various amounts of the expression vector for the I κ B α mutant were co-transfected into Ag876 cells (Figure 4.2A) and IB4 cells (Figure 4.2B) along with the *bfl-1* promoter reporter -1374/+81-Luc. In the absence of the mutant, transfection of the *bfl-1* promoter reporter construct (-1374/+81-Luc) into both cell lines resulted in a high level of luciferase activity relative to that obtained upon transfection with the empty vector (pGL2-Basic). This value was set at 100 %. A high level of luciferase activity was expected, as the *bfl-1* promoter is responsive to both LMP1 and EBNA2, which are present in both Ag876 and IB4 cells. When the *bfl-1* promoter was co-transfected with the expression vector for the I κ B α mutant, only a marginal decrease in promoter activity (<20 %) was observed in either Ag876 or IB4 cells.

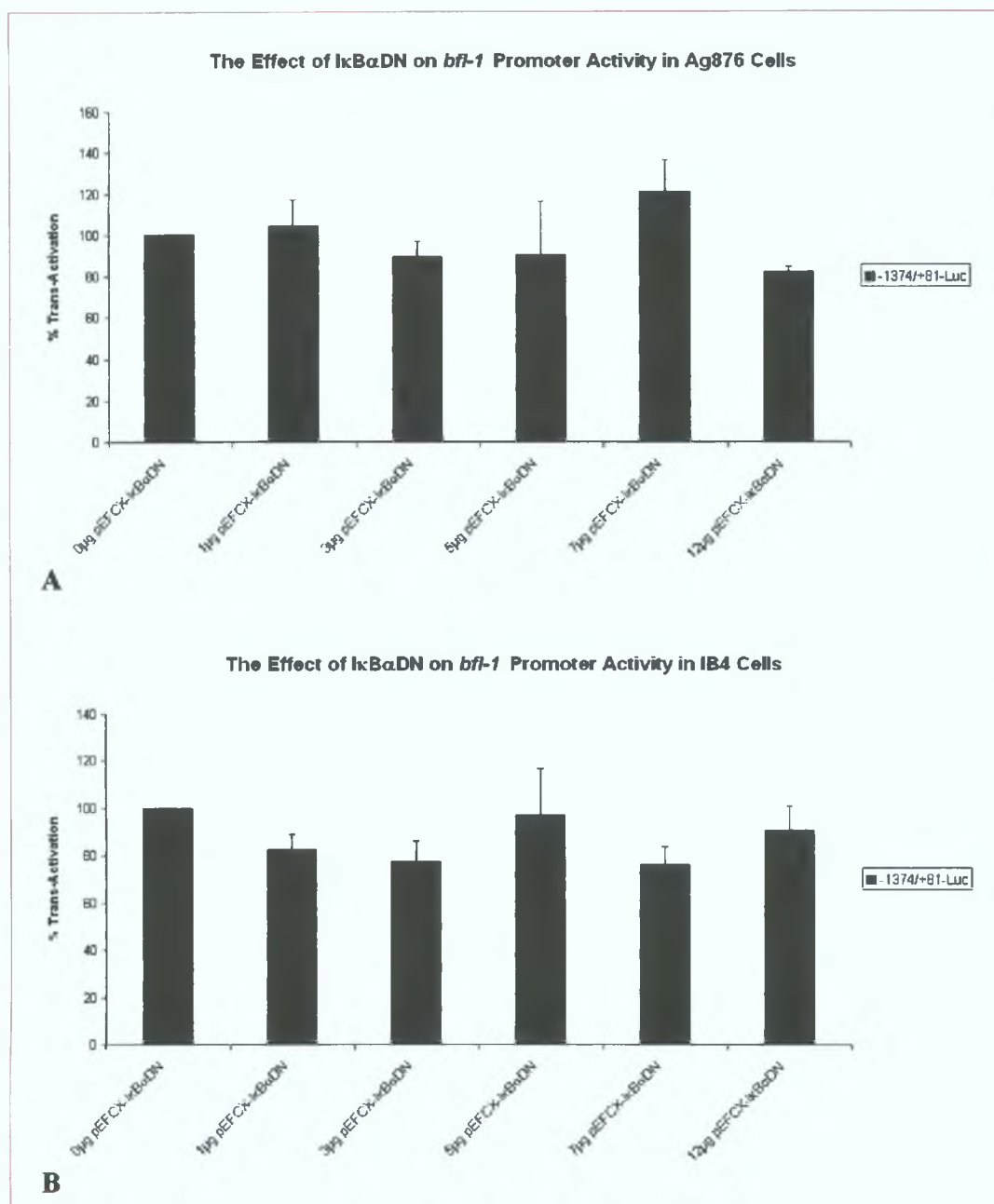


Figure 4.2: Over-expression of a dominant negative form of IkBa does not dramatically affect *bfl-1* promoter activity in EBV-infected cell lines Ag876 and IB4. Co-transfections were carried out with Ag876 cells (A) and IB4 cells (B) using various amounts of pEFCX-IkBaDN and 2 µg of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the *bfl-1* reporter construct and the empty expression vector for IkBaDN (pEFCX). Luciferase activity obtained upon co-transfection with the reporter construct along with the empty vector was arbitrarily assigned a value of 100 %.

This data showed that although the mutant form of IkBa effectively blocks activation of the NF-κB-responsive reporter construct 3 x enh κB luc, it does not affect *bfl-1* promoter activity in the EBV-infected cell lines Ag876 and IB4. It is possible that although LMP1-mediated activation of the *bfl-1* promoter is inhibited by expression of

the I κ B α mutant, EBNA2 is maintaining the level of *bfl-1* promoter activity by signalling independently through the CBF1/Notch pathway.

4.2.2 EBNA2-mediated *bfl-1* promoter activation in the cell lines Ag876 and IB4

4.2.2.1 The effect of the RBP-J κ /CBF1 mutant on *bfl-1* promoter activity in the cell lines Ag876 and IB4

It was shown in Chapter 3, using the EBV-negative cell line DG75, that an RBP-J κ /CBF1 mutant effectively blocked EBNA2-mediated responsiveness on three different promoters: an RBP-J κ /CBF1-regulated control promoter (pGa981-6), the *bfl-1* promoter (-1374/+81-Luc) and the *LMP1* promoter (pLLO-luc). To determine whether this mutant could also inhibit *bfl-1* promoter activation in EBV-infected cells, co-transfections were performed using an expression vector for this mutant (pEFBOSneo-R218H) along with pGa981-6. When this reporter construct was transfected into either Ag876 cells (Figure 4.3A) or IB4 cells (Figure 4.3B) in the absence of the RBP-J κ /CBF1 mutant, a high level of luciferase activity was observed, most likely due to the fact that this construct is responsive to EBNA2, which is present in both Ag876 and IB4 cells. Expression of the RBP-J κ /CBF1 mutant inhibited trans-activation of pGa981-6 in both Ag876 cells (Figure 4.3A) and IB4 cells (Figure 4.3B). The inhibitory effect was found to be dose-dependent with maximum inhibition of approximately 70 % in both cell lines when 12 μ g of pEFBOSneo-R218H was used during co-transfection experiments. It was noted that inhibition was to the same degree as that observed during similar co-transfection experiments performed in the EBV-negative cell line DG75 (Figure 3.5).

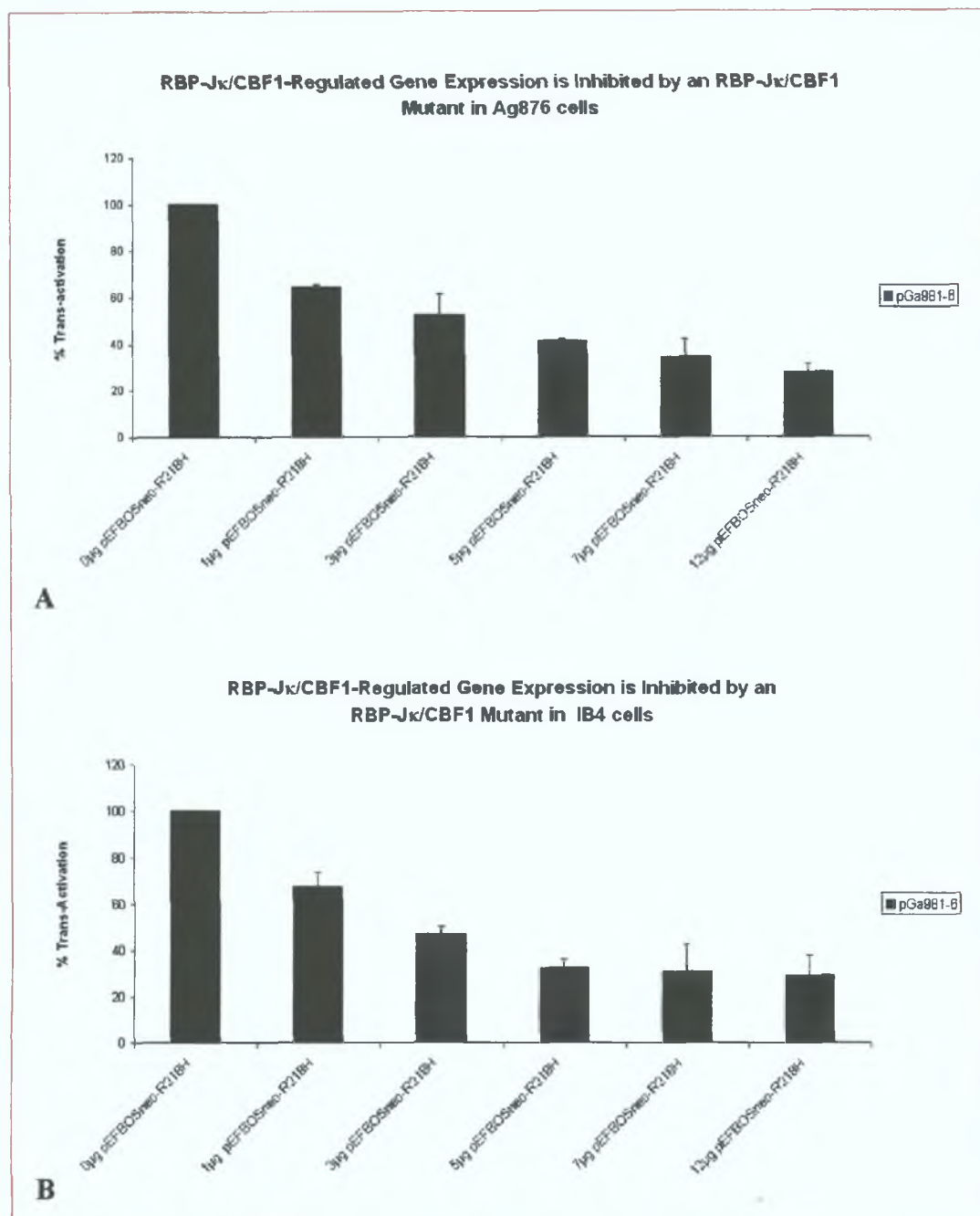


Figure 4.3: Over-expression of the RBP-Jκ/CBF1 mutant inhibits activation of the luciferase reporter construct pGa981-6 in EBV-infected cell lines. Co-transfections were carried out using Ag876 cells (A) and IB4 cells (B) with various amounts of pEFBOSneo-R218H and 2 μg of the RBP-Jκ/CBF1 responsive control promoter pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the reporter construct and the empty expression vector for the RBP-Jκ/CBF1mutant (pEFBOSneo). Luciferase activity obtained upon co-transfection with the reporter construct and the empty vector was arbitrarily assigned a value of 100 %.

These data showed that expression of the RBP-Jκ/CBF1 mutant, pEFBOSneo-R218H, effectively inhibits trans-activation of the luciferase reporter construct pGa981-6 in both Ag876 and IB4 cells.

To determine whether expression of the RBP-J κ /CBF1 mutant could affect the levels of *bfl-1* promoter activity in the EBV-infected cells, transient co-transfections were performed using various amounts of the expression vector for the RBP-J κ /CBF1 mutant along with the *bfl-1* promoter reporter construct -1374/+81-Luc. When the *bfl-1* promoter reporter construct was transfected into the cells in the absence of the RBP-J κ /CBF1 mutant, high levels of promoter activity were observed in both Ag876 cells (Figure 4.4A) and IB4 cells (Figure 4.4B). Upon co-transfection of the RBP-J κ /CBF1 mutant along with the *bfl-1* promoter, a small decrease in *bfl-1* promoter activity was observed (30 % in Ag876 cells, Figure 4.4A and 10 % in IB4 cells, Figure 4.4B). This effect on *bfl-1* promoter activity was not as potent as the effect observed in transfection experiments using the RBP-J κ /CBF1 regulated control promoter pGa981-6, where inhibition of approximately 70 % was demonstrated. It was also noted that inhibition of EBNA2-mediated activation of the *bfl-1* promoter in the EBV-negative BL-derived cell line DG75 by the RBP-J κ /CBF1 mutant was far more effective (approximately 75 %, Figure 3.7).

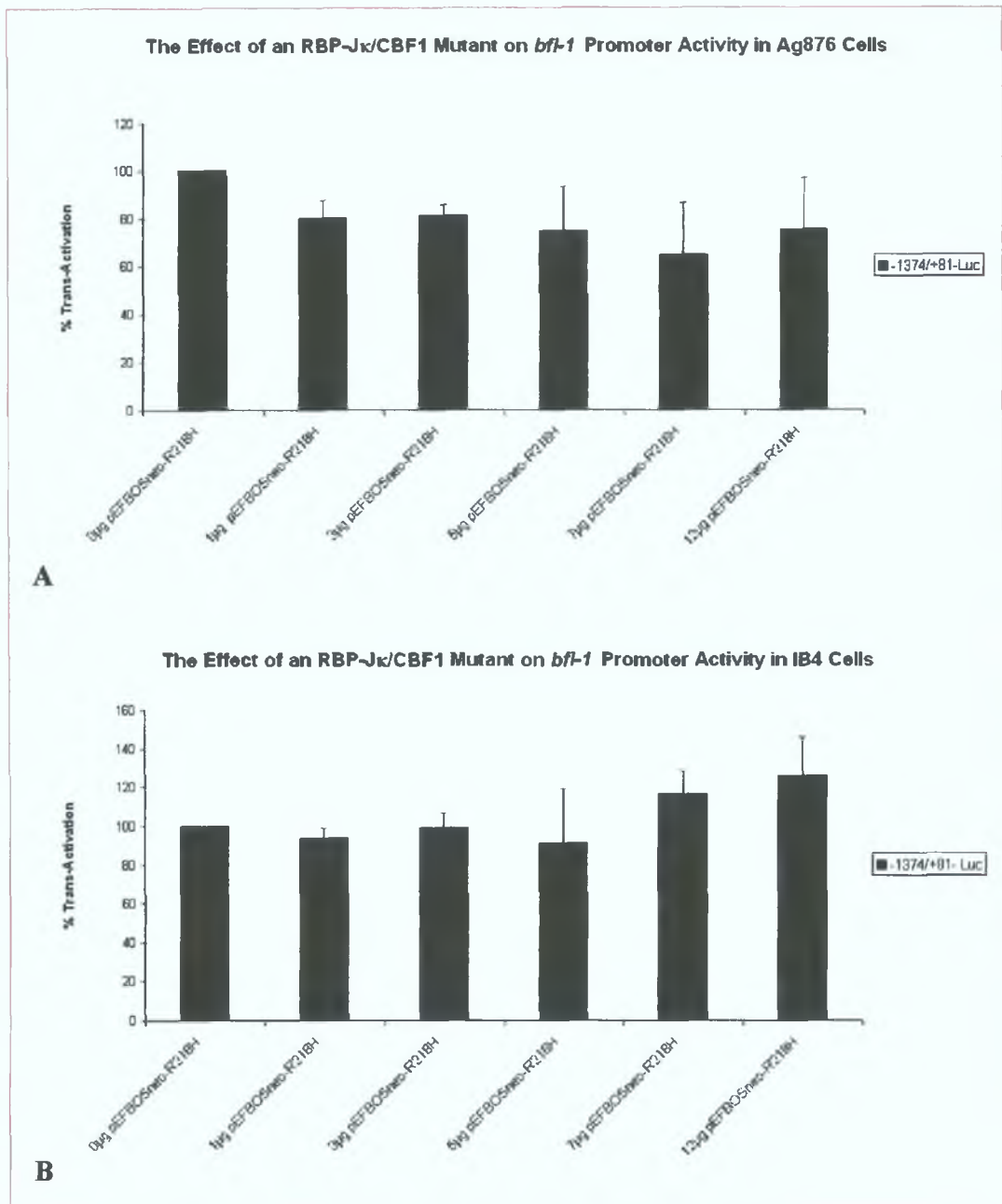


Figure 4.4: Over-expression of an RBP-J κ /CBF1 mutant does not dramatically affect *bfl-1* promoter activity in EBV-infected cell lines. Co-transfections were performed in Ag876 cells (A) and IB4 cells (B) using various amounts of pEFBOSneo-R218H and 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the *bfl-1* promoter reporter construct and the empty expression vector for the RBP-J κ /CBF1 mutant (pEFBOSneo). Luciferase activity obtained upon co-transfection with the reporter construct and the empty vector was arbitrarily assigned a value of 100 %.

This data shows that although over-expression of the RBP-J κ /CBF1 mutant inhibits trans-activation of the control luciferase reporter construct pGa981-6 in Ag876 and IB4 cells, it does not significantly affect *bfl-1* promoter activity.

It was demonstrated in Chapter 3 that over-expression of the RBP-J κ /CBF1 mutant inhibited EBNA2-mediated activation of the *LMP1* promoter in the EBV-negative BL-derived cell line DG75 (Figure 3.10). If EBNA2-mediated activation of the *LMP1* promoter was inhibited by the RBP-J κ /CBF1 mutant in EBV-infected cells, it is likely that there would be a decrease in NF- κ B activity as a result. In order to test this hypothesis, co-transfections were carried using various amounts of the expression vector for the RBP-J κ /CBF1 mutant along with the NF- κ B-responsive reporter construct 3 x enh κ B luc. Over-expression of the mutant did indeed affect the level of NF- κ B-responsive promoter activity in both Ag876 cells (Figure 4.5A) and IB4 cells (Figure 4.5B). A maximum inhibition in luciferase activity of approximately 40 % was observed in both cell lines. Because this decrease in NF- κ B activity was observed, it is difficult to conclude whether RBP-J κ /CBF1 mutant-mediated partial inhibition of *bfl-1* promoter activity observed in the Ag876 cells (Figure 4.4A) was due to (i) inhibition of EBNA2-mediated activation of the *bfl-1* promoter by over-expression of the RBP-J κ /CBF1 mutant, (ii) inhibition of EBNA2-mediated trans-activation of the *LMP1* promoter due to over-expression of the RBP-J κ /CBF1 mutant, and as a result lower levels of LMP1 in the cells, resulting in a decrease in *bfl-1* promoter activity or (iii) a combination of these two factors. Although over-expression of the RBP-J κ /CBF1 mutant affected NF- κ B levels in IB4 cells (Figure 4.5B), no decrease in *bfl-1* promoter activity was observed (Figure 4.4A).

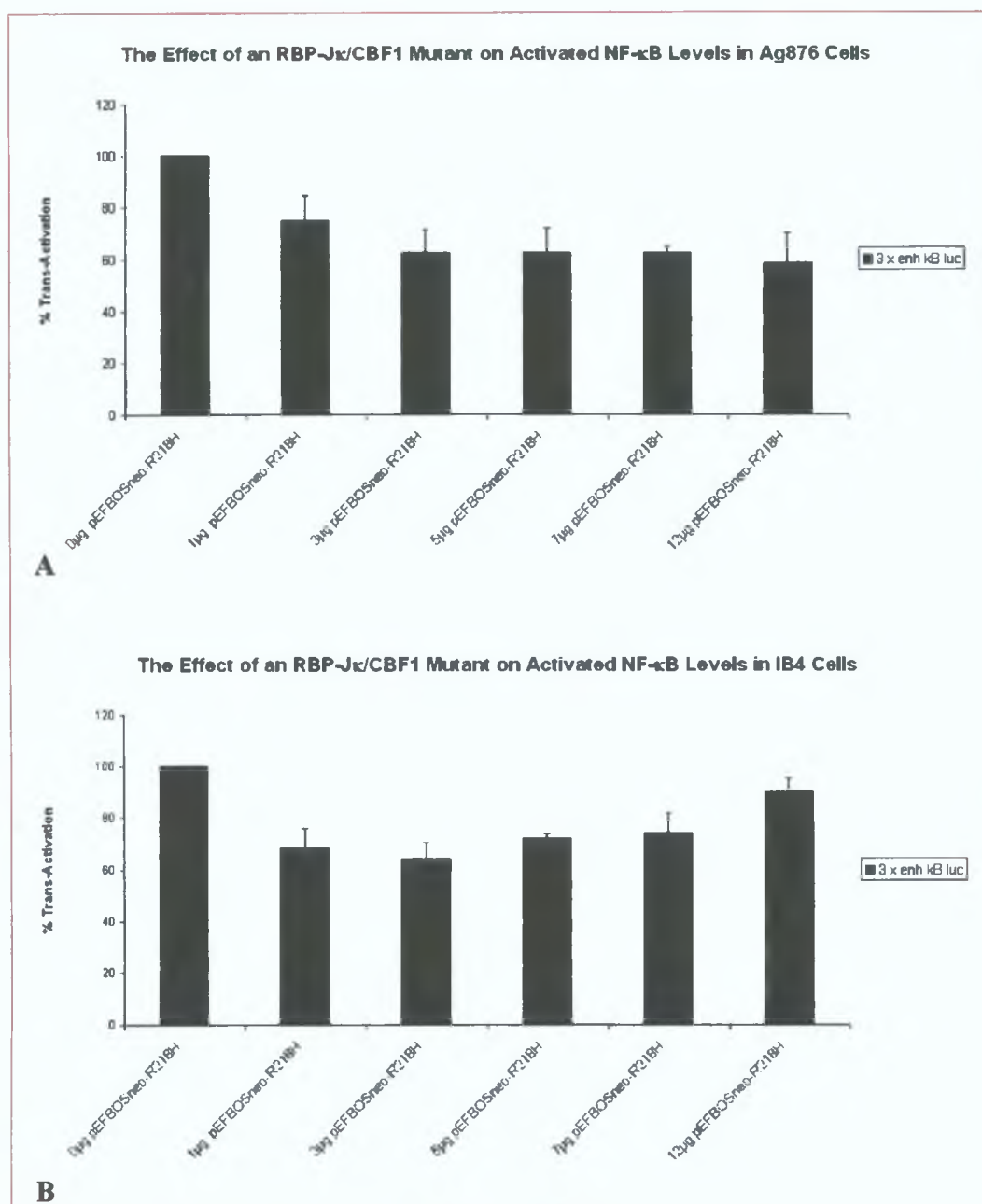


Figure 4.5: The effect of the RBP-J κ /CBF1 mutant on activated NF- κ B levels in EBV-infected cell lines. Co-transfections were performed using Ag876 cells (A) and IB4 cells (B) with various amounts of pEFBOSneo-R218H and 2 μ g of the NF- κ B-responsive luciferase reporter construct 3 x enh κ B luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the reporter construct and the empty expression vector for the RBP-J κ /CBF1 mutant (pEFBOSneo). Luciferase activity obtained upon co-transfection with the reporter construct and the empty vector was arbitrarily assigned a value of 100 %.

4.2.2.2 The effect of the EBNA2 mutant on *bfl-1* promoter activity in the cell lines Ag876 and IB4

In a second attempt to block EBNA2-mediated trans-activation of the *bfl-1* promoter in EBV-positive cell lines, co-transfections were carried out using an EBNA2 mutant with a deletion in its trans-activation domain. This mutant was shown to dramatically inhibit EBNA2-mediated trans-activation of the control reporter construct pGa981-6 (Figure 3.31B), the viral *LMP1* promoter (Figure 3.32B) and the cellular *bfl-1* promoter in the EBV-negative cell line DG75 (Figure 3.32A) in the previous chapter. In a preliminary experiment, transfections were carried out using the RBP-J κ /CBF1-responsive control promoter pGa981-6. As before, high levels of promoter activity were observed when this reporter construct was transfected into Ag876 cells (Figure 4.6A) and IB4 cells (Figure 4.6B). However, upon co-transfection of this reporter construct with different quantities of the expression vector for the EBNA2 mutant, a decrease in promoter activity was observed. This decrease was dose-dependent with maximum inhibition observed when 12 μ g of EBNA2 Mut (437-477) expression vector was used. Co-transfections using this quantity of EBNA2 mutant resulted in 90 % inhibition in luciferase activity in experiments using Ag876 cells (Figure 4.6A). A similar degree of inhibition was observed during co-transfection experiments using EBNA2 Mut (437-477) in the EBV-negative cell line DG75 (Figure 3.31B). A maximum inhibition of approximately 70 % was noted when IB4 cells were used (Figure 4.6B).

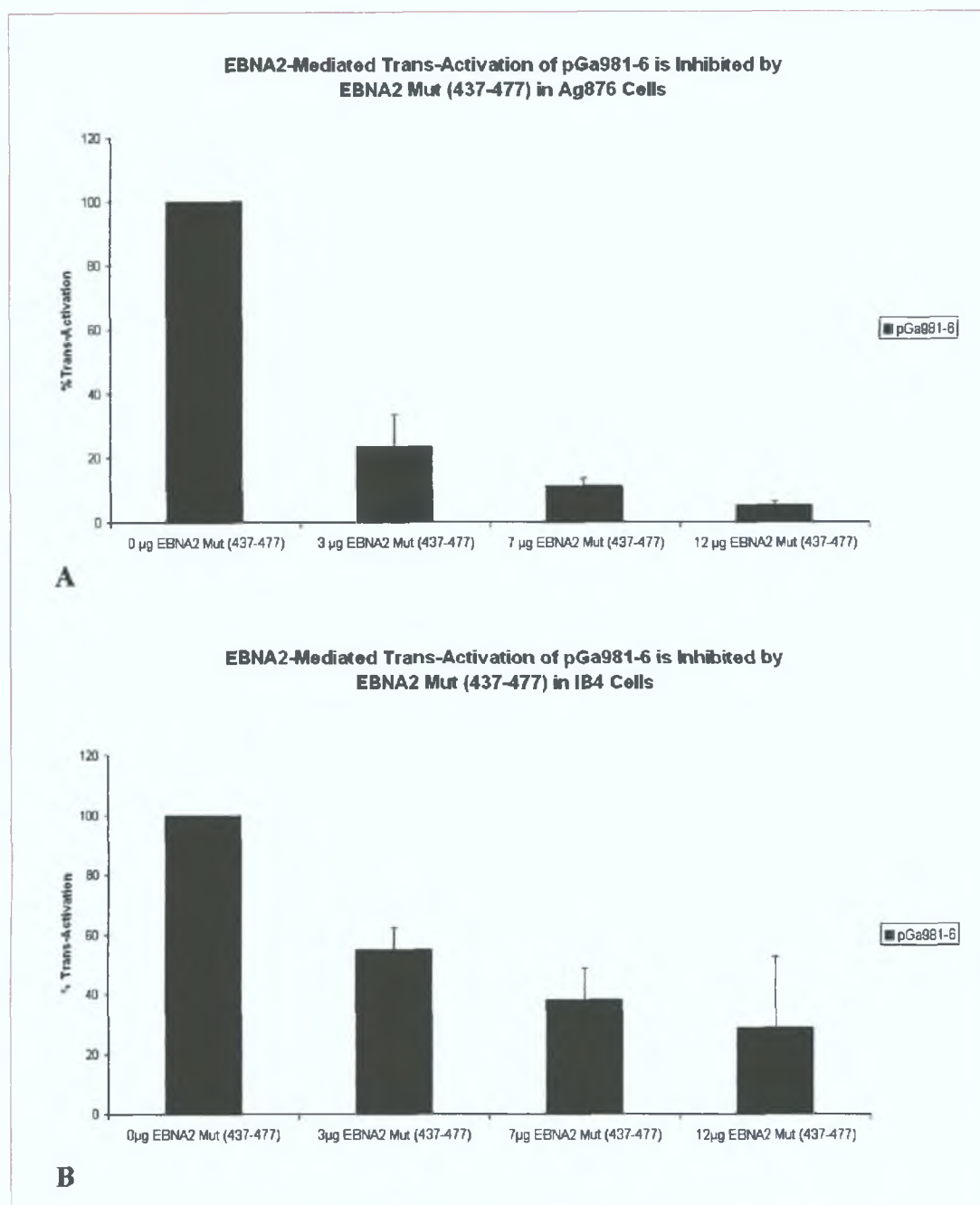


Figure 4.6: Over-expression of an EBNA2 mutant inhibits activation of pGa981-6 in EBV-positive cell lines. Co-transfections were performed using Ag876 cells (A) and IB4 cells (B) using various amounts of and expression vector for EBNA2 Mut (437-477) and 2 µg of the luciferase reporter construct pGa981-6. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the reporter and the empty expression vector for the EBNA2 mutant (pSG5). Luciferase activity obtained upon co-transfection with the reporter and the empty vector was arbitrarily assigned a value of 100 %.

These results indicated that the EBNA2 mutant (with a deletion in its trans-activation domain) effectively inhibited EBNA2-mediated trans-activation of the control luciferase reporter construct pGa981-6 in Ag876 and IB4 cells.

In order to determine whether EBNA2 Mut (437-477) could inhibit EBNA2-mediated activation of the *bfl-1* promoter, co-transfections were carried out using either Ag876 cells (Figure 4.7A) or IB4 cells (Figure 4.7B) and the expression vector for the EBNA2 mutant, along with the *bfl-1* promoter reporter construct -1374/+81-Luc. As expected a high level of *bfl-1* promoter activity was observed upon transfection of the reporter into both cell lines. When increasing quantities of the expression vector for EBNA2 Mut (437-477) were co-transfected along with the *bfl-1* promoter, a small decrease in promoter activity was observed. Promoter activity was inhibited by approximately 10 % in experiments using Ag876 cells (Figure 4.7A) and approximately 30 % in experiments using IB4 cells (Figure 4.7B). This degree of inhibition is not as great as that observed during transfections using the control luciferase reporter construct pGa981-6 (Figure 4.6, 90 % inhibition in Ag876 cells and 70 % in IB4 cells) or in EBV-negative cell line DG75 (Figure 3.32A, 90 % inhibition)

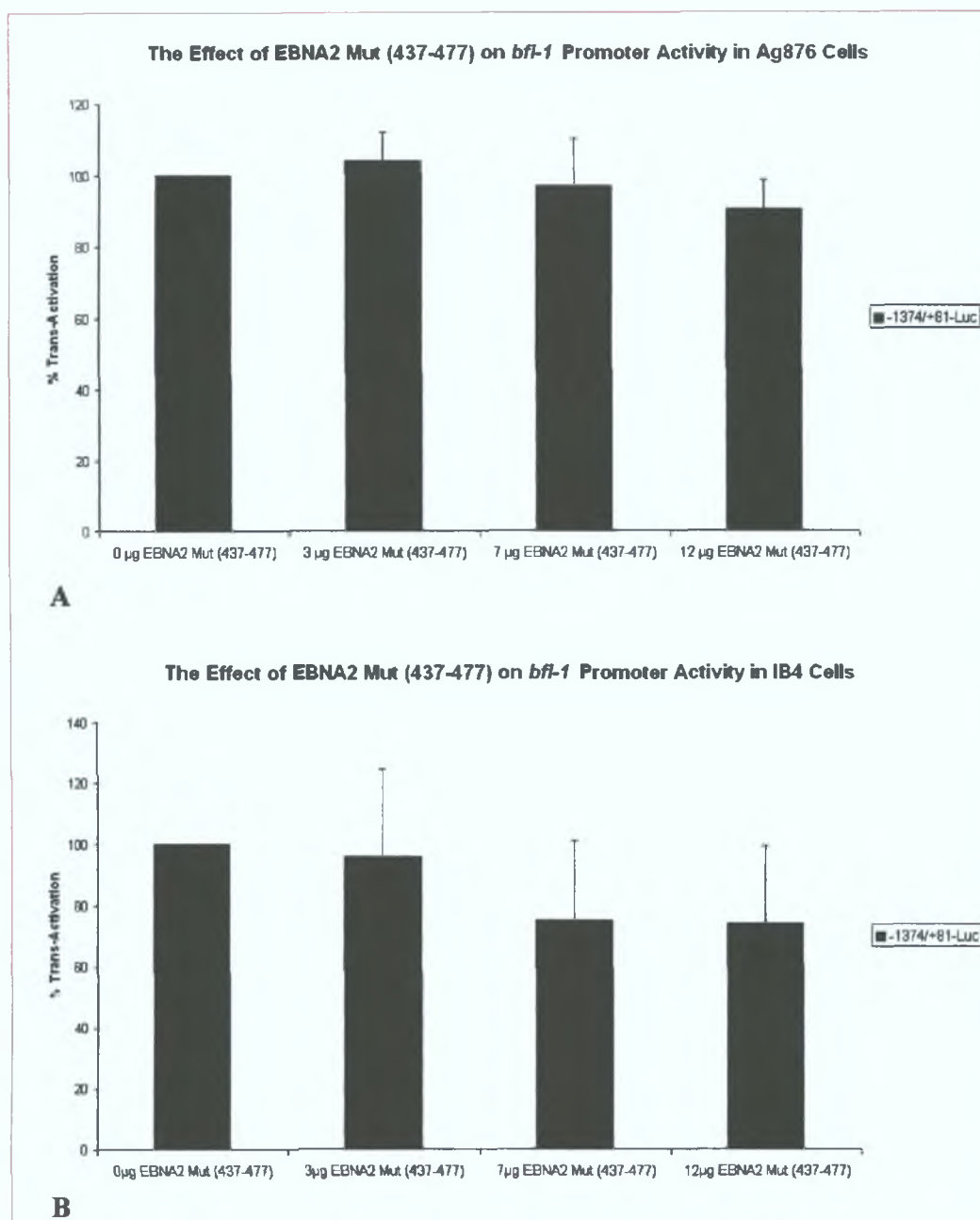


Figure 4.7: Over-expression of an EBNA2 mutant does not dramatically affect EBNA2-mediated activation of the *bfl-1* promoter in EBV-positive cell lines. Co-transfections were carried out with Ag876 cells (A) and IB4 cells (B) using various amounts of EBNA2 Mut (437-477) and 2 µg of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the reporter construct and the empty expression vector for the EBNA2 mutant (pSG5). Luciferase activity obtained upon co-transfection with the reporter and the empty vector was arbitrarily assigned a value of 100 %.

This data shows that although over-expression of EBNA2 Mut (437-477) inhibits trans-activation of the control luciferase reporter construct pGa981-6, it does not inhibit *bfl-1* promoter activity as efficiently.

It was demonstrated in Chapter 3 that over-expression of EBNA2 Mut (437-477) inhibited EBNA2-mediated activation of the *LMP1* promoter in the EBV-negative BL-derived cell line DG75 (Figure 3.32B). If EBNA2-mediated activation of the *LMP1* promoter was inhibited by over-expression of the EBNA2 mutant in EBV-infected cells, it is likely that there would be a decrease in NF- κ B activity as a result. In order to investigate this, co-transfections were carried using various amounts of the expression vector for EBNA2 Mut (437-477) along with the NF- κ B-responsive reporter construct 3 x enh κ B luc. Over-expression of the mutant was found to affect the level of NF- κ B-responsive promoter activity in both Ag876 cells (Figure 4.8A) and IB4 cells (Figure 4.8B). A maximum inhibition in luciferase activity of approximately 20 % was observed in Ag876 cells and 40 % in IB4 cells. Similarly to the experiments using the RBP-J κ /CBF1 mutant, it is difficult to conclude whether EBNA2 Mut (437-477)-mediated inhibition of *bfl-1* promoter activity observed in the IB4 cells (Figure 4.7B) was due to (i) inhibition of EBNA2-mediated activation of the *bfl-1* promoter by over-expression of the EBNA2 mutant, (ii) inhibition of EBNA2-mediated trans-activation of the *LMP1* promoter by over-expression of the EBNA2 mutant, and as a result lower levels of LMP1 in the cells, resulting in a decrease in *bfl-1* promoter activity or (iii) a combination of these two factors. Over-expression of the EBNA2 mutant affected NF- κ B levels in Ag876 cells (Figure 4.8A) to a lesser extent than in the IB4 cells. This correlates with the decrease in *bfl-1* promoter activity observed in Figure 4.7.

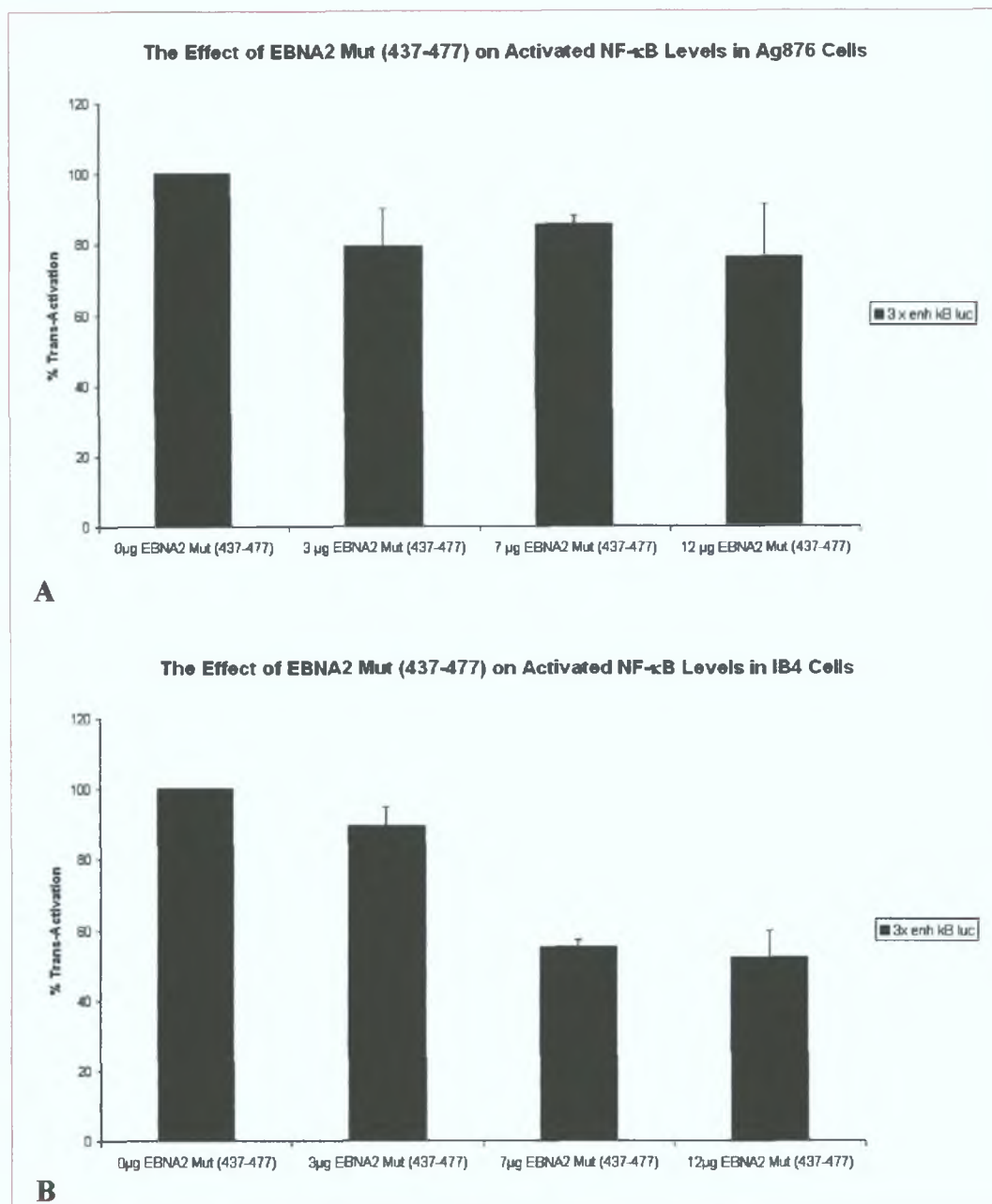


Figure 4.8: The effect of the EBNA2 mutant on activated NF- κ B levels in EBV-infected cell lines. Co-transfections were performed using Ag876 cells (A) and IB4 cells (B) using various amounts of the expression vector for the EBNA2 mutant and 2 μ g of the NF- κ B-responsive luciferase reporter construct 3 x enh kB luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the reporter construct and the empty expression vector for the EBNA2 mutant (pSG5). Luciferase activity obtained upon co-transfection with the reporter construct and the empty vector was arbitrarily assigned a value of 100 %.

4.2.3 Co-expression of the RBP-J κ /CBF1 and I κ B α mutants in the cell lines Ag876 and IB4 inhibits *bfl-1* promoter activation

It has already been demonstrated that over-expression of either the RBP-J κ /CBF1 mutant or the I κ B α mutant alone (Figure 4.4 and Figure 4.2 respectively) does not lead to a potent inhibition in *bfl-1* promoter activity using the cell lines Ag876 and IB4. These results are summarised in Figure 4.9. Consequently, transient co-transfections were performed to investigate whether co-expression of these two mutants at the same time would affect the level of *bfl-1* promoter activity. Co-transfection with 12 μ g of the expression vector for the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H) together with 12 μ g of the the vector expressing the I κ B α mutant (pEFCX-I κ B α DN) resulted in approximately 50 % inhibition in the level of *bfl-1* promoter activity in Ag876 cells (Figure 4.9A) and approximately 40 % inhibition in IB4 cells (Figure 4.9B).

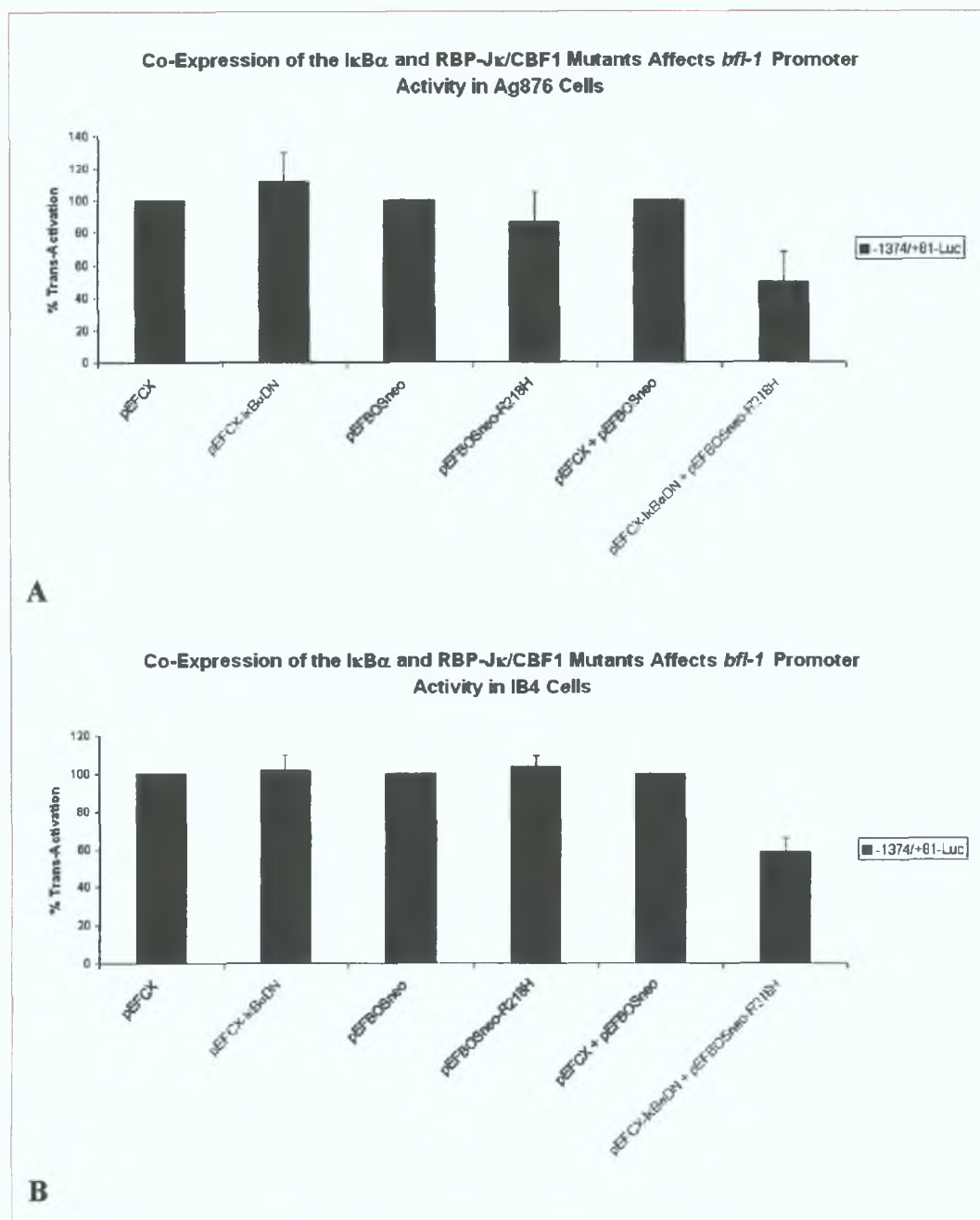


Figure 4.9: Co-expression of the RBP-J κ /CBF1 and $\text{I}\kappa\text{B}\alpha$ mutants affects *bfl-1* promoter activity in the cell lines Ag876 and IB4. Co-transfections were performed using Ag876 cells (A) and IB4 cells (B) using 12 μg of the expression vector indicated underneath the graph along with 2 μg of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the empty expression vector. Luciferase activity obtained upon co-transfection with the empty vector was arbitrarily assigned a value of 100 %.

These results demonstrated that blocking both EBNA2- and LMP1-mediated activation of the *bfl-1* promoter by co-expression of the RBP-J κ /CBF1 and $\text{I}\kappa\text{B}\alpha$ mutants simultaneously resulted in stronger inhibition in *bfl-1* promoter activity than expression of either of these mutants alone.

4.2.4 Co-expression of the EBNA2 and IκBα mutants in the cell lines Ag876 and IB4 inhibits *bfl-1* promoter activation

It was demonstrated in Section 4.2.2 that over-expression of the EBNA2 mutant with a trans-activation domain deletion does not inhibit *bfl-1* promoter activity as efficiently as the control reporter construct in Ag876 and IB4 cells (Figure 4.7). In order to investigate whether co-expression of this mutant along with the IκBα mutant would have a greater inhibitory effect on the level of *bfl-1* promoter trans-activation, transient co-transfections were performed using the expression vector for the EBNA2 mutant (EBNA2 Mut (437-477)) together with the vector expressing the IκBα mutant (pEFCX-IκBαDN). Co-expression of the two mutants at the same time resulted in approximately 60 % inhibition in the level of *bfl-1* promoter activity in Ag876 cells (Figure 4.10A) and 40 % inhibition in IB4 cells (Figure 4.10B). The levels of inhibition were similar to those obtained upon co-transfection with the RBP-Jκ/CBF1 and IκBα mutants (Figure 4.9).

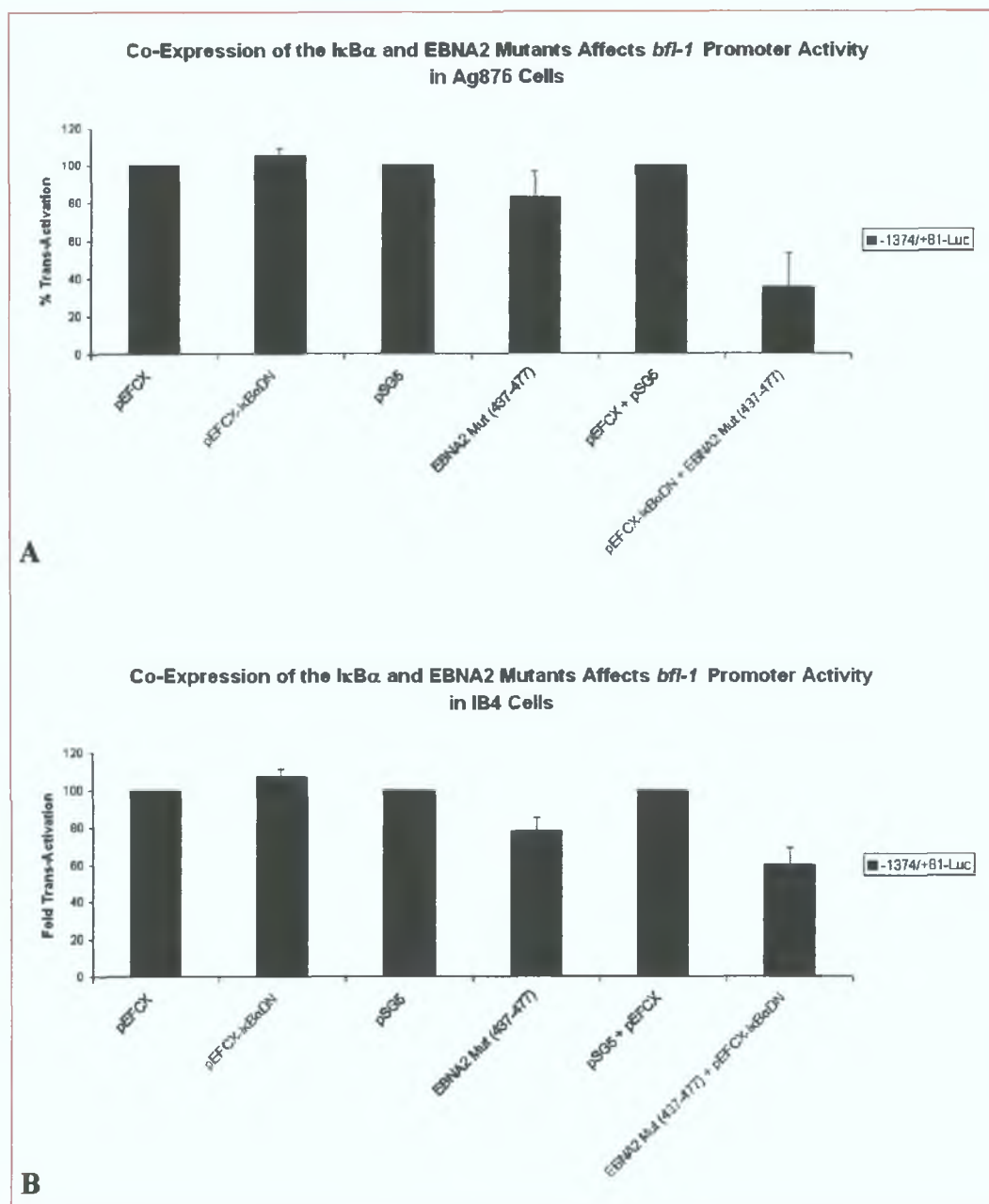


Figure 4.10: Co-expression of the EBNA2 and $\text{IkB}\alpha$ mutants affects *bfl-1* promoter activity in EBV-infected cell lines Ag876 and IB4. Co-transfections were performed using Ag876 cells (A) and IB4 cells (B) using 12 μg of the expression vector indicated underneath the graph and 2 μg of the *bfl-1* promoter reporter construct -1374/+81-Luc. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the empty expression vector. Luciferase activity obtained upon co-transfection with the empty vector was arbitrarily assigned a value of 100 %.

These results demonstrated for a second time that inhibition of both EBNA2- and LMP1-mediated trans-activation of the *bfl-1* promoter, in this case by co-expression of the EBNA2 and $\text{IkB}\alpha$ mutants simultaneously, resulted in a significant decrease in the level of *bfl-1* promoter activity in Ag876 and IB4 cells.

4.2.5 The effect of an I κ B α DN on promoter activity in the cell lines Ag876 and IB4

In order to further investigate inhibition of *bfl-1* promoter activity by blocking both EBNA2- and LMP1-mediated activation simultaneously, experiments were performed using a *bfl-1* promoter construct containing a mutation in the putative RBP-J κ /CBF1 binding site at position -343 to -249 relative to the transcription start site (-1374/+81 (mCBF1), Pegman *et al* , submitted December 2004) and the expression vector for the I κ B α mutant. In a preliminary control experiment, the NF κ B-responsive reporter construct 3 x enh κ B luc was transfected into Ag876 cells (Figure 4 11A) or IB4 cells (Figure 4 11B), resulting in a high level of luciferase activity. This finding was not surprising as LMP1 is known to trigger signalling pathways that result in the activation of NF- κ B. Upon co-transfection of the NF- κ B-responsive reporter along with the expression vector for the I κ B α mutant (pEFCX-I κ B α DN), a significant decrease in luciferase activity (approximately 95 %) was observed. This finding was similar to that observed in experiments using the EBV-negative BL-derived cell line DG75 (Figure 3 9A). When the I κ B α mutant was co-transfected along with the *bfl-1* promoter reporter construct -1374/+81-Luc, only a small decrease in luciferase activity was detected in experiments carried out using both cell lines. Ag876 and IB4 cells were also transfected with the *bfl-1* promoter construct containing a mutation in the putative RBP-J κ /CBF1 binding site at position -343 to -249 relative to the transcription start site [-1374/+81 (mCBF1)]. This mutation was not found to significantly inhibit *bfl-1* promoter activity in either cell line. However, upon co-transfection of the I κ B α mutant together with the promoter bearing the mutation in the putative RBP-J κ /CBF1 binding site, a 45 % decrease in luciferase activity was observed in Ag876 cells and a 50 % decrease was observed in IB4 cells.

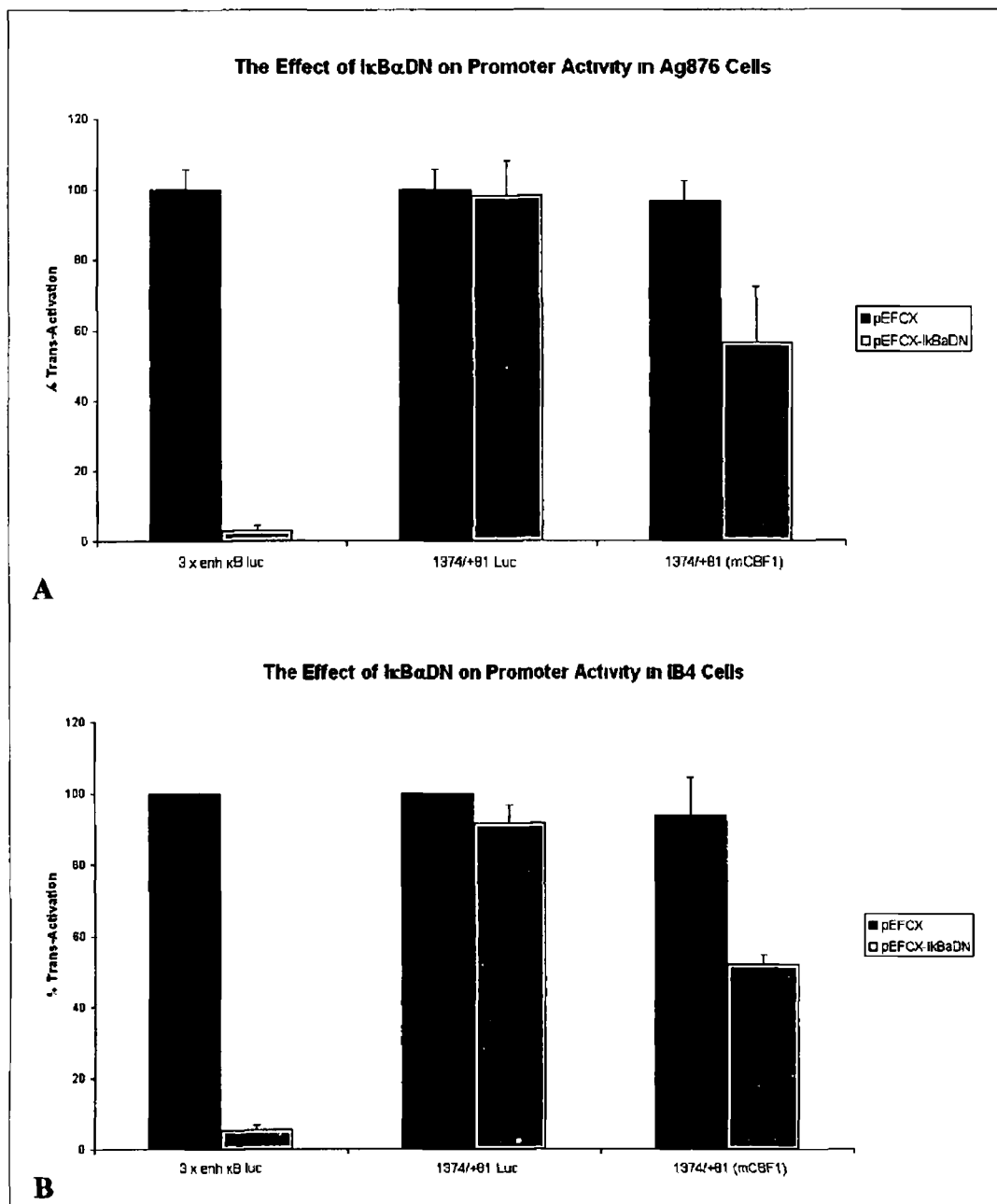


Figure 4 11 The effect of expression of the κ Ba mutant on promoter activity in Ag876 and IB4 cells. Co-transfections were performed using Ag876 cells (A) and IB4 cells (B) using 12 μ g of either pEFCX or pEFCX- κ BaDN and 2 μ g of the reporter construct indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the empty vector. Luciferase activity obtained upon co-transfection with the empty vector was arbitrarily assigned a value of 100 %.

These results indicated that blocking LMP1-mediated *bfl-1* promoter activity by over-expression of the κ Ba mutant as well as blocking EBNA2-mediated *bfl-1* promoter activity by mutating the putative RBP-J κ /CBF1 binding site on the promoter, results in inhibition in *bfl-1* promoter trans-activation in the cell lines Ag876 and IB4.

4.2.6 The effect of RBP-J κ /CBF1 mutant on promoter activity in the cell lines Ag876 and IB4

The effect of the RBP-J κ /CBF1 mutant on the activity of the *bfl-1* promoter construct containing a mutation in the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site was also investigated using Ag876 and IB4 cells. Initially, the RBP-J κ /CBF1-regulated reporter construct (pGa981-6) was transfected into Ag876 cells (Figure 4 12A) or IB4 cells (Figure 4 12B). A high level of luciferase activity was observed. This was an expected finding as the reporter construct is known to be responsive to EBNA2, which is present in both these cell lines. When pGa981-6 was co-transfected along with the expression vector for the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H), a 70 % decrease in luciferase activity was detected in Ag876 cells and a 60 % decrease was detected in IB4 cells. This finding was similar to that observed in experiments performed using the EBV-negative BL-derived cell line DG75 (Figure 3 5). When the *bfl-1* promoter reporter construct -1374/+81-Luc was co-transfected along with the RBP-J κ /CBF1 mutant, no decrease in luciferase activity was observed in either of the EBV-infected cell lines. Ag876 and IB4 cells were also transfected with the *bfl-1* promoter construct containing a mutation in the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site (-1374/+81 m κ B (-52), D'Souza *et al*, 2004). This mutation was not found to inhibit *bfl-1* promoter activity in either of the cell lines. However, upon co-transfection with the RBP-J κ /CBF1 mutant together with the promoter containing the NF- κ B-like binding site mutation, a decrease of approximately 50 % in luciferase activity was observed in both cell lines.

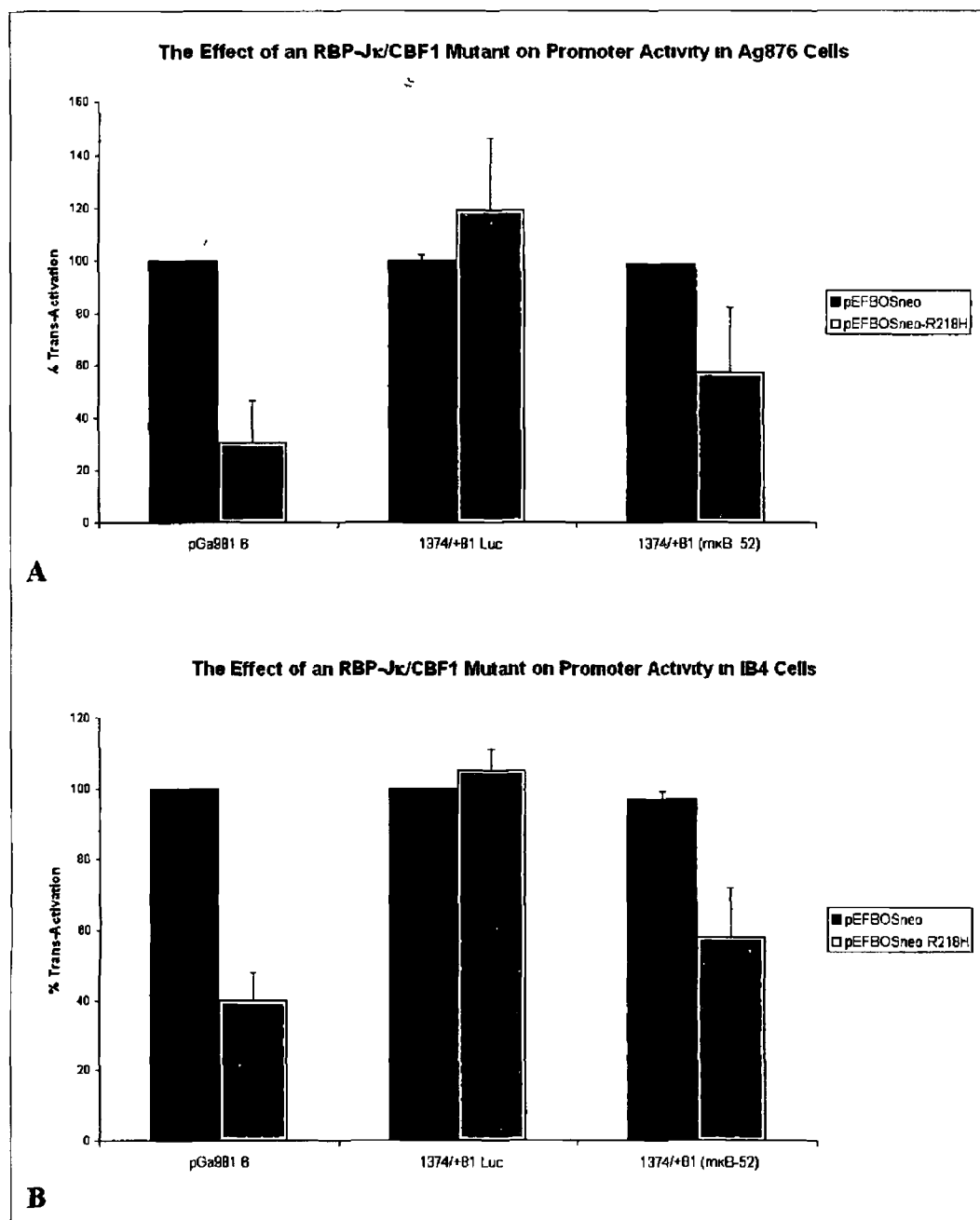


Figure 4.12 The effect of expression of the RBP-J κ /CBF1 mutant on promoter activity in the cell lines Ag876 and IB4. Co-transfections were performed using Ag876 cells (A) and IB4 cells (B) using 12 μ g of either pEFBOSneo or pEFBOSneo-R218H together with 2 μ g of the reporter construct indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the empty expression vector (pEFBOSneo). Luciferase activity obtained upon co-transfection with the empty vector was arbitrarily assigned a value of 100 %.

These results indicated that blocking LMP1-mediated *bfl-1* promoter activity by mutating the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site on the *bfl-1* promoter as well as blocking EBNA2-mediated *bfl-1* promoter

activity by over-expression of the RBP-J κ /CBF1 mutant, results in inhibition in *bfl-1* promoter trans-activation in both Ag876 and IB4 cells

4.2.7 The effect of the EBNA2 mutant with a trans-activation domain deletion on promoter activity in the cell lines Ag876 and IB4

The effect of the EBNA2 mutant on the activity of the *bfl-1* promoter construct containing a mutation in the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site was also investigated using Ag876 and IB4 cells. Initially, the RBP-J κ /CBF1-regulated reporter construct (pGa981-6) was transfected into either Ag876 or IB4 cells. As before, a high level of luciferase activity was observed in both Ag876 cells (Figure 4 13A) and IB4 cells (Figure 4 13B). When pGa981-6 was co-transfected along with the expression vector for the EBNA2 mutant (EBNA2 Mut (437-477)), a dramatic decrease (approximately 80 %) in luciferase activity was detected in both cell lines. This finding was similar to that observed in experiments using the EBV-negative BL-derived cell line DG75 (Figure 3 31B). Upon co-transfection of the EBNA2 mutant together with the wild-type *bfl-1* promoter reporter construct (-1374/+81-Luc) a 10 % decrease in luciferase activity was observed in Ag876 cells (Figure 4 13A) and a 20 % decrease was observed in IB4 cells (Figure 4 13B). The two EBV-infected cell lines were also transfected with the *bfl-1* promoter construct containing a mutation in the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site [-1374/+81 m κ B (-52)]. This mutation was not found to inhibit the level of promoter activity in either cell line. However, upon co-transfection with the EBNA2 mutant together with the promoter bearing the NF- κ B-like binding site mutation, a 40 % decrease in luciferase activity was observed in Ag876 cells and a 50 % decrease in IB4 cells.

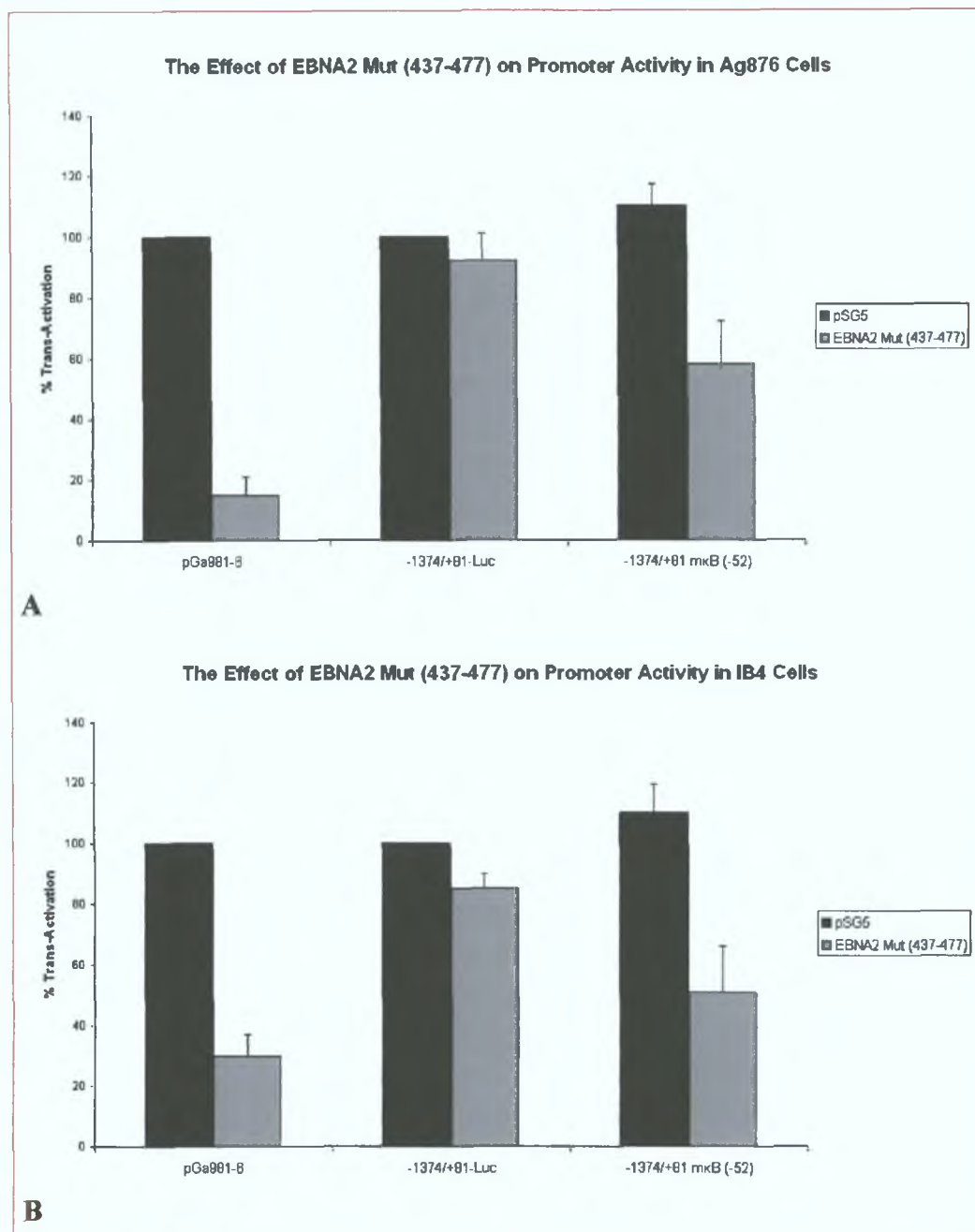


Figure 4.13: The effect of expression of the EBNA2 mutant on promoter activity in the cell lines Ag876 and IB4. Co-transfections were performed with Ag876 cells (A) and IB4 cells (B) using 12 μ g of either pSG5 or EBNA2 Mut (437-477) together with 2 μ g of the reporter construct indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon co-transfection with the empty expression vector (pSG5). Luciferase activity obtained upon co-transfection with the empty vector was arbitrarily assigned a value of 100 %.

These results indicated that blocking LMP1-mediated *bfl-1* promoter activity by mutating the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site on the *bfl-1* promoter, as well as blocking EBNA2-mediated *bfl-1* promoter

activity by over-expression of the EBNA2 mutant, results in inhibition in *bfl-1* promoter trans-activation in the cell lines Ag876 and IB4

4.3 DISCUSSION

Modulation of the expression of *bfl-1* has been studied extensively in our laboratory using the EBV-negative Burkitt's lymphoma-derived cell line DG75. It has been demonstrated that two EBV latent proteins, LMP1 and EBNA2, usurp the NF- κ B and Notch signalling pathways respectively, to affect transcription of the anti-apoptotic *bfl-1* gene in this cell context. It is widely accepted that the oncogenic EBV protein LMP1 induces survival signals through the up-regulation of anti-apoptotic proteins, such as A20, Bcl-2, Mcl-1 and cIAPs (Henderson *et al*, 1991, Fries *et al*, 1996, Wang *et al*, 1996, Laherty *et al*, 1992, Hong *et al*, 2000). The ability of EBNA2 to up-regulate *bfl-1* implies that EBNA2 may also contribute to cell survival, as well as to the proliferative response that occurs upon EBV infection. It is not yet clear whether the anti-apoptotic effect of EBNA2 is to simply complement that of LMP1, and/or to provide initial protection from apoptosis before LMP1 is expressed. The contribution of EBNA2 to the trans-activation of the *bfl-1* gene in EBV-infected cells was investigated here. Because both EBNA2 and LMP1 are expressed in EBV-infected cell lines, and EBNA2 is responsible for inducing expression of LMP1 (Abbot *et al*, 1990, Fahraeus *et al*, 1990, Laux *et al*, 1994a, Wang *et al*, 1990), experiments were performed to uncouple the roles of EBNA2 and LMP1 in the trans-activation of the *bfl-1* promoter in the EBV-positive type III (latency III) BL cell line Ag876 and the LCL IB4, both of which are typical of EBV-infected cells that express all the latent gene products.

It has already been demonstrated using DG75 cells that over-expression of an I κ B α super-repressor dramatically inhibits LMP1-mediated trans-activation of the *bfl-1* promoter (D'Souza *et al*, 2004). However, this was not found to be the case in the EBV-infected cell lines Ag876 and IB4. Although co-expression of the I κ B α super-repressor was seen to significantly decrease the level of activated NF- κ B in these cell lines, *bfl-1* promoter activity was not dramatically affected in this cell context, where inhibition of less than 20 % was observed. It is possible that the level of *bfl-1* promoter activity is maintained by some other factor, EBNA2 being a likely candidate, as the *bfl-*

I promoter was found to be responsive to this protein in EBV-negative BL-derived cells, and EBNA2 is present in both the EBV-infected cell lines Ag876 and IB4

The observation that expression of an I κ B α super-repressor did not have a dramatic effect on the level of *bfl-1* promoter activity is in discordance with the findings of another study, in which tetracycline-inducible expression of an I κ B α mutant (with a deletion from amino acids 1 to 36) in the LCL IB4 resulted in a 50 % decrease in *bfl-1* mRNA, as determined by Northern blot analysis 2 days post induction (Cahir-McFarland *et al* , 2000) Although this mutant form of I κ B α differs from the one used in the current study, in which serine residues at positions 32 and 36 have been replaced with alanines (Liljeholm *et al* , 1998), both mutants can no longer be phosphorylated and subsequently ubiquitinated, resulting in the detainment of the NF- κ B transcription factor in the cytoplasm (Brockman *et al* , 1995) Furthermore, both mutants have been shown to inhibit NF- κ B activation (Figures 3 9A and 4 1, Cahir-McFarland *et al* , 2000) Thus, the discrepancies between the two studies may lie in the fact that two different approaches were taken to monitor activity of the *bfl-1* gene Results provided from the current study reflect the rate of transcription initiation from the *bfl-1* promoter, as determined by transfection of *bfl-1* promoter reporter constructs followed by luciferase assay On the other hand, *bfl-1* mRNA levels were monitored in the study carried out Cahir-McFarland *et al* (2000) It has been demonstrated that although the transcription rates and mRNA abundance of most genes change with the same direction, they usually have different ranges in variation (Shu and Hong-Hui, 2004) *bfl-1* has been classified as an immediate-early response gene (Karsan *et al* , 1996a, Lin *et al* , 1996, Moreb and Schweber, 1997), many of which are thought to be regulated at the level of mRNA It has previously been shown in our laboratory that induction of LMP1 expression in the cell line DG75-tTA-LMP1 (Floettmann *et al* , 1996) results in a significant increase in the stability of *bfl-1* mRNA (D'Souza *et al* , 2000) Expression of an I κ B α super-repressor/mutant inhibits LMP1-mediated signalling, and as a result, could possibly reduce the ability of LMP1 to increase *bfl-1* mRNA stability This could explain the 50 % decrease in *bfl-1* mRNA observed by Northern blot analysis following induction of the I κ B α mutant (Cahir-McFarland *et al* , 2000), but would not affect the level of transcription initiated from the *bfl-1* promoter, observed during the current study

EBNA2-mediated trans-activation of the control reporter construct pGa981-6, the viral *LMP1* promoter and the cellular *bfl-1* promoter was seen to be significantly inhibited in DG75 cells, upon co-expression of an RBP-J κ /CBF1 mutant or an EBNA2 mutant with a deletion in the trans-activation domain [EBNA2 Mut (437-477)]. By contrast, expression of either of these mutants was not found to affect the level of *bfl-1* promoter activity as efficiently in the EBV-infected cell lines Ag876 or IB4. Although both mutants were found to inhibit EBNA2-mediated trans-activation of the control reporter pGa981-6 in the EBV-infected cells, the maximum inhibition observed, by either of these mutants, was 30 %. It has been established that the *LMP1* promoter is EBNA2-responsive (Abbot *et al* , 1990, Fahraeus *et al* , 1990, Laux *et al* , 1994a, Wang *et al* , 1990), and it was demonstrated in Chapter 3 that both the dominant-negative RBP-J κ /CBF1 mutant and EBNA2 Mut (437-477) blocked EBNA2-mediated trans-activation of the *LMP1* promoter in DG75 cells. If EBNA2-mediated activation of LMP1 expression were to be inhibited by these mutants in the context of the EBV-infected cell lines, it is likely that there would be a decrease in the level of activated NF- κ B as a result. This would, however, be indirect and take longer to take effect, unlike when I κ B α DN is expressed and can sequester NF- κ B subunits directly in the cytoplasm. Both the RBP-J κ /CBF1 and EBNA2 mutants were indeed seen to affect the level of NF- κ B activity, albeit to a small degree. The degree of inhibition in activated NF- κ B levels generally mirrored that observed during RBP-J κ /CBF1- or EBNA2-mutant-mediated inhibition of *bfl-1* promoter activity. Therefore, it is difficult to conclude whether the slight inhibition in *bfl-1* promoter activity as a result of co-expression with the RBP-J κ /CBF1 or EBNA2 mutants, is due to (i) inhibition of EBNA2-mediated activation of *bfl-1*, (ii) inhibition of EBNA2-mediated trans-activation of the *LMP1* promoter, and as a result lower levels of LMP1 in the cells, leading to a decrease in *bfl-1* promoter activity or (iii) a combination of these two factors.

Because blocking either LMP1 by over-expression of an I κ B α super-repressor, or blocking EBNA2 by over-expression of either an RBP-J κ /CBF1 mutant or an EBNA2 Mut (437-477), did not result in a significant decrease in *bfl-1* promoter activity, it was subsequently tested whether blocking both LMP1- and EBNA2-mediated trans-activation simultaneously would affect the level of *bfl-1* promoter activity in the EBV-infected cell lines Ag876 and IB4. To this end, co-transfections were carried out using the I κ B α super-repressor together with either the RBP-J κ /CBF1 mutant or EBNA2 Mut (437-477). Co-expression of the I κ B α super-repressor with either the RBP-J κ /CBF1 or

EBNA2 mutant resulted in between 40 % to 60 % inhibition in *bfl-1* promoter activity in both cell lines, suggesting that blocking both LMP1- and EBNA2-mediated activation significantly affects the level of *bfl-1* promoter activity. This implies that both these EBV latent proteins play a role in maintaining the level of *bfl-1* promoter activity in the context of the EBV-infected B lymphocyte.

In a second attempt to block both LMP1- and EBNA2-mediated trans-activation of the *bfl-1* promoter, co-transfections were carried out using either the RBP-J κ /CBF1 or EBNA2 mutant along with a *bfl-1* promoter reporter construct containing a mutation in the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site of the *bfl-1* gene. The mutation in this NF- κ B-like binding site has been found to almost completely abolish LMP1-responsiveness on the *bfl-1* promoter in the EBV-negative BL-derived cell line DG75 (D'Souza *et al* , 2004). By contrast, transfection of this *bfl-1* promoter reporter construct containing the -52 to -43 deletion was not found to affect the level of *bfl-1* promoter activity in Ag876 or IB4 cells, strengthening the observation that blocking LMP1-mediated signalling does not affect the level of *bfl-1* promoter activity in Ag876 or IB4 cells. However, by blocking LMP1-mediated *bfl-1* promoter activity by mutating the NF- κ B-like binding site, and blocking EBNA2-mediated promoter activity by co-expression with either the RBP-J κ /CBF1 or EBNA2 mutants, the level of promoter activity was reduced by approximately 50 %. The level of inhibition was similar to that observed after blocking both LMP1 and EBNA2 signalling by co-expression of the I κ B α super-repressor along with the RBP-J κ /CBF1 or EBNA2 mutants. The observation that blocking both LMP1- and EBNA2-mediated trans-activation of the *bfl-1* promoter at the same time results in approximately 50 % decrease in promoter activity was reinforced during transfection experiments using the I κ B α super-repressor and a *bfl-1* promoter reporter construct containing a mutation in putative RBP-J κ /CBF1 binding site at position -343 to -249 relative to the transcription start site of the *bfl-1* gene. Mutation of this putative RBP-J κ /CBF1 binding site has been shown to significantly inhibit EBNA2-mediated trans-activation of the *bfl-1* promoter in DG75 cells (Pegman *et al* , submitted December, 2004). It was observed in the current study that the presence of this mutation did not affect the level of *bfl-1* promoter activity in the EBV-infected cell lines Ag876 and IB4. However, upon co-transfection of the RBP-J κ /CBF1 mutated promoter reporter construct together with the I κ B α super-repressor, an approximate 50 % decrease in *bfl-1* promoter activity was observed. This novel finding implies a role for the putative RBP-J κ /CBF1 binding site in the trans-

activation of the *bfl-1* promoter in the context of EBV-infected cell. Although blocking both EBNA2- and LMP1-mediated trans-activation results in a significant decrease in *bfl-1* promoter activity, inhibition was not found to be as efficient as that observed by blocking either pathway in the EBV-negative B cell line DG75. It is possible that the *bfl-1* promoter is responsive to other, as yet unidentified, factors present in these cell contexts.

Chapter 5

Regulation of the Anti-Apoptotic Cellular *bfl-1* Gene in Conditional Lymphoblastoid Cell Lines

5.1 INTRODUCTION

The EBV-infected cell lines (Ag876 and IB4) used up to this point express LMP1 and EBNA2 simultaneously, and LMP1 expression is induced by EBNA2. In order to uncouple the roles of LMP1 and EBNA2 in the regulation of the *bfl-1* gene, a series of cell lines were used in which EBNA2 could be expressed conditionally. Fusion of a given protein with the hormone-binding domain of a steroid receptor can render its function dependent on the presence of hormone (Picard *et al*, 1988, Eilers *et al*, 1989). Kempkes *et al* (1995b) exploited this approach to develop a conditional system in which the function of EBNA2 could be switched on and off. Firstly, they generated a chimeric protein, in which the N-terminus of EBNA2 was fused with the hormone-binding domain of the estrogen receptor. Conditional function of this construct in the presence or absence of hormone was verified by testing the trans-activation of the *LMP* genes, which are known target genes for transcriptional activation by EBNA2 (Abbot *et al*, 1990, Fahraeus *et al*, 1990, Wang *et al*, 1990, Zimmer-Strobl *et al*, 1991, Laux *et al*, 1994a). Both the trans-activation of these genes by estrogen responsive (ER)-EBNA2 and interaction between the chimeric EBNA2 and RBP-J κ /CBF1, were found to be dependent on the presence of estrogen (Kempkes *et al*, 1995a).

Kempkes *et al* (1995b) subsequently cloned the gene encoding the conditional EBNA2 into a mini-EBV vector carrying all the *cis*-acting elements, which are essential for propagation of the plasmid in all phases of the EBV life cycle. The mini-EBV plasmid (Figure 5 1A) carries the origin of replication (*oriP*), which is required for the maintenance of the EBV plasmid in target cells, the lytic origin of replication (*oriLyt*), which allows high copy number amplification upon induction of the lytic cycle, and the terminal repeats (TR), which permit packaging of the mini-EBV into virions. The non-transforming P3HR1 strain of EBV (Figure 5 1B), in which the EBNA2 open reading frame has been deleted served as a helper virus for amplification and packaging of these mini-EBV plasmids. P3HR1 cells were transfected with the mini-EBV plasmid expressing the chimeric EBNA2 and simultaneously induced for lytic cycle replication (Kempkes *et al*, 1995b). Supernatants of transfected cells containing the mini-EBV plasmid, together with the P3HR1 helper virus, were harvested and used to co-infect primary B-cells, which were cultivated in the presence of estrogen (Kempkes *et al*, 1995b). The resulting LCL, designated EREB 2.5, contained mini-EBV plasmid expressing the chimeric EBNA2 protein as an autonomously replicating plasmid, plus

the P3HR1 viral genome, thus complementing the EBNA2 deletion of the P3HR1 virus (Kempkes *et al.*, 1995b). Therefore, in the presence of estrogen, functional EBNA2 trans-activates the P3HR1 viral promoters of the three latent membrane proteins (LMP1, LMP2A and LMP2B) and the Cp promoter, which regulates the expression of the EBV nuclear antigens. In the absence of estrogen EBNA2 is non-functional and is unable to trans-activate P3HR1 virus genes. Therefore LMP1 expression is dependent on the presence of functional EBNA2 (i.e. the presence of estrogen in the culture medium).

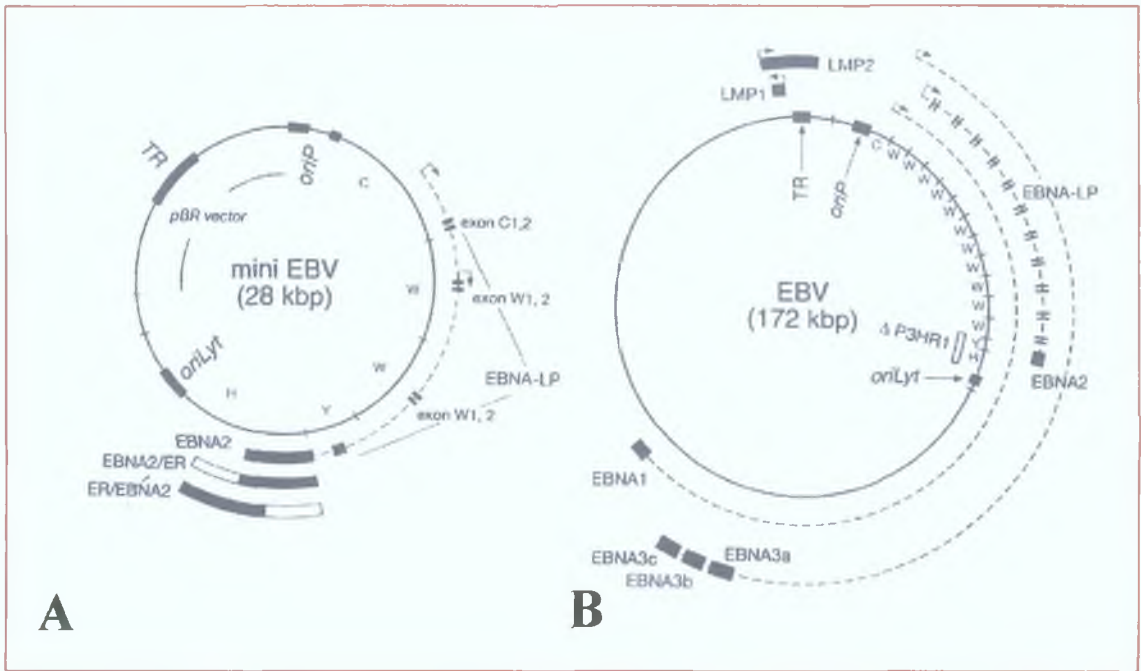


Figure 5.1: Strategy for expression of conditional EBNA2 mutants in EREB 2.5 cells. (A) Mini-EBV plasmids are composed of *cis*-acting elements, which are essential for the propagation of these plasmids in all phases of the EBV life cycle. They carry the oriP necessary for the maintenance of the EBV plasmids in target cells, the oriLyt, which allows high copy number amplification upon induction of lytic replication of the virus, and the TRs, which allow packaging of the mini-EBV plasmids into virions. The hormone-binding domain of the estrogen receptor (white boxes) is fused to the N-terminus of the wild type EBNA2 ORF (black boxes) yielding ER-EBNA2. The EBNA2 expression cassettes are flanked by EBNA2 sequences, the *Bam*HI restriction fragments C, W, Y, and H of the EBV genome, which represent in part the genomic organisation of the EBV DNA and encompass the deletion present in the non-transforming P3HR1 EBV strain (Δ P3HR1 open bar in B). EBNA2 and ER-EBNA2 can be transcribed from two alternative promoters situated in the C or W fragments. (B) The EBV map shows the position of EBNAs and LMPs transcribed and the location of their promoters relative to the *Bam*HI restriction map of EBV strain B95.8 (adapted from Kempkes *et al.*, 1995b).

5.2 RESULTS

5.2.1 Regulation of *bfl-1* mRNA levels in EREB 2.5 stable transfectants

To uncouple the roles of EBNA2 and LMP1 with regard to the regulation of *bfl-1* mRNA levels in the context of an EBV-infected cell, experiments using a panel of EREB 2.5 cell derivatives were performed. Zimmer-Strobl *et al.* (1996) established a system in which LMP1 is expressed constitutively from a stably transfected vector in EREB 2.5 cells. This group cloned the LMP1 gene, expressed from the SV40 early promoter/enhancer, onto an episomal oriP vector encoding the gene for hygromycin phosphotransferase (Sugden *et al.*, 1985). This construct, termed p581, was then introduced into EREB 2.5 cells, resulting in the establishment of two stably transfected cell lines, namely EREB 2.5 SV LMP clone 2C and EREB 2.5 SV LMP clone 11C (Zimmer-Strobl *et al.*, 1996). As negative controls, a p581-based plasmid with a truncated non-functional LMP1 gene or the parental oriP vector (pHEBo) were introduced into EREB 2.5 cells, generating the cell lines EREB 2.5 SV LMP Mut 2 and EREB 2.5 pHEBo. These EREB 2.5 stable transfectants allow dissection of the role of LMP1 in the regulation of the *bfl-1* gene in the absence of EBNA2. The LMP1 and EBNA2 expression profile of these cell lines grown in the presence or absence of estrogen is summarised in Table 5.1.

Table 5.1: EBNA2 & LMP1 expression profile of EREB 2.5 cell stable transfectants

Cell line	+ Estrogen	- Estrogen
pHEBo	EBNA2 & LMP1	no EBNA2, no LMP1
SV LMP clone 2C	EBNA2 & LMP1	no EBNA2, plasmid LMP1
SV LMP clone 11C	EBNA2 & LMP1	no EBNA2, plasmid LMP1
SV LMP Mut 2	EBNA2 & LMP1	no EBNA2, LMP1 mutant

In order to monitor *bfl-1* mRNA levels in the presence of EBNA2 and/or LMP1 a series of time courses were performed with the various EREB 2.5 cell line derivatives, cultivated in the presence or absence of estrogen. All cell lines were washed four times in PBS, followed by another two washes 24 hours later. At this point each cell line was

divided, with one half seeded into medium supplemented with 1 μ M estrogen and the other half seeded into medium without estrogen. Protein and RNA extracts were prepared at various time points over a 96-hour period. The level of EBNA2 and LMP1 protein expressed during the time-course was analysed by western blot, and *bfl-1* mRNA levels were assayed by reverse transcription followed by real-time PCR.

5.2.1.1 Regulation of *bfl-1* mRNA levels in EREB 2.5 pHEBo cells

Western blot analysis on samples isolated during the time course using EREB 2.5 pHEBo cells is shown in Figure 5.2A. EREB 2.5 pHEBo cells grown in the presence of estrogen expressed chimeric EBNA2 with an apparent molecular weight of 120 kDa (in contrast to 86 kDa, which is the molecular weight of wild type EBNA2). An increase in EBNA2 expression was observed over time. This result is compatible with a functional role for EBNA2 in positive regulation of its own promoter (Sung *et al* , 1991, Rooney *et al* , 1992). It was noted that LMP1 expression also increased over time, indicating that the *LMP1* gene is under strict control of functional EBNA2. In the absence of estrogen, only non-functional EBNA2 was detected and LMP1 levels were seen to deteriorate over time, reinforcing the idea that functional EBNA2 regulates LMP1 expression. Removal of estrogen also affected the electrophoretic mobility of the ER-EBNA2 fusion protein. Chimeric EBNA2 was seen to migrate more rapidly and homogeneously in the absence of hormone, indicating that switching ER-EBNA2 function on and off is associated with post-translational modification or profound changes in the tertiary structure of ER-EBNA2. Furthermore, the amount of ER-EBNA2 fusion protein appeared to decrease in the absence of hormone, indicating once again that functional EBNA2 positively regulates its own promoter.

Initially RNA isolated during all the time-courses performed was analysed by agarose gel electrophoresis. Analysis of the RNA extracted from EREB 2.5 pHEBo cells is shown in Figure 5.2B. The presence of two strongly stained bands represent the 28 S and 18 S ribosomal RNAs, which indicated intact RNA.

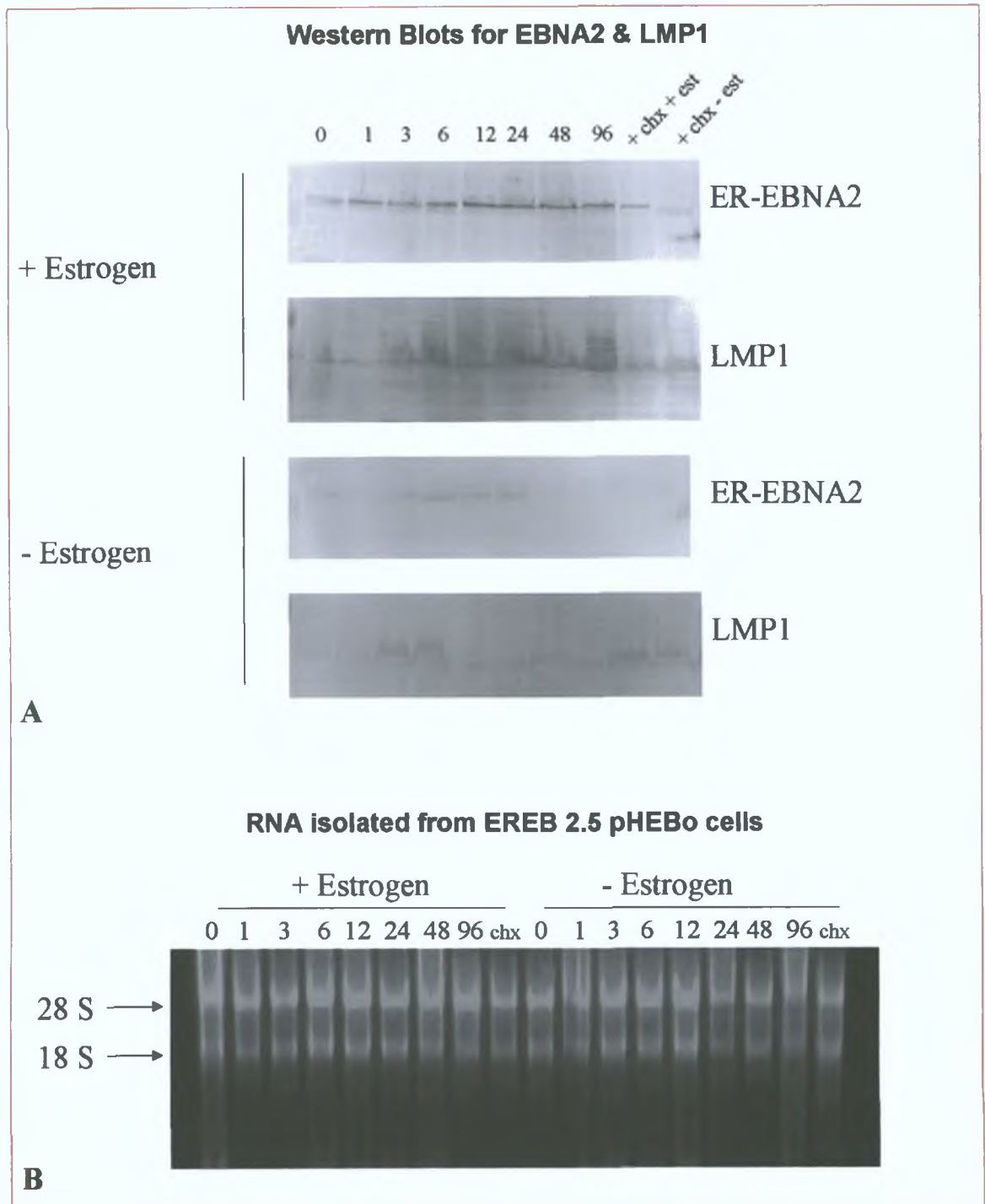


Figure 5.2: Protein and RNA analysis on samples isolated during the time-course using EREB 2.5 pHEBo cells. EREB 2.5 pHEBo cells were washed 4 times in PBS, followed by another 2 washes 24 hours later. Cells were re-seeded into medium with or without estrogen (est) and/or cyclohexamide (chx). Protein and RNA were isolated at various time-points over a 96-hour period. Protein samples were analysed for EBNA2 or LMP1 expression (A). Isolated RNA stained with ethidium bromide was analysed by agarose gel electrophoresis (B). The presence of two strongly stained bands represent the 28 S and 18 S ribosomal RNAs, which indicated intact RNA.

Once it was established that the RNA was of good integrity, reverse transcription (RT) reactions were carried out to generate cDNA, which was subsequently used in a real-

time PCR assay to determine the level of *bfl-1* mRNA present. mRNA levels of the house keeping gene *gapdh* were also monitored, and the resulting data obtained was used to normalise data acquired for *bfl-1*. Following real-time PCR assays, PCR products were analysed by agarose gel electrophoresis to determine whether the PCR products migrated at the expected size. The real-time PCR products generated from samples acquired during the time-course using EREB 2.5 pHEBo cells cultivated in the presence of estrogen are shown in Figure 5.3. The PCR products for *bfl-1* and *gapdh* migrated at the expected band sizes of 150 bp and 168 bp respectively, and only one PCR product was found in each reaction, indicating the specificity of the PCR assay.

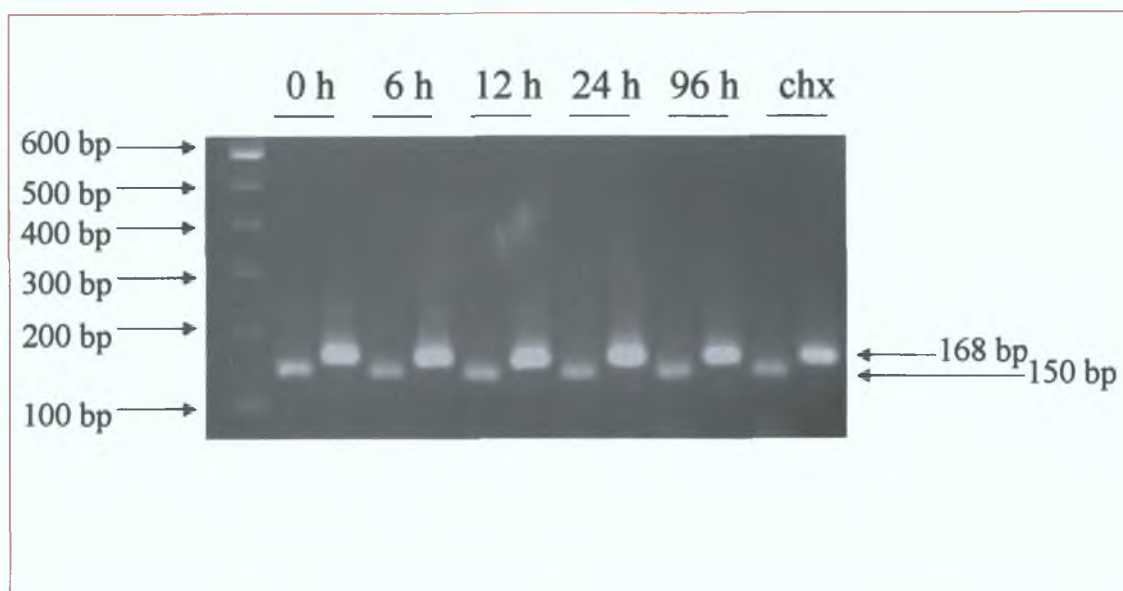


Figure 5.3: PCR products amplified from cDNA generated from the RNA isolated from EREB 2.5 pHEBo cells. cDNA was generated from RNA isolated from EREB 2.5 pHEBo cells by reverse transcription PCR. The cDNA was then used as template for real time PCR to quantitate the amount of *bfl-1* and *gapdh* present. Figure 2 shows real-time PCR products amplified from cDNA generated from EREB 2.5 pHEBo cells grown in the presence of estrogen over a time-course (0 - 96 hours). PCR products for *bfl-1* and *gapdh* migrated at the expected band sizes of 150 bp and 168 bp respectively.

Figure 5.4 shows the differences in *bfl-1* mRNA levels over time in the EREB 2.5 pHEBo cells. A dramatic increase in *bfl-1* levels (approximately 4-fold) was observed 6 hours following the addition of estrogen to the culture medium. Both functional EBNA2 and LMP1 were expressed at high levels at this time point (Figure 5.2A). The amount of *bfl-1* decreased gradually over time, with the level of *bfl-1* mRNA at 96 hours similar to that observed at time 0. *bfl-1* mRNA levels were seen to decrease significantly over time in EREB 2.5 pHEBo cells grown in the absence of estrogen. This correlated with a decrease in the expression of functional EBNA2 and LMP1.

These results indicated that turning on functional EBNA2, and therefore LMP1, by addition of estrogen, led to a transient increase in the level of *bfl-1* mRNA in the LCL EREB 2.5 pHEBo

In order to determine whether up-regulation of *bfl-1* mRNA was a direct effect of functional EBNA2, EREB 2.5 pHEBo cells were cultivated in medium containing 50 µg/ml cyclohexamide (chx), which is a well-known protein synthesis inhibitor. It should be noted that some chimeric EBNA2 is already present in the cells and estrogen simply activates its function, thus the expression of existing ER-EBNA2 is not inhibited by cyclohexamide. However, because EBNA2 positively regulates its own promoter, a lot more functional EBNA2 is expressed in the absence of cyclohexamide. Translation of genes trans-activated by functional EBNA2 (i.e. *LMP* and *Cp* genes) is significantly inhibited by the presence of cyclohexamide in the culture medium.

Cells cultivated in the presence of cyclohexamide were harvested after 6 hours in order to prepare protein and RNA extracts. The level of *bfl-1* mRNA prepared from cells grown in the presence of both estrogen and cyclohexamide for 6 hours was compared with that of cells grown in estrogen only for the same period of time. It was observed that the level of *bfl-1* mRNA in cells cultivated in the presence of estrogen and cyclohexamide was 1.5 fold lower than that of cells grown in estrogen only after 6 hours in culture (Figure 5.4). This was not due to instability of the *bfl-1* mRNA, as there was no difference between the levels of *bfl-1* observed in cells grown in the absence of estrogen and cells grown in the absence of estrogen plus cyclohexamide after 6 hours. One possible reason for the decrease in the level of *bfl-1* mRNA in cells grown in the presence of both estrogen and cyclohexamide may lie in the fact that less EBNA2 is expressed in the presence of cyclohexamide (Figure 5.2A). As cyclohexamide is a protein synthesis inhibitor, it would inhibit EBNA2 expression resulting from functional EBNA2 positively regulating its own promoter. There was also a notable decrease in the level of LMP1 expression in cells grown in cyclohexamide, relative to cells grown in the absence of cyclohexamide harvested at the same time-point (Figure 5.2A), suggesting that LMP1 plays a possible role in up-regulating *bfl-1*. Furthermore, the fact that *bfl-1* levels in the presence of estrogen and cyclohexamide is greater than the level of *bfl-1* at time 0 implies a direct role for EBNA2 in the up-regulation of *bfl-1*.

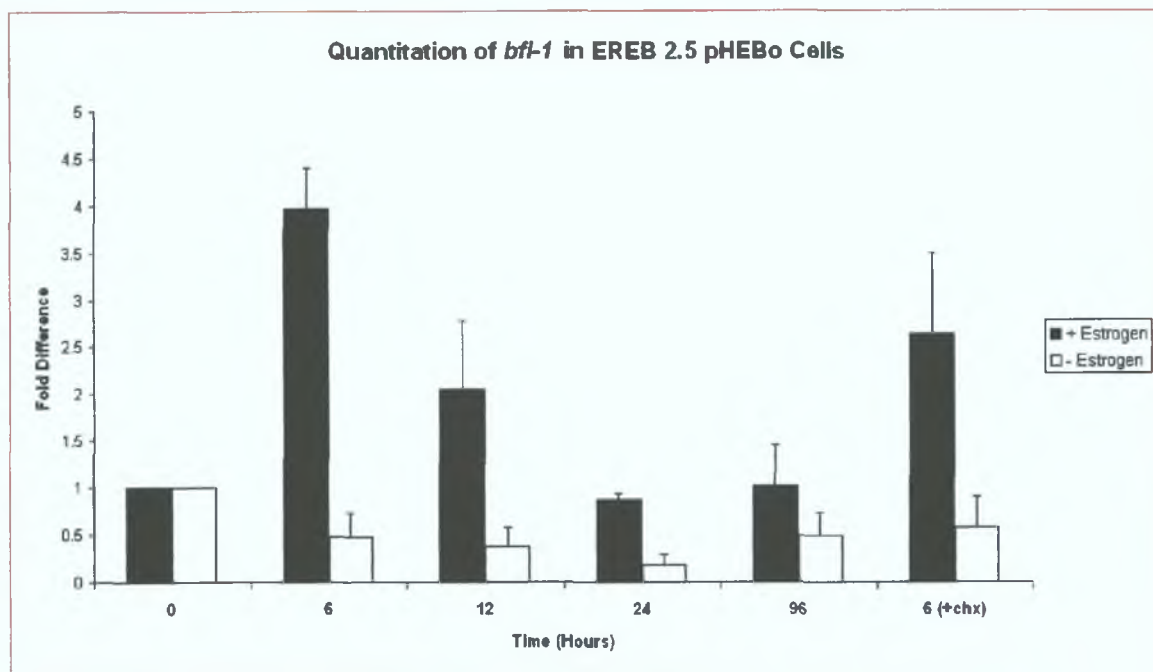


Figure 5.4: *bfl-1* mRNA quantitation in EREB 2.5 pHEBo cells. EREB 2.5 pHEBo cells were washed 4 times with PBS, followed by another 2 washes 24 hours later. Cells were re-seeded into medium with or without estrogen, and in some cases in the presence of cyclohexamide (chx). RNA was isolated at intervals over a period of time (0 - 96 hours) and quantitated by reverse transcription followed by real-time PCR. The level of *bfl-1* mRNA was assayed in triplicate and normalised by *gapdh* level. Fold differences were calculated relative to *bfl-1* levels at time zero.

These data indicated that turning on functional EBNA2, and as a result LMP1, leads to a transient increase in the level of *bfl-1* mRNA in EREB 2.5 pHEBo cells. Inhibition of protein synthesis, including that of LMP1 causes a decrease in the level of *bfl-1* mRNA relative to cells in which protein synthesis has not been blocked. In cells where LMP1 translation is inhibited, however, an increase in the level of *bfl-1* is observed after 6 hours, implying a direct role for EBNA2 in the up-regulation of *bfl-1* in this cell context.

5.2.1.2 Regulation of *bfl-1* mRNA levels in EREB 2.5 SV LMP clone 11C cells

Protein expression and *bfl-1* mRNA levels observed during the time-course performed using EREB 2.5 SV LMP clone 11C cells is shown in Figure 5.5. Western blot analysis for EBNA2 and LMP1 may be seen in Figure 5.5A. Similarly to EREB 2.5 pHEBo cells grown in the presence of estrogen, an increase in chimeric EBNA2 expression was observed over the 96-hour time-course. When estrogen was withdrawn from the culture

medium, only non-functional EBNA2 was detected. As for EREB 2.5 pHEBo cells, it was observed that ER-EBNA2 migrated more rapidly and homogenously in the absence of hormone. In contrast to the EREB 2.5 pHEBo cell line, cells transfected with the p581 vector expressing the wild type LMP1 gene expressed from the SV40 promoter/enhancer, revealed a level of LMP1 that was not down-regulated after estrogen withdrawal. The LMP1 levels were comparable or even slightly higher than in EREB 2.5 pHEBo cells in the presence of estrogen, which is not unexpected because in the transfectants there is probably expression of LMP1 both from the resident viral genome and from the introduced vector.

Figure 5.5B shows the differences in *bfl-1* mRNA levels over time in the EREB 2.5 SV LMP clone 11C cells. A dramatic increase in *bfl-1* levels was observed 6 hours after addition of estrogen to the culture medium. A further increase in the level of *bfl-1* mRNA was observed at the 12-hour time point. Both functional EBNA2 and LMP1 were expressed at high levels at these time points (Figure 5.5A). The amount of *bfl-1* decreased gradually over time, with the level of *bfl-1* mRNA at 96 hours slightly higher than that observed at time 0. In the absence of estrogen, the level of *bfl-1* mRNA was maintained at a level higher than that recorded at time 0, with a 2.5-fold increase in the level of *bfl-1* after 96 hours in culture. In the absence of estrogen only non-functional chimeric EBNA2 was expressed, but LMP1 was expressed from the p581 vector (Figure 5.5A). The observation that *bfl-1* levels are maintained in the absence of estrogen contrasts with the levels of *bfl-1* mRNA recorded using the EREB 2.5 pHEBo cell line, which has been transfected with the empty expression vector for LMP1. When these cells were grown in the absence of estrogen, the level of *bfl-1* mRNA decreased over time (Figure 5.4). These results demonstrate that LMP1 is capable of maintaining elevated *bfl-1* mRNA levels in the absence of functional EBNA2 in the LCL EREB 2.5 SV LMP clone 11C.

EREB 2.5 SV LMP clone 11C cells cultivated in cyclohexamide were again used to determine whether up-regulation of *bfl-1* mRNA was a direct effect of functional EBNA2. The level of *bfl-1* mRNA prepared from cells grown in the presence of both estrogen and cyclohexamide for 6 hours was compared with that of cells grown in estrogen only for the same period of time. It was observed that the level of *bfl-1* mRNA in cells cultivated in the presence of estrogen and cyclohexamide was lower than that of cells grown in estrogen only after 6 hours in culture (Figure 5.5B). No difference in the

level of *bfl-1* was observed between cells grown in the absence of estrogen at 6 hours and cells grown in the absence of estrogen and cyclohexamide, indicating that *bfl-1* mRNA remains stable for this period of time. The level of LMP1 expression was seen to decrease in cells grown in cyclohexamide, relative to cells grown in the absence of cyclohexamide harvested at the same time-point (Figure 5.5A), suggesting that LMP1 plays a possible role in up-regulating *bfl-1* in this cell context. The level of *bfl-1* mRNA at time 0 was lower than that of cells grown in the presence of estrogen and cyclohexamide. Although the level of LMP1 was observed to decrease in the presence of the translation inhibitor, some LMP1 was still detectable. As a result, it is difficult to conclude whether EBNA2 plays a direct role in *bfl-1* up-regulation in this case.

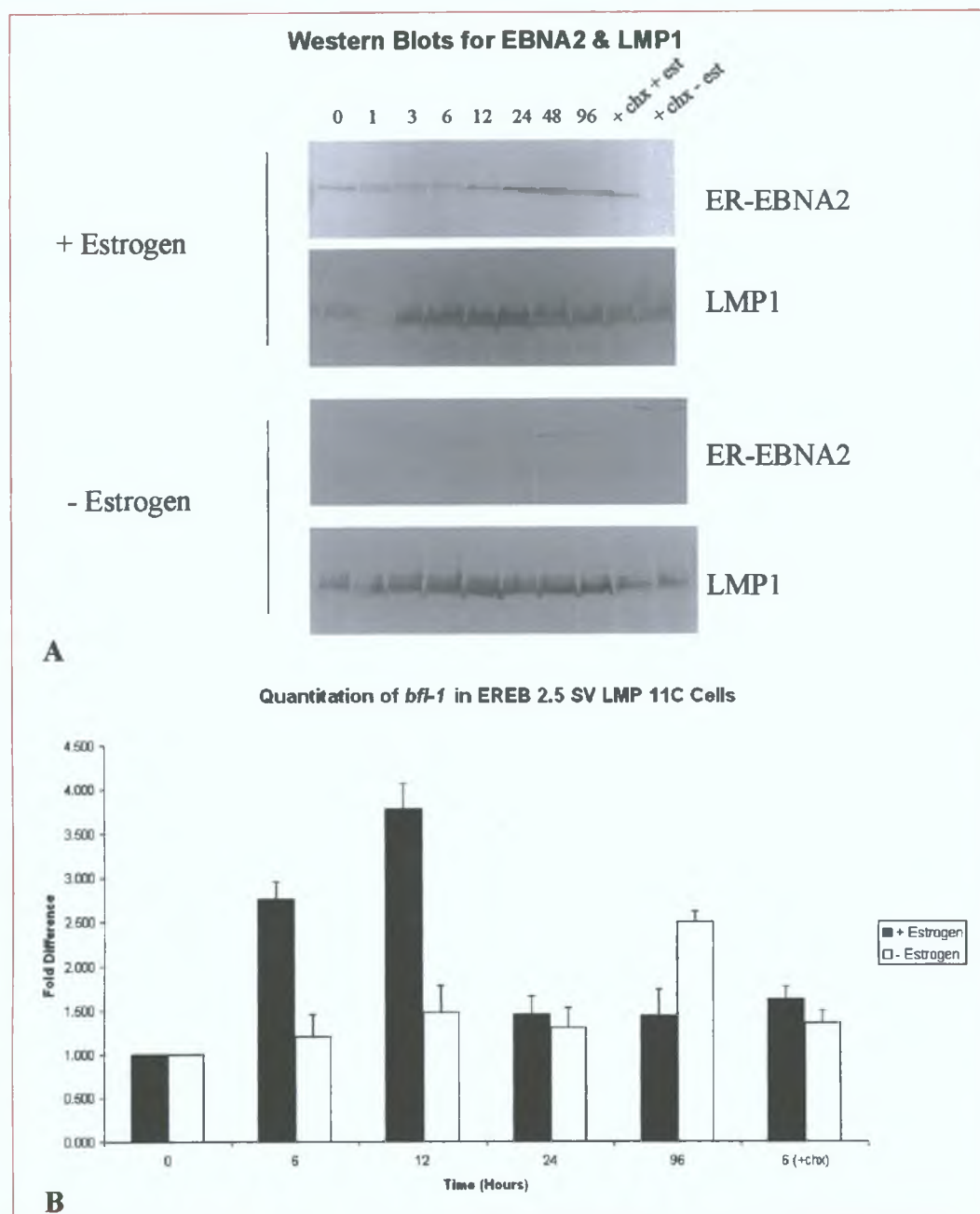


Figure 5.5: Protein expression and *bfl-1* quantitation in EREB 2.5 SV LMP clone 11C cells. EREB 2.5 SV LMP clone 11C cells were washed 4 times in PBS, followed by another 2 washes 24 hours later. Cells were re-seeded into medium with or without estrogen. Protein and RNA were isolated at various time-points over a 96-hour period. Protein samples were analysed for EBNA2 or LMP1 expression (A). The amount of *bfl-1* mRNA present in the cells at individual time-points over the 96-hour period was quantitated by reverse transcription followed by real time PCR. *bfl-1* levels were assayed in triplicate and normalised by *gapdh* levels (B). Fold differences were calculated relative to *bfl-1* levels at time zero.

Taken together, these results indicated once again that turning on functional EBNA2, and as a result LMP1, leads to a transient increase in *bfl-1* mRNA in the cell line EREB 2.5 SV LMP clone 11C. Inhibition of protein translation, resulting in a lower level of LMP1 expression, causes a decrease in the level of *bfl-1* mRNA relative to cells in

which protein translation has not been blocked. Importantly, it was also observed that LMP1 is capable of maintaining elevated *bfl-1* mRNA levels in the absence of functional EBNA2 in this cell context.

5.2.1.3 Regulation of *bfl-1* mRNA levels in EREB 2.5 SV LMP clone 2C cells

The data obtained from the time-course carried out using EREB 2.5 SV LMP clone 2C cells is shown in Figure 5.6. Western blot analysis for EBNA2 and LMP1 may be seen in Figure 5.6A. As was the case for the other EREB 2.5 transfectants, chimeric EBNA2 and LMP1 expression increased over time in cells grown in the presence of estrogen, strengthening the observation that turning on functional EBNA2 positively regulates its own expression, as well as that of LMP1. When estrogen was withdrawn from the culture medium, only non-functional EBNA2 was detected. LMP1 levels were seen to deteriorate by 12 hours post-withdrawal of estrogen from the culture medium. This result was unexpected, as this cell line (similarly to EREB 2.5 SV LMP clone 11C) has been transfected with the p581 vector that expresses wild type LMP1 from the SV40 promoter/enhancer and should, therefore, express LMP1 from the plasmid, independent of EBNA2. It is possible that this cell line has lost the LMP1 expression vector.

Figure 5.6B shows the differences in *bfl-1* mRNA levels over time in the EREB 2.5 SV LMP clone 2C cell line. An increase in *bfl-1* levels was observed 6 hours after addition of estrogen to the culture medium, which was increased further after 12 hours. The increase in *bfl-1* mRNA coincided with an increase in the expression of functional EBNA2 and LMP1 (Figure 5.6A). The amount of *bfl-1* decreased gradually over time, with the level of *bfl-1* mRNA at 96 hours slightly lower than that observed at time 0. *bfl-1* mRNA levels were seen to decrease over time in cells grown in the absence of estrogen. This correlated with a decrease in the expression of functional EBNA2 and LMP1. These results indicated for the second time that turning on functional EBNA2, and therefore LMP1, up-regulates the level of *bfl-1* mRNA in an LCL.

In order to determine whether up-regulation of *bfl-1* mRNA was a direct effect of functional EBNA2, EREB 2.5 SV LMP clone 2C cells were cultivated in medium containing cyclohexamide. The level of *bfl-1* mRNA prepared from cells grown in the presence of both estrogen and cyclohexamide for 6 hours was compared with that of

cells grown in estrogen only for the same period of time. It was observed that the level of *bfl-1* mRNA in cells cultivated in the presence of estrogen and cyclohexamide was slightly lower than that of cells grown in estrogen only after 6 hours in culture (Figure 5.6B). The level of LMP1 expression also decreased significantly in cells grown in cyclohexamide, relative to cells grown in the absence of cyclohexamide harvested at the same time-point (Figure 5.6A). It is possible that the decrease in the level of *bfl-1* mRNA is due to inhibition of LMP1 expression by cyclohexamide, implying a role for LMP1 in up-regulation of *bfl-1*. The fact that level of *bfl-1* mRNA in the presence of estrogen and cyclohexamide is greater than at time 0 indicates a direct role for EBNA2 in the up-regulation of *bfl-1*.

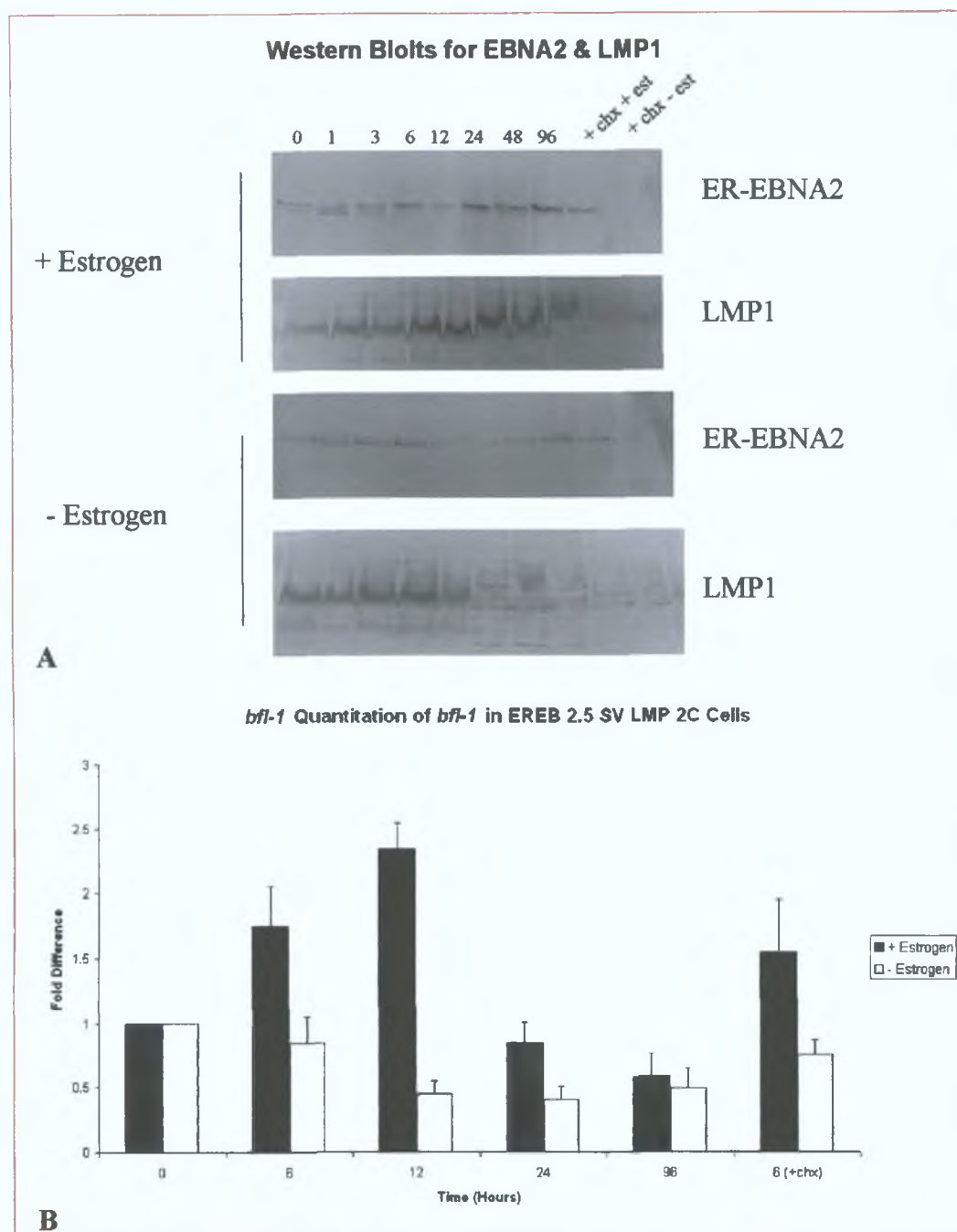


Figure 5.6: Protein expression and *bfl-1* quantitation in EREB 2.5 SV LMP clone 2C cells. EREB 2.5 SV LMP clone 2C cells were washed 4 times in PBS, followed by another 2 washes 24 hours later. Cells were re-seeded into medium with or without estrogen. Protein and RNA extracts were prepared at various time-points over a 96-hour period. Protein samples were analysed for the expression of EBNA2 or LMP1 (A). The amount of *bfl-1* mRNA present in the EREB 2.5 SV LMP clone 2C cells at individual time-points over the 96-hour period was quantitated by reverse transcription followed by real time PCR. *bfl-1* levels were assayed in triplicate and normalised by *gapdh* levels (B). Fold differences were calculated relative to *bfl-1* levels at time zero.

These results indicated that turning on functional EBNA2, and as a result LMP1, results in a transient increase in *bfl-1* mRNA in EREB 2.5 SV LMP clone 2C cells. Inhibition of protein translation causes a decrease in the level of *bfl-1* mRNA relative to cells in

which protein translation has not been blocked. In cells where protein translation has been inhibited, however, an increase in the level of *bfl-1* is observed after 6 hours, implying a direct role for EBNA2 in the up-regulation of *bfl-1* in this cell context.

5.2.1.4 Regulation of *bfl-1* mRNA levels in EREB 2.5 SV LMP Mut 2 cells

The data obtained from the time-course carried out using the EREB 2.5 SV LMP Mut 2 cell line is shown in Figure 5.7. Expression of both ER-EBNA2 and LMP1 was seen to increase over the 96-hour time-course when the cells were grown in the presence of estrogen (Figure 5.7A). In the absence of estrogen only non-functional EBNA2 was detected. In contrast with the EREB 2.5 SV LMP clone 11C cells, LMP1 levels were seen to deteriorate over time in the EREB 2.5 SV LMP Mut 2 cells grown without estrogen. These cells have been transfected with a vector expressing a non-functional truncated LMP1, which is undetectable with the LMP1 antibody (CS1-4).

Figure 5.7B shows the differences in *bfl-1* mRNA levels over time in the EREB 2.5 SV LMP Mut 2 cells. An increase in *bfl-1* levels was observed 6 hours after addition of estrogen to the culture medium, which was increased further after 12 hours. The increase in *bfl-1* mRNA coincided with an increase in the expression of functional EBNA2 and LMP1 (Figure 5.7A). The amount of *bfl-1* decreased gradually over time, with the level of *bfl-1* mRNA at 96 hours slightly higher than that observed at time 0. *bfl-1* mRNA levels were seen to decrease over time in cells grown in the absence of estrogen. This correlated with a decrease in the expression of functional EBNA2 and LMP1. Only non-functional truncated LMP1 is expressed from the SV40 promoter enhancer.

EREB 2.5 SV LMP Mut 2 cells were cultivated in cyclohexamide to determine whether up-regulation of *bfl-1* mRNA was a direct effect of functional EBNA2. The level of *bfl-1* mRNA prepared from cells grown in the presence of both estrogen and cyclohexamide for 6 hours was compared with that of cells grown in estrogen only for the same period of time. It was observed that the level of *bfl-1* mRNA in cells cultivated in the presence of estrogen and cyclohexamide was slightly higher than that of cells grown in estrogen only after 6 hours in culture (Figure 5.7B). Western blot analysis of cells grown in cyclohexamide and estrogen revealed a decrease in LMP1

expression after 6 hours (Figure 5.7A). These results indicate a direct role for EBNA2 in the up-regulation of *bfl-1*.

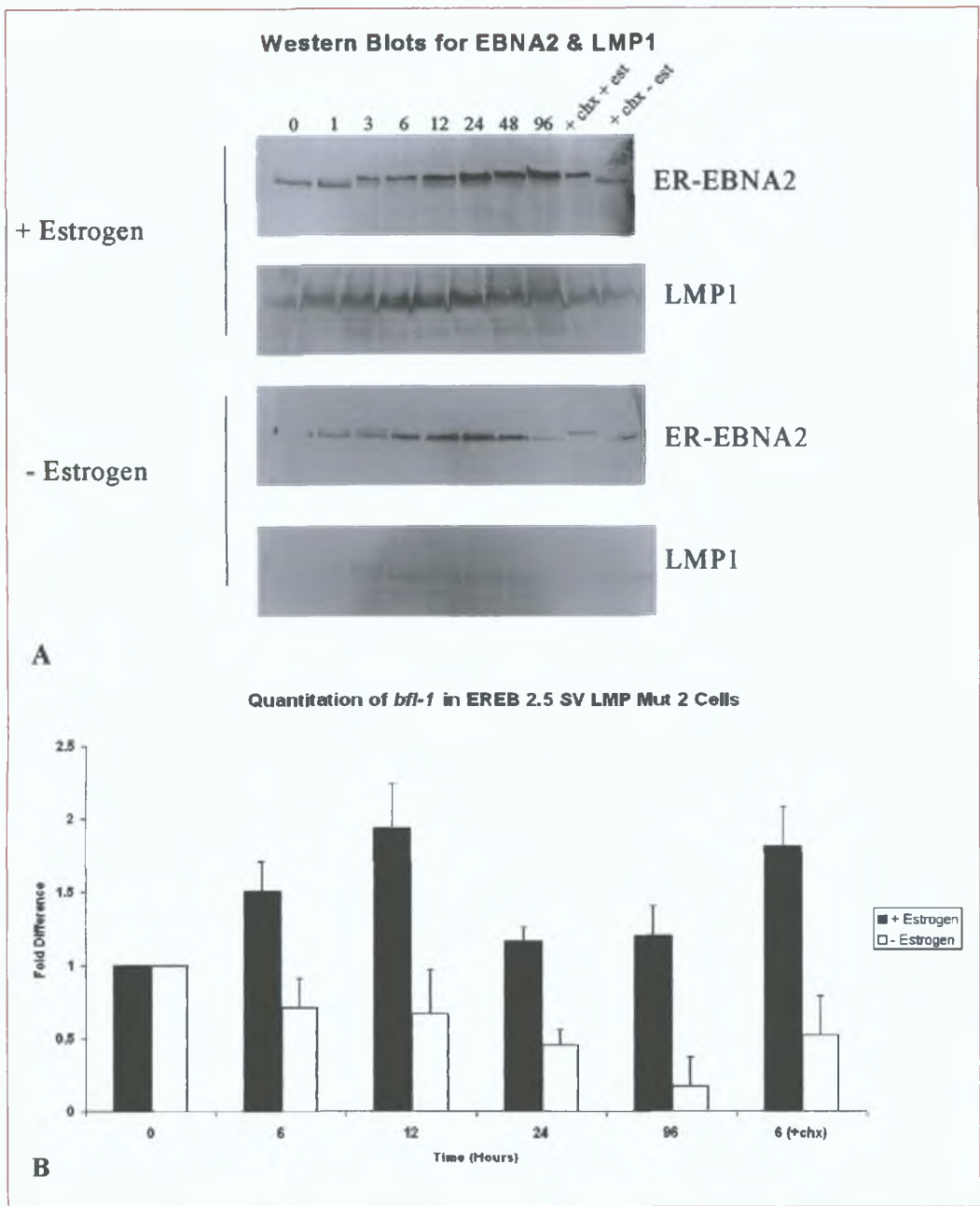


Figure 5.7: Protein expression and *bfl-1* quantitation in EREB 2.5 SV LMP Mut2 cells. EREB 2.5 SV LMP Mut2 cells were washed 4 times in PBS, followed by another 2 washes 24 hours later. Cells were re-seeded into medium with or without estrogen. Protein and RNA were isolated at various time-points over a 96-hour period. Protein samples were analysed for EBNA2 or LMP1 expression (A). The amount of *bfl-1* mRNA present in the EREB 2.5 SV LMP Mut2 cells at individual time-points over the 96-hour period was quantitated by reverse transcription followed by real time PCR. *bfl-1* levels were assayed in triplicate and normalised by *gapdh* levels (B). Fold differences were calculated relative to *bfl-1* levels at time zero.

These data indicated once again that turning on functional EBNA2, and therefore LMP1, leads to a transient up-regulation in the level *bfl-1* mRNA in EREB 2.5 SV LMP Mut 2 cells. Inhibition of protein translation, including that of LMP1 causes a decrease in the level of *bfl-1* mRNA relative to cells in which protein translation has not been blocked. In cells where LMP1 translation has been inhibited, however, an increase in the level of *bfl-1* is observed after 6 hours, implying a direct role for EBNA2 in the up-regulation of *bfl-1*. In contrast to EREB 2.5 SV LMP clone 11C cells, the non-functional truncated LMP1 is unable to maintain the level of *bfl-1* mRNA in the absence of functional EBNA2.

5.2.1.5 Regulation of *bfl-1* mRNA levels in EREB 2.5 Tet LMP clone 3A cells

An additional cell line, in which LMP1 expression could be inducibly regulated, was generated by Zimmer-Strobl *et al.*, namely EREB 2.5 Tet LMP clone 3A (unpublished). This cell was established by transfection of EREB 2.5 cells with a tetracycline regulatable LMP1 construct. LMP1 expression is off in the presence of 1 µg/ml tetracycline, and can be switched on by removal of tetracycline from the culture medium. This cell line allows further dissection of the role of LMP1 in the regulation *bfl-1* mRNA levels, in the absence of EBNA2. The LMP1 and EBNA2 expression profile of this cell line, grown in the presence and absence of estrogen and/or tetracycline is summarised in Table 5.2.

Table 5.2: EBNA2 and LMP1 expression profile of EREB 2.5 Tet LMP clone 3A

Cell line	+ Estrogen		- Estrogen	
	+ Tetracycline	- Tetracycline	+ Tetracycline	- Tetracycline
Tet LMP clone 3A	EBNA2 & LMP1	EBNA2 & LMP1	no EBNA2 no LMP1	no EBNA2, plasmid LMP1

Protein expression and *bfl-1* mRNA levels observed during the time-course performed using EREB 2.5 Tet LMP clone 3A cells grown in the presence of tetracycline is shown in Figure 5.8. LMP1 expression from the transfected expression vector is off in the presence of tetracycline. Western blot analysis for EBNA2 and LMP1 is shown in Figure 5.8A. As for the other EREB 2.5 cell derivatives, chimeric EBNA2 and LMP1

expression increased over time in cells grown in the presence of estrogen. In the absence of estrogen only non-functional ER-EBNA2 was detected, and as a result no LMP1 was expressed from the viral genome (Figure 5 8A). Because tetracycline was present in the medium no LMP1 was expressed from the transfected plasmid either

Figure 5 8B shows the differences in *bfl-1* mRNA levels over time in the EREB 2 5 Tet LMP clone 3A cells grown in the presence of tetracycline. A dramatic increase in *bfl-1* levels was observed 6 hours after addition of estrogen to the culture medium. A further increase in the level of *bfl-1* mRNA was observed at the 12-hour time point. Both functional EBNA2 and LMP1 were expressed at high levels at these time points (Figure 5 8A). The amount of *bfl-1* decreased gradually over time, with the level of *bfl-1* mRNA at 96 hours still higher than that observed at time 0. In the absence of estrogen, the level of *bfl-1* mRNA decreased over time.

EREB 2 5 Tet LMP clone 3A cells cultivated in cyclohexamide were used to determine whether up-regulation of *bfl-1* mRNA was a direct effect of functional EBNA2. The level of *bfl-1* mRNA prepared from cells grown in the presence of both estrogen and cyclohexamide for 6 hours was compared with that of cells grown in estrogen only for the same period of time. It was observed that the level of *bfl-1* mRNA in cells cultivated in the presence of estrogen and cyclohexamide was lower than that of cells grown in estrogen only after 6 hours in culture (Figure 5 8B). One possible reason for this decrease could be due to the fact that less EBNA2 is expressed in the presence of cyclohexamide, which would block EBNA2 expression resulting from EBNA2 positively regulating its own promoter. The level of LMP1 expression also decreased significantly in cells grown in cyclohexamide (Figure 5 8A), relative to cells grown in the absence of cyclohexamide harvested at the same time-point, suggesting that LMP1 plays a possible role in up-regulating *bfl-1*. The level of *bfl-1* mRNA at time 0 was lower than that of cells grown in the presence of estrogen and cyclohexamide, implying a direct role for EBNA2 in the up-regulation of *bfl-1*.

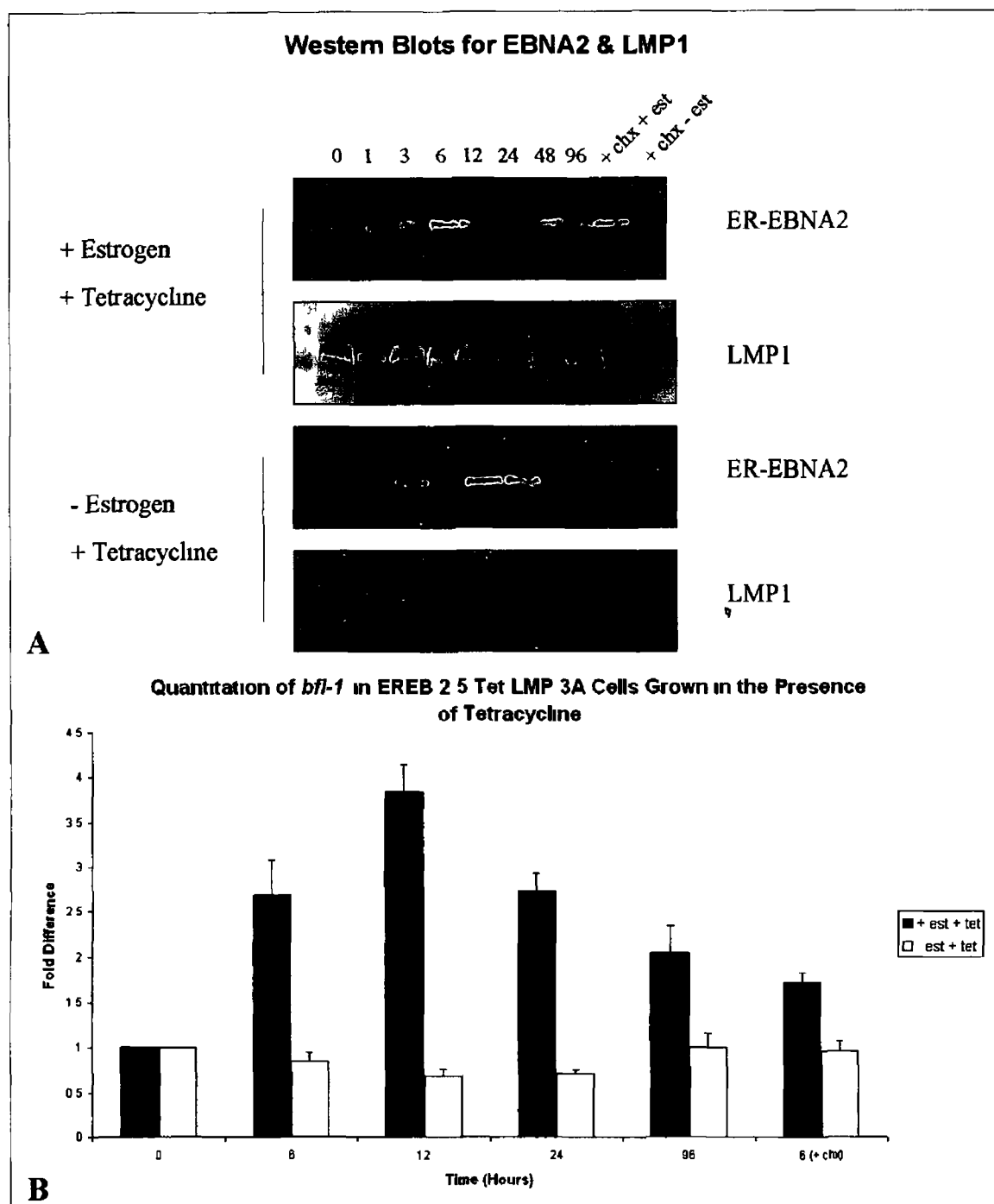


Figure 5.8 Protein expression and *bfl-1* quantitation in EREB 2.5 Tet LMP clone 3A cells grown in the presence of tetracycline. EREB 2.5 Tet LMP clone 3A cells were washed 4 times in PBS, followed by another 2 washes 24 hours later. Cells were re-seeded into medium containing 1 µg/ml tetracycline, with or without estrogen. Protein and RNA extracts were prepared at various time-points over a 96-hour period. Protein samples were analysed for EBNA2 or LMP1 expression (A). The amount of *bfl-1* mRNA present in the EREB 2.5 Tet LMP clone 3A cells at individual time-points over the 96-hour period was quantitated by reverse transcription followed by real time PCR. *bfl-1* levels were assayed in triplicate and normalised by *gapdh* levels (B). Fold differences were calculated relative to *bfl-1* levels at time zero.

Protein expression and *bfl-1* mRNA levels observed during the time-course performed using EREB 2.5 Tet LMP clone 3A cells cultivated in the absence of tetracycline is

shown in Figure 5 9 LMP1 expression from the transfected plasmid is turned on in the absence of tetracycline Western blot analysis for EBNA2 and LMP1 is shown in Figure 5 9A Similarly to western blot analysis performed using protein extracts from the other EREB 2 5 cell derivatives, chimeric EBNA2 and LMP1 expression increased over time in cells grown in the presence of estrogen In the absence of estrogen only non-functional ER-EBNA2 was detected In contrast to EREB 2 5 Tet LMP clone 3A cells grown in the presence of tetracycline, LMP1 expression was not down regulated upon withdrawal of estrogen from the culture medium

Figure 5 9B shows the differences in *bfl-1* mRNA levels over time in the EREB 2 5 Tet LMP clone 3A cells cultivated in the absence of tetracycline A significant increase in *bfl-1* levels was observed 6 hours after addition of estrogen to the culture medium A further increase in the level of *bfl-1* mRNA was observed at the 12-hour time point Both functional EBNA2 and LMP1 were expressed at high levels at these time points (Figure 5 9A) The amount of *bfl-1* decreased gradually over time, with the level of *bfl-1* mRNA at 96 hours slightly higher than that observed at time 0 In the absence of estrogen, the level of *bfl-1* mRNA was maintained at a level greater than or equal to that recorded at time 0, with a 3 7-fold increase in the level of *bfl-1* after 96 hours in culture In the absence of estrogen only non-functional chimeric EBNA2 was expressed, but LMP1 was expressed from the tetracycline-regulatable vector (Figure 5 9A) The observation that *bfl-1* levels are maintained in the absence of estrogen contrasts with the levels of *bfl-1* mRNA recorded using the EREB 2 5 Tet LMP clone 3A cells, in which LMP1 expression has been switched off by the presence of tetracycline When these cells were grown in the absence of estrogen, the level of *bfl-1* mRNA decreased over time (Figure 5 9B) These results demonstrate that LMP1 is capable of maintaining elevated *bfl-1* mRNA levels in the absence of functional EBNA2 in the LCL EREB 2 5 Tet LMP clone 3A

Cyclohexamide was included in some cultures of EREB 2 5 Tet LMP clone 3A cells, to inhibit protein translation As before, the level of *bfl-1* mRNA prepared from cells grown in the presence of both estrogen and cyclohexamide for 6 hours was compared with that of cells grown in estrogen only for the same period of time It was observed that the level of *bfl-1* mRNA in cells cultivated in the presence of estrogen and cyclohexamide was slightly higher than that of cells grown in estrogen only after 6 hours in culture (Figure 5 9B) Although the level of LMP1 expression decreased

slightly in cells grown in cyclohexamide, relative to cells grown in the absence of cyclohexamide harvested at the same time-point, a strong band for LMP1 was observed following treatment with cyclohexamide (Figure 5 9A), indicating that protein expression was not significantly inhibited in this case. As a result it cannot be concluded whether the up-regulation in the level of *bfl-1* mRNA is a direct result of functional EBNA2. The level of *bfl-1* observed between cells grown in the absence of estrogen was higher than that of cells grown in the absence of estrogen plus cyclohexamide.

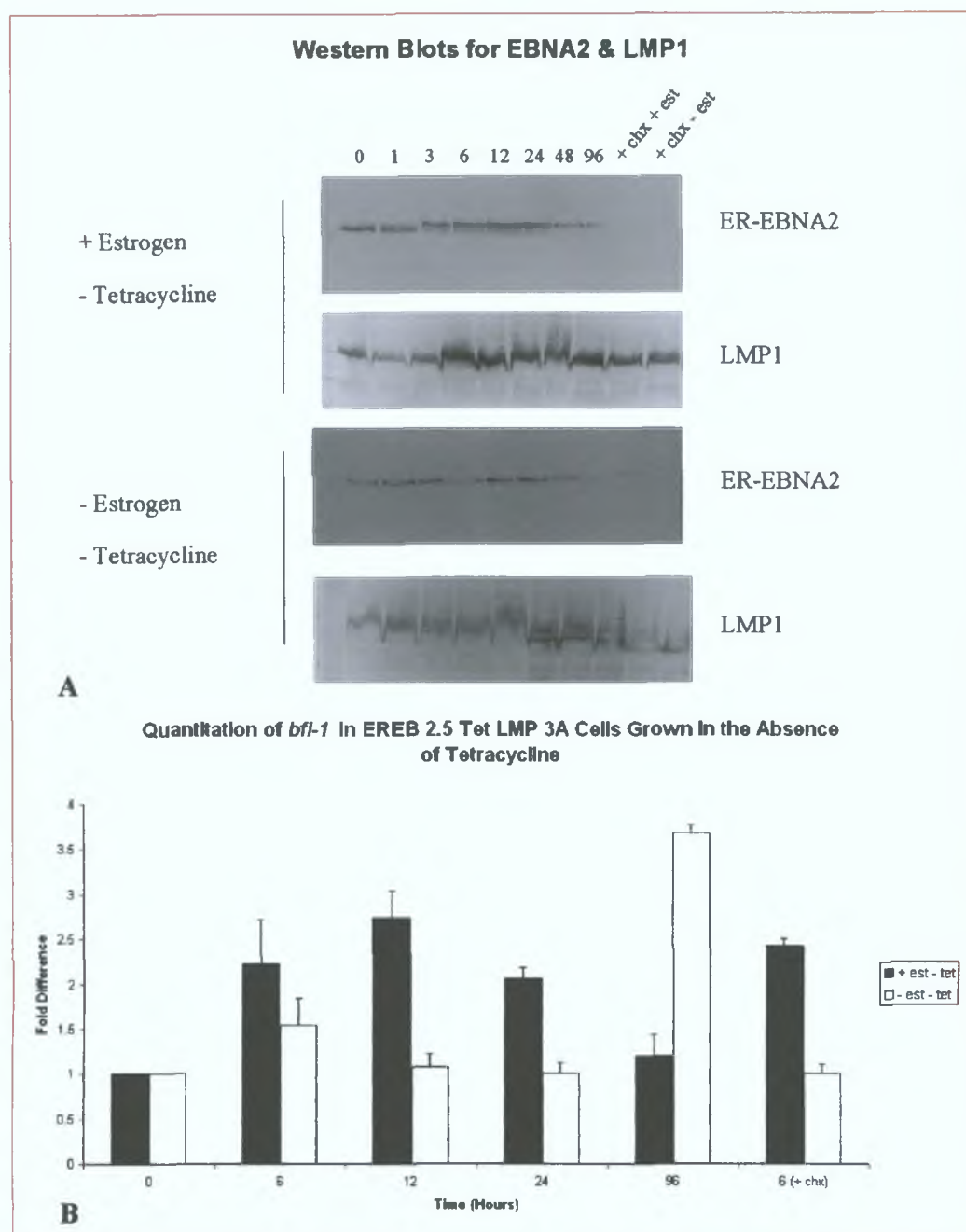


Figure 5.9: Protein expression and *bfl-1* quantitation in EREB 2.5 Tet LMP clone 3A cells grown in the absence of tetracycline. EREB 2.5 Tet LMP clone 3A cells were washed 4 times in PBS, followed by another 2 washes 24 hours later. Cells were re-seeded into medium supplemented with or without estrogen. Protein and RNA extracts were prepared at various time-points over a 96-hour period. Protein samples were analysed for EBNA2 or LMP1 expression (A). The amount of *bfl-1* mRNA present in the EREB 2.5 Tet LMP clone 3A cells at individual time-points over the 96-hour period was quantitated by reverse transcription followed by real time PCR. *bfl-1* levels were assayed in triplicate and normalised by *gapdh* levels (B). Fold differences were calculated relative to *bfl-1* levels at time zero.

Taken together, these results indicated as before that turning on functional EBNA2, and as a result LMP1, up-regulates the expression of *bfl-1* mRNA in the cell line EREB 2.5 Tet LMP clone 3A. It was also observed that LMP1 is capable of maintaining *bfl-1*

mRNA levels in the absence of functional EBNA2 in this cell context. This result is in keeping with results from the time-course carried out using EREB 2.5 SV LMP clone 11C cells.

5.2.2 The regulation of the *bfl-1* promoter in EREB 2.5 cells

Having established that both EBNA2 and LMP1 are both likely to play a role in the up-regulation in the level of *bfl-1* mRNA in derivatives of the EREB 2.5 cell line, experiments were conducted to investigate their role in the regulation of the *bfl-1* promoter in the parental cell line EREB 2.5. EREB 2.5 is an LCL, in which all the EBV latent proteins are expressed during cell culture in the presence of estrogen. Transient transfections were performed to investigate the effect of the expression vectors for (i) the I κ B α super-repressor (pEFCX-I κ B α DN), (ii) the RBP-J κ /CBF1 mutant (pERBOSneo-R218H) and (iii) the EBNA2 mutant [EBNA2 Mut (437-477)] on *bfl-1* promoter activity in this cell context. Because EREB 2.5 is an LCL, it was expected that co-transfections using the I κ B α , RBP-J κ /CBF1 and EBNA2 mutants, would yield similar results to those obtained in Chapter 4 using the IB4 LCL.

5.2.2.1 The effect of the I κ B α mutant on promoter activity in EREB 2.5 cells

In a preliminary control experiment, the NF κ B-responsive reporter construct 3 x enh κ B luc was transfected into EREB 2.5 cells, resulting in a high level of luciferase activity (Figure 5.10). This finding was not surprising as LMP1, which is present in EREB 2.5 cells grown in the presence of estrogen, is known to trigger signalling pathways that result in the activation of NF- κ B. Upon co-transfection of the NF- κ B-responsive reporter along with the expression vector for the I κ B α mutant (pEFCX-I κ B α DN), a significant decrease in luciferase activity (approximately 98%) was observed. This finding was similar to that observed in similar experiments using the EBV-negative BL-derived cell line DG75 and the EBV-infected cell lines Ag876 and IB4. When the I κ B α mutant was co-transfected along with the *bfl-1* promoter reporter construct -1374/+81-Luc, only a small decrease (approximately 10 %) in luciferase activity was detected. EREB 2.5 cells were also transfected with the *bfl-1* promoter construct containing a mutation in the putative RBP-J κ /CBF1 binding site at position -343 to -249 relative to

the transcription start site [-1374/+81 (mCBF1)]. This mutation was not found to inhibit *bfl-1* promoter activity in EREB 2.5 cells. However, upon co-transfection with the I κ B α mutant together with the promoter bearing mutation in the putative RBP-J κ /CBF1 binding site, a 50 % decrease in luciferase activity was observed.

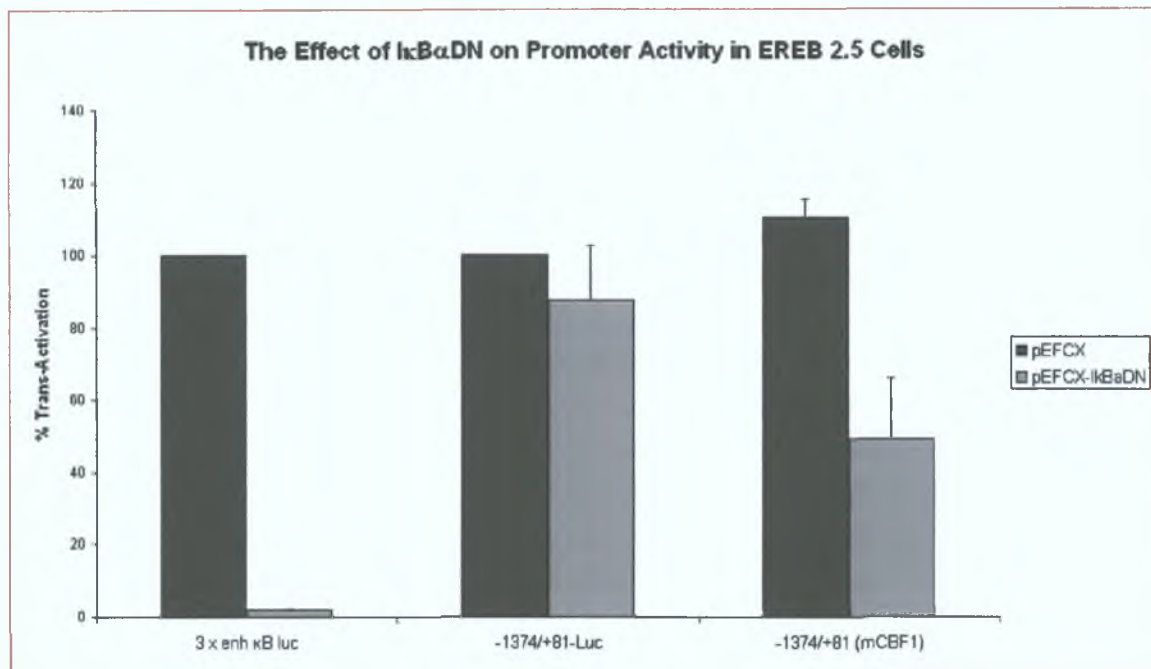


Figure 5.10: The effect of a dominant negative form of I κ B α on promoter activity in EREB 2.5 cells. EREB 2.5 cells grown in the presence of estrogen were transiently co-transfected with either 12 μ g of the expression vector I κ B α DN or 12 μ g of the empty vector (pEFCX) together with 2 μ g of the reporter construct indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value upon co-transfection with the empty vector and the reporter construct.

These results indicated that blocking LMP1-mediated *bfl-1* promoter activity by over-expression of the I κ B α mutant as well as blocking EBNA2-mediated *bfl-1* promoter activity by mutating the putative RBP-J κ /CBF1 binding site on the promoter, results in significant inhibition in the level of *bfl-1* promoter trans-activation in EREB 2.5 cells.

5.2.2.2 The effect of the RBP-J κ /CBF1 mutant on promoter activity in EREB 2.5 cells

The effect of the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H) on the activity of various promoters in EREB 2.5 cells was investigated. Initially, the RBP-J κ /CBF1-regulated

reporter construct (pGa981-6) was transfected into EREB 2.5 cells. A high level of luciferase activity was observed (Figure 5.11). This was expected as the reporter construct is known to be responsive to EBNA2, which is present in this cell context. When pGa981-6 was co-transfected along with the expression vector for the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H), a dramatic decrease (approximately 80 %) in luciferase activity was detected. This finding was similar to that observed in experiments using the EBV-negative BL-derived cell line DG75 and the EBV-infected cell lines Ag876 and IB4. The effect of the RBP-J κ /CBF1 mutant on *bfl-1* promoter activity was not as potent. When the *bfl-1* promoter reporter construct -1374/+81-Luc was co-transfected along with the RBP-J κ /CBF1 mutant, a decrease in luciferase activity of only 20 % was observed. EREB 2.5 cells were also transfected with the *bfl-1* promoter construct containing a mutation in the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site [-1374/+81 m κ B (-52)]. This mutation was not found to inhibit *bfl-1* promoter activity in EREB 2.5 cells. However, upon co-transfection with the RBP-J κ /CBF1 mutant together with the promoter containing the NF- κ B-like binding site mutation, a 65 % decrease in luciferase activity was observed.

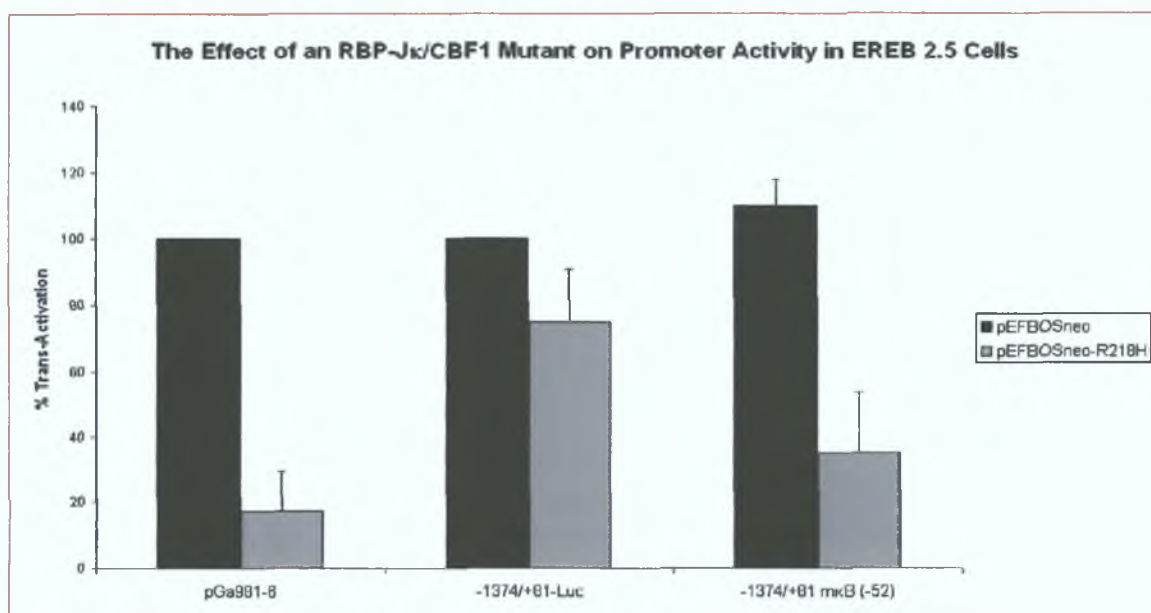


Figure 5.11: The effect of an RBP-J κ /CBF1 mutant on promoter activity in EREB 2.5 cells. EREB 2.5 cells cultivated in estrogen were transiently co-transfected with either 12 μ g of the expression vector the RBP-J κ /CBF1 mutant (pEFBOSneo-R218H) or 12 μ g of the empty vector (pEFBOSneo) together with 2 μ g of the reporter construct indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value upon co-transfection with the empty vector and the reporter construct.

These data showed that blocking EBNA2-mediated *bfl-1* promoter activity by over-expression of the RBP-J κ /CBF1 mutant as well as blocking LMP1-mediated *bfl-1* promoter activity by mutating the NF- κ B-like binding site at -52 to -43 on the promoter, results in inhibition in *bfl-1* promoter -activity in the cell line EREB 2.5.

5.2.2.3 The effect of the EBNA2 mutant with a trans-activation domain deletion on promoter activity in EREB 2.5 cells

The effect of the EBNA2 mutant, with a trans-activation domain deletion, on the activity of various promoters in EREB 2.5 cells was also investigated. Initially, the RBP-J κ /CBF1-regulated reporter construct (pGa981-6) was transfected into EREB 2.5 cells. As before, a high level of luciferase activity was observed (Figure 5.12). When pGa981-6 was co-transfected along with the expression vector for the EBNA2 mutant (EBNA2 Mut (437-477)), a dramatic decrease (approximately 80 %) in luciferase activity was detected. This finding was reminiscent of observations in similar experiments using the EBV-negative BL-derived cell line DG75 and the EBV-infected cell lines Ag876 and IB4. EREB 2.5 cells were also transfected with the *bfl-1* promoter construct containing a mutation in the NF- κ B-like binding site at position -52 to -43 relative to the transcription start site [-1374/+81 m κ B (-52)]. This mutation was not found to inhibit *bfl-1* promoter activity in EREB 2.5 cells. However, upon co-transfection with the EBNA2 mutant together with the promoter bearing the NF- κ B-like binding site mutation, a 65 % decrease in luciferase activity was observed.

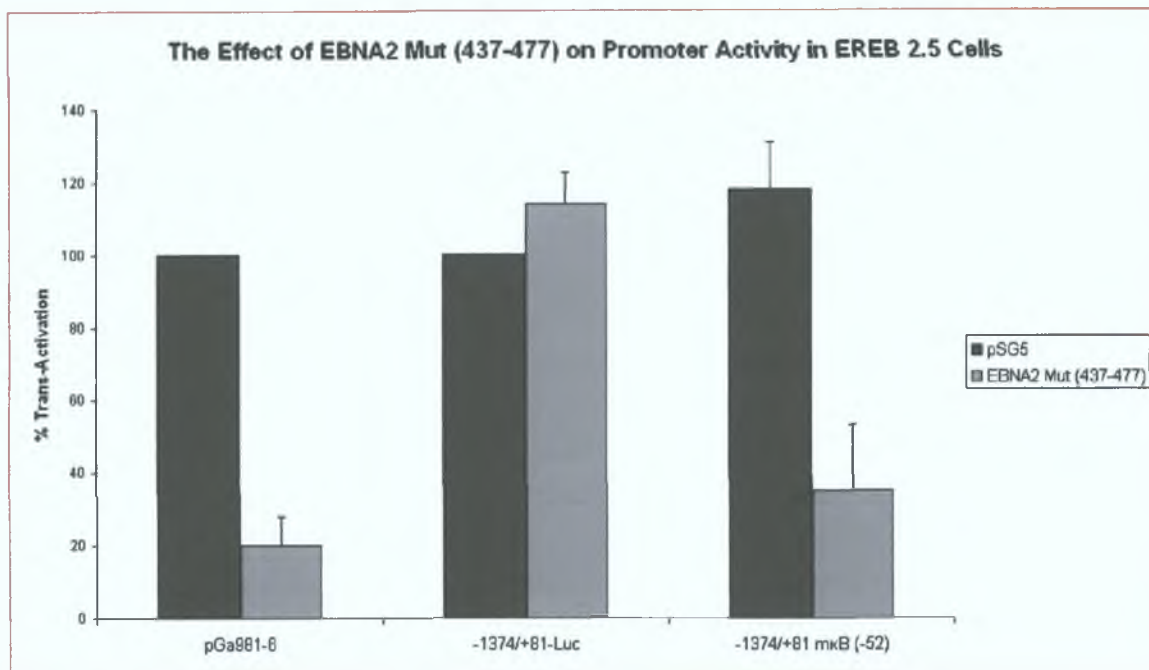


Figure 5.12: The effect of an EBNA2 mutant on promoter activity in EREB 2.5 cells. EREB 2.5 cells cultivated in the presence of estrogen were transiently co-transfected with either 12 μ g of the expression vector an EBNA2 mutant with a deletion in its trans-activation domain (EBNA2 Mut (437-477)) or 12 μ g of the empty vector (pSG5) together with 2 μ g of the reporter construct indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value upon co-transfection with the empty vector and the reporter construct.

These results indicated that blocking EBNA2-mediated *bfl-1* promoter activity, in this case by over-expression of the EBNA2 mutant, as well as blocking LMP1-mediated *bfl-1* promoter activity by mutating the NF- κ B-like binding site at position -52 to -43 on the promoter, results in inhibition in the level of *bfl-1* promoter trans-activation in EREB 2.5 cells.

5.2.2.4 Co-expression of the RBP-J κ /CBF1 and I κ B α mutants inhibits *bfl-1* promoter activation

It has already been demonstrated that over-expression of either the I κ B α super-repressor or the RBP-J κ /CBF1 mutant does not lead to a potent inhibition in *bfl-1* promoter activity using EREB 2.5 cells (Figure 5.10 and Figure 5.11). These results are summarised in Figure 5.13. Consequently, transient co-transfections were performed to investigate whether co-expression of these two mutants at the same time would affect the level of *bfl-1* promoter activation. Co-transfection with the expression vector for the

RBP-J κ /CBF1 mutant (pEFBOSneo-R218H) together with the vector expressing the I κ B α mutant (pEFCX-I κ B α DN) resulted in approximately 80 % inhibition in the level of *bfl-1* promoter activity in EREB 2.5 cells (Figure 5.13).

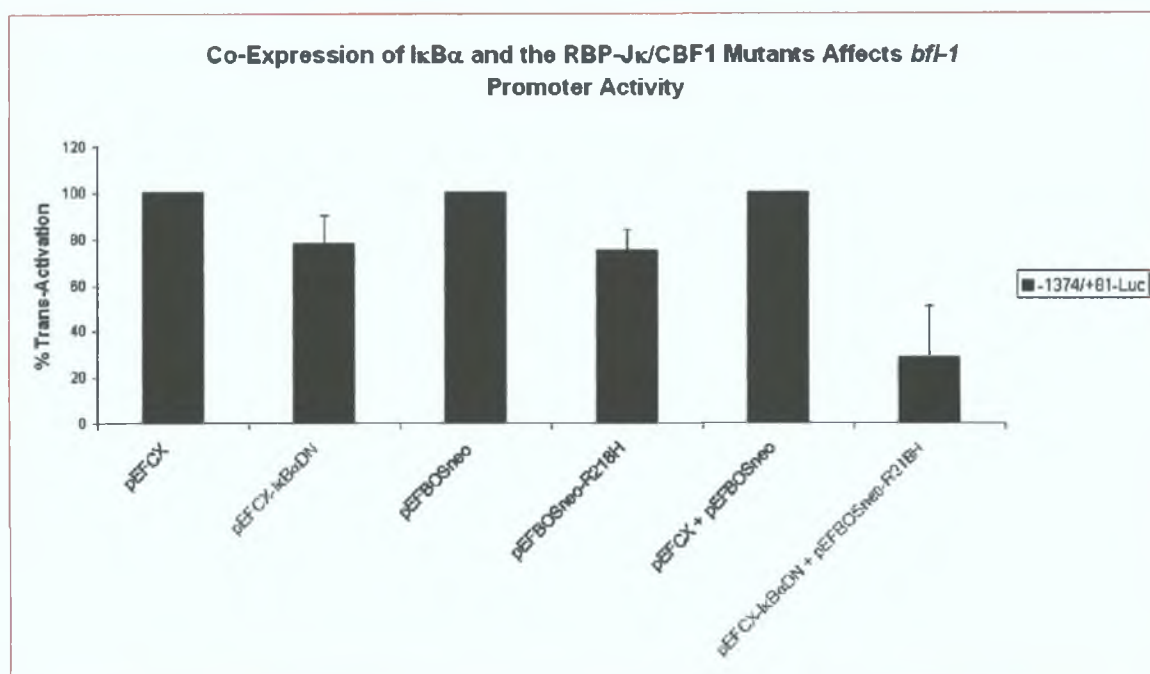


Figure 5.13: Co-expression of the I κ B α and RBP-J κ /CBF1 mutants affects *bfl-1* promoter activity in EREB 2.5 cells. Transient transfections were performed in EREB 2.5 cells grown in estsrogen with 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc along with 12 μ g of the expression vector indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon transfection of the reporter construct and the empty vector.

These results demonstrated that inhibition of both EBNA2- and LMP1-mediated trans-activation of the *bfl-1* promoter by co-expression of the RBP-J κ /CBF1 and I κ B α mutants simultaneously resulted in stronger inhibition in *bfl-1* promoter activity than expression of either of these mutants alone. Blocking both LMP1- and EBNA2-mediated signalling was required to significantly decrease *bfl-1* promoter activity in EREB 2.5 cells.

5.2.2.5 Co-expression of the EBNA2 and I κ B α mutants inhibits *bfl-1* promoter activity

It has previously been demonstrated in this study, that over-expression of the EBNA2 mutant with a deletion in its trans-activation domain does not inhibit in *bfl-1* promoter

activity using EREB 2.5 cells (Figure 5.12). To determine whether co-expression of this mutant along with the I κ B α super-repressor would affect *bfl-1* promoter trans-activation, transient co-transfections were performed using the expression vector for the EBNA2 mutant [EBNA2 Mut (437-477)] together with the vector expressing the I κ B α super-repressor (pEFCX-I κ B α DN). Co-expression of the two mutants at the same time resulted in approximately 80 % inhibition in the level of *bfl-1* promoter activity in EREB 2.5 cells (Figure 5.14).

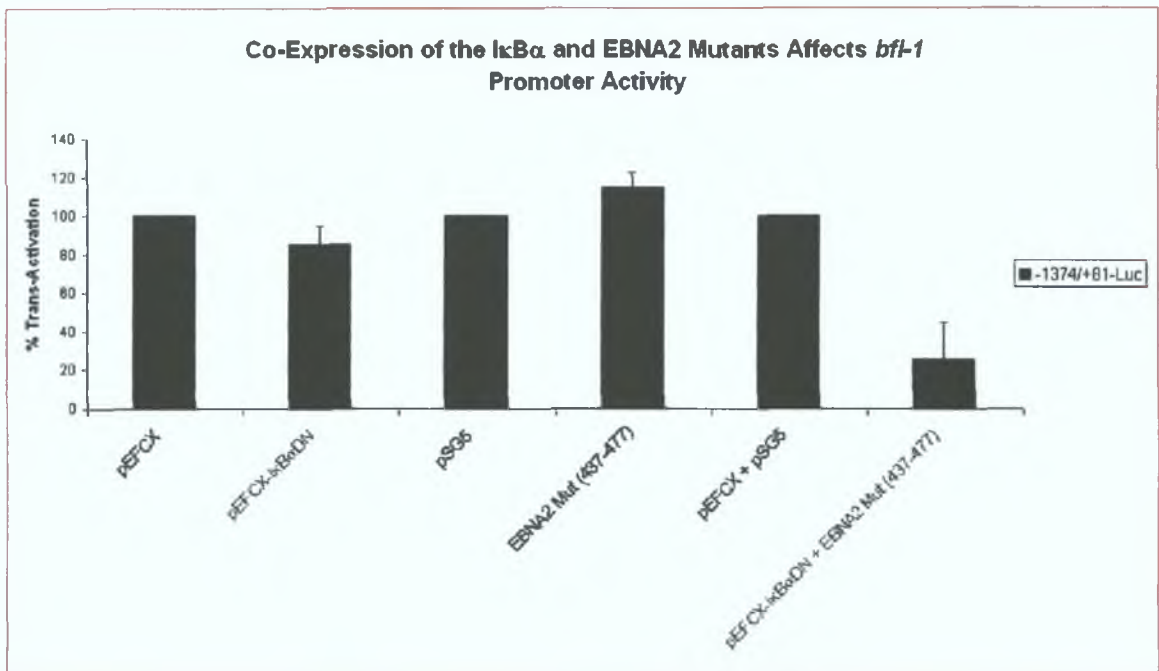


Figure 5.14: Co-expression of the I κ B α and RBP-J κ /CBF1 mutants affects *bfl-1* promoter activity in EREB 2.5 cells. Transient transfections were performed in EREB 2.5 cells cultivated in the presence of estrogen using 2 μ g of the *bfl-1* promoter reporter construct -1374/+81-Luc along with 12 μ g of the expression vector indicated underneath the graph. Cells were harvested at 48 hours post-transfection and normalised luciferase values were expressed as % activation relative to the corresponding value obtained upon transfection of the reporter construct and the empty vector

These results demonstrated that inhibition of both EBNA2- and LMP1-mediated trans-activation of the *bfl-1* promoter by co-expression of the EBNA2 and I κ B α mutants simultaneously resulted in stronger inhibition in *bfl-1* promoter activity than expression of either of these mutants alone.

5.3 DISCUSSION

The regulation of *bfl-1* activity in the EBV-infected cell lines Ag876 and IB4 was investigated in Chapter 4. Because these EBV-infected cell lines express EBNA2 and LMP1 simultaneously, with trans-activation of the LMP1 gene under the strict control of EBNA2 (Abbot *et al.*, 1990; Fahraeus *et al.*, 1990; Laux *et al.*, 1994a; Wang *et al.*, 1990), it is difficult to assess the contribution of each of these individual EBV latent proteins to the modulation of *bfl-1* expression. To overcome this obstacle, experiments were carried out using a panel of cell lines, based a cell system established by Kempkes *et al.* (1995b), in which primary B cells have been co-infected with a mini-EBV plasmid that expresses estrogen-responsive EBNA2 (ER-EBNA2), together with the EBV strain P3HR1, which is a non-immortalising viral strain that is deleted for it's own EBNA2 (Figure 5.1). As a result the function of EBNA2 in the resulting cell line, EREB 2.5, may be switched on and off by addition or removal of estrogen from the culture medium. Therefore, in the presence of estrogen, functional EBNA2 trans-activates the P3HR1 viral promoters, including that of LMP1. Thus, addition of estrogen, and the subsequent induction of functional EBNA2, results in EBV-infected cells with a phenotype similar to those found in healthy individuals upon primary infection with the virus, or EBV-infected cells found in immuno-suppressed patients.

Experiments performed in the current study involved a panel of derivatives of the parental EREB 2.5 cell line, which had been stably transfected with additional expression vectors (Zimber-Strobl *et al.*, 1996). This panel of cell lines allowed (i) investigation into the effects of the induction of functional EBNA2 (and as a result LMP1) on *bfl-1* mRNA levels in EBV-infected cells and (ii) dissection of the contribution of the LMP1 protein, in the absence of EBNA2, to maintenance of *bfl-1* activity in this cell context. Five cell lines were used in total. Two of these cell lines, namely EREB 2.5 SV LMP clone 2C and EREB 2.5 SV LMP clone 11C, describe EREB 2.5 cells, which have been stably transfected with a vector expressing LMP1 from the SV40 early promoter/enhancer (Zimber-Strobl *et al.*, 1996). An additional LMP1-inducible cell line, (EREB 2.5 Tet LMP 3A), which has been transfected with a tetracycline regulatable LMP1 expression vector, was also used in the study (Zimber-Strobl *et al.*, unpublished). EREB 2.5 cells transfected with a plasmid expressing a truncated non-functional LMP1 gene (EREB 2.5 SV LMP Mut 2) or the empty vector (EREB 2.5 pHEBo) served as negative controls.

These experiments demonstrated that turning on functional EBNA2 (by addition of estrogen) leads to an increase in EBNA2 expression over time (Figure 5.2A). This observation is compatible with a functional role for EBNA2 in the positive regulation of its own promoter (Sung *et al.*, 1991; Rooney *et al.*, 1992). LMP1 expression coincided with an increase in EBNA2 expression in all the cell lines tested, indicating that the LMP1 gene is under the strict control of EBNA2. Expression of functional EBNA2, and the resulting induction of LMP1, led to a transient increase in the level of *bfl-1* mRNA at 6 hours post-addition of estrogen, as quantitated by reverse transcription real time PCR. Removal of estrogen from the culture medium, and therefore “switching off” functional EBNA2 resulted in a decrease in both functional EBNA2 and LMP1 levels, as determined by Western blot analysis, reinforcing the idea that EBNA2 regulates its own expression, as well as that of LMP1. In the absence of estrogen, the ER-EBNA2 protein was observed to migrate more rapidly and homogenously, suggesting that switching on EBNA2 function is associated with a post-translational modification or profound changes in the tertiary structure of ER-EBNA2, possible due to phosphorylation (Grasser *et al.*, 1991). Switching off functional EBNA2 also coincided with a decrease in the level of *bfl-1* mRNA.

As both EBNA2 and LMP1 were expressed rapidly following activation of functional EBNA2, it is difficult to assess which protein was responsible for the up-regulation in *bfl-1* mRNA levels in the EREB 2.5 cell lines. To address the question of whether LMP1 maintains *bfl-1* mRNA levels in the absence of EBNA2, EREB 2.5 cells stably transfected with LMP1 expression vectors were used. In experiments performed using the negative control cell lines transfected with either the empty vector (EREb 2.5 pHEBo) or the truncated non-functional LMP1 mutant (EREb 2.5 SV LMP Mut 2), LMP1 expression was significantly down-regulated after removal of hormone, indicating that the LMP1 gene is under strict control of EBNA2 in these cell lines. It was also observed that *bfl-1* mRNA levels deteriorated significantly in the absence of functional EBNA2 and LMP1. In contrast, experiments using cells transfected with the LMP1 expression vector (EREb 2.5 SV LMP clone 11C) or cells in which LMP1 was induced from the tetracycline regulatable vector (EREb 2.5 Tet LMP clone 3A), revealed LMP1 levels that were not down-regulated following estrogen withdrawal. These results clearly demonstrate that LMP1 expression from either the SV40 promoter or the tetracycline inducible vector maintains elevated *bfl-1* mRNA levels, independently of functional EBNA2. However, in the case of the cell line EREB 2.5

SV LMP clone 2C, LMP1 levels were seen to decrease following removal of estrogen from the culture medium. This suggests that this particular clone was unstable, and had possibly lost the expression vector for LMP1.

Because EBNA2 trans-activates the *LMP1* promoter, it is believed that the primary role for EBNA2 is to maintain LMP1 levels in EBV-infected cells. In order to determine whether functional EBNA2 plays a direct role in the up-regulation of *bfl-1* mRNA levels, the various stably transfected EREB 2.5 derivatives were cultivated in medium containing cyclohexamide, which is a known inhibitor of protein synthesis.

It should be noted that chimeric ER-EBNA2 is already present in the EREB 2.5 derivatives, thus the expression of functional EBNA2 is not subject to inhibition by cyclohexamide. However, because EBNA2 positively regulates its own promoter, a lot more functional EBNA2 is expressed in the absence of cyclohexamide. Translation of genes trans-activated by functional EBNA2 (i.e. genes from the LMP and Cp promoters), is inhibited by the presence of cyclohexamide in the culture medium. During experiments where translation of EBNA2-responsive genes (including *LMP1*), expressed from the viral genome was inhibited by cyclohexamide, an increase in *bfl-1* mRNA was observed 6 hours post-induction of functional EBNA2 in all of the cell lines tested, implying a possible direct role for EBNA2 in the regulation of *bfl-1* in the context of an LCL. In the majority of cases, Western blot analysis revealed that LMP1 expression was almost completely abolished following 6 hours culture in the presence of cyclohexamide. It was noted however, in experiments using two of the cell lines (EREB 2.5 SV LMP clone 11C and EREB 2.5 Tet LMP 3A grown in the absence of tetracycline) that although LMP1 expression decreased somewhat in cells cultured in the presence of cyclohexamide for 6 hours, LMP1 was still detectable by Western blot analysis, so it was difficult to conclude whether EBNA2 and/or LMP1 was responsible for the increase in *bfl-1* mRNA levels in these cases. For more effective inhibition of translation a combination of protein synthesis inhibitors may have proved more effective, for example a mixture of cyclohexamide and anisomycin has been demonstrated to be effective in other studies (Spender *et al.*, 2001).

It was observed that the level of *bfl-1* mRNA prepared from cells grown in the presence of both estrogen and cyclohexamide (where only functional EBNA2 is expressed) was generally lower than the level of mRNA in cells grown in estrogen only (where

functional EBNA2 at pre-existing levels and all its responsive genes are expressed). The decrease in mRNA levels was not due to mRNA instability, as no difference in *bfl-1* mRNA levels was observed between cells grown in the absence of estrogen and cells grown in the absence of estrogen plus cyclohexamide. One possible reason for the decrease in *bfl-1* mRNA levels observed when cyclohexamide is present, could be that less EBNA2 is expressed in the presence of cyclohexamide, as the protein synthesis inhibitor would result in a decrease in EBNA2 expression that would result from EBNA2 positively regulating its own promoter. It is also possible that an additional factor plays a role in the maintenance of *bfl-1* levels in this cell context. LMP1 is a likely candidate for such an additional factor, as western blot analysis revealed it was down-regulated in the presence of cyclohexamide. Furthermore, LMP1 expression from the plasmid in the absence of functional EBNA2 was demonstrated to be sufficient for maintaining elevated *bfl-1* mRNA levels independently of functional EBNA2, indicating a key role for LMP1 in the regulation of *bfl-1* in EREB cell lines. It must be taken into consideration that cyclohexamide would inhibit translation of other EBNA2-responsive viral and cellular genes expressed from the P3HR1 viral genome present in the EREB 2.5 stable transfectants and the effects of inhibition of these genes on *bfl-1* regulation have not been analysed in this study.

Having established that both EBNA2 and LMP1 are both likely to play a role in the up-regulation of *bfl-1* mRNA in derivatives of the EREB 2.5 cell line, experiments were conducted to investigate the role of these two EBV latent proteins in the regulation of the *bfl-1* promoter in the parental cell line EREB 2.5. Transient transfections were performed to investigate the effects of the I κ B α super-repressor, the RBP-J κ /CBF1 mutant and the EBNA2 mutant on *bfl-1* promoter activity in this cell context. Since EREB 2.5 cells grown in the presence of estrogen is in fact an LCL, it was expected that co-transfections using the I κ B α , RBP-J κ /CBF1 and EBNA2 mutants, would yield similar results to those obtained in using the IB4 LCL in Chapter 4.

As was the case for experiments carried out using IB4 cells, blocking both EBNA2-and LMP1-mediated signalling was required in order to significantly decrease *bfl-1* promoter activity in EREB 2.5 cells. This was demonstrated using *bfl-1* promoter reporter constructs containing mutations in either the NF- κ B-like binding site at position -52 to -43, or the putative RBP-J κ /CBF1 binding site at -343 to -249 relative to the transcription start site on the *bfl-1* promoter. Firstly, co-transfections using either the

RBP-J κ /CBF1 or EBNA2 mutant along with a *bfl-1* promoter reporter construct containing a mutation in the NF- κ B-like binding site were performed. This mutation in this NF- κ B-like binding site has been found to almost completely abolish LMP1-responsiveness on the *bfl-1* promoter in the EBV-negative BL-derived cell line DG75 (D'Souza *et al.*, 2004). However, the mutation was not found to affect the level of *bfl-1* promoter in EREB 2.5 cells. On the other hand, by mutating the NF- κ B-like binding site, and blocking EBNA2-mediated promoter activity by co-expression with either the RBP-J κ /CBF1 or EBNA2 mutants, the level of promoter activity was reduced by up to 70 %. The observation that blocking both LMP1- and EBNA2-mediated trans-activation of the *bfl-1* promoter simultaneously, results in a significant decrease in promoter activity, was reinforced during transfection experiments using the I κ B α super-repressor and the *bfl-1* promoter reporter construct containing a mutation in putative RBP-J κ /CBF1 binding site. Mutation of this putative binding site has been shown to significantly inhibit EBNA2-mediated trans-activation of the *bfl-1* promoter in EBV-negative BL-derived cell lines (Pegman *et al.*, submitted December, 2004). It was observed in the current study that the presence of this mutation did not affect the level of *bfl-1* promoter activity in EREB 2.5 cells. However, upon co-transfection of the RBP-J κ /CBF1 mutated promoter reporter construct together with the I κ B α super-repressor, an approximate 50 % decrease in *bfl-1* promoter activity was observed. These results imply a role for the putative RBP-J κ /CBF1 binding site in the trans-activation of the *bfl-1* promoter in the context of the EREB 2.5 cell line and are in keeping with results obtained during similar experiments carried out in the IB4 LCL, and the type III BL cell line Ag876.

To further investigate the effect of blocking both LMP1- and EBNA2-mediated signalling, co-transfections were carried out using the I κ B α super-repressor together with either the RBP-J κ /CBF1 mutant or the EBNA2 mutant with the deletion in the trans-activation domain. Co-expression of the I κ B α super-repressor with either the RBP-J κ /CBF1 or EBNA2 mutant resulted in up to 80 % inhibition in *bfl-1* promoter activity in both cell lines, further strengthening the observation that blocking both LMP1- and EBNA2-mediated activation significantly affects the level of *bfl-1* promoter activity. This implies that pathways leading to NF- κ B activation and CBF1 work simultaneously and in a mutually redundant fashion to maintain the level of *bfl-1* promoter activity in EBV-infected cell lines with a type III latency programme and that

EBNA2 and LMP1 are the key EBV latent proteins that drive the expression of this cellular gene in the cell line EREB 2.5, in addition to the cell lines Ag876 and IB4.

Chapter 6

General Discussion

6.1 General discussion

A central component of the overall EBV strategy and its role in the development of related malignant disease is the ability of the viral proteins to suppress the cellular apoptotic program (Allday, 1996; Klein, 1994). One well-documented mechanism by which EBV can protect against apoptosis is through the LMP1-mediated up-regulation of anti-apoptotic proteins, including Bcl-2, A20, Mcl-1 and c-IAP2 (Henderson *et al.*, 1991; Fries *et al.*, 1996; Wang *et al.*, 1996; Laherty *et al.*, 1992; Hong *et al.*, 2000), thus raising the apoptotic threshold of the infected cell and also providing protection against a wide range of apoptosis-inducing stimuli. It has previously been shown in our laboratory that elevated mRNA levels of the anti-apoptotic *bfl-1* gene are a feature of EBV-infected B lymphocytes exhibiting type III latency, and that expression of either LMP1 or EBNA2 as the sole EBV protein in an EBV-negative BL cell line coincides with a dramatic increase in *bfl-1* mRNA levels (D'Souza *et al.*, 2000; Pegman *et al.*, submitted December, 2004). In addition, Bfl-1 protects against serum depletion-induced apoptosis when expressed in the same cell context (D'Souza *et al.*, 2000), and both LMP1 and EBNA2 stimulate *bfl-1* promoter activity in EBV-negative BL-derived cell lines by a mechanism that involves components of the NF- κ B and Notch signalling pathways respectively (D'Souza *et al.*, 2004; Pegman *et al.*, submitted December, 2004).

Here it was shown that NF- κ B composed of p65 subunits trans-activated the *bfl-1* promoter in the EBV-negative BL-derived cell line DG75, and that an NF- κ B-like binding site at position -52 to -43 relative to the transcription start site was essential for this effect. The crucial role for the DNA-binding protein RBP-J κ /CBF1 in EBNA2-mediated trans-activation of *bfl-1* was demonstrated, as an RBP-J κ /CBF1 mutant (pEFBOSneo-R218H) blocked trans-activation of *bfl-1* by EBNA2. In contrast to that of LMP1, EBNA2-mediated trans-activation of *bfl-1* was not associated with an increase in the level of activated NF- κ B. The cellular functional equivalent of EBNA2, Notch1C, did not trans-activate the *bfl-1* promoter in DG75 cells, and this was not due to the fact that the Notch1C protein has a weaker trans-activation domain than that of EBNA2. An EBNA2 mutant, with a trans-activation domain deletion [EBNA2 Mut (437-477)], significantly inhibited wild type EBNA2-mediated trans-activation of both the *LMP1* and *bfl-1* promoters in DG75 cells. The signalling pathways that link EBV to the up-regulation of Bfl-1 are summarised in Figure 6.1. The inhibition of both

EBNA2- and LMP1-mediated activity by the mutant constructs used in the study is also indicated.

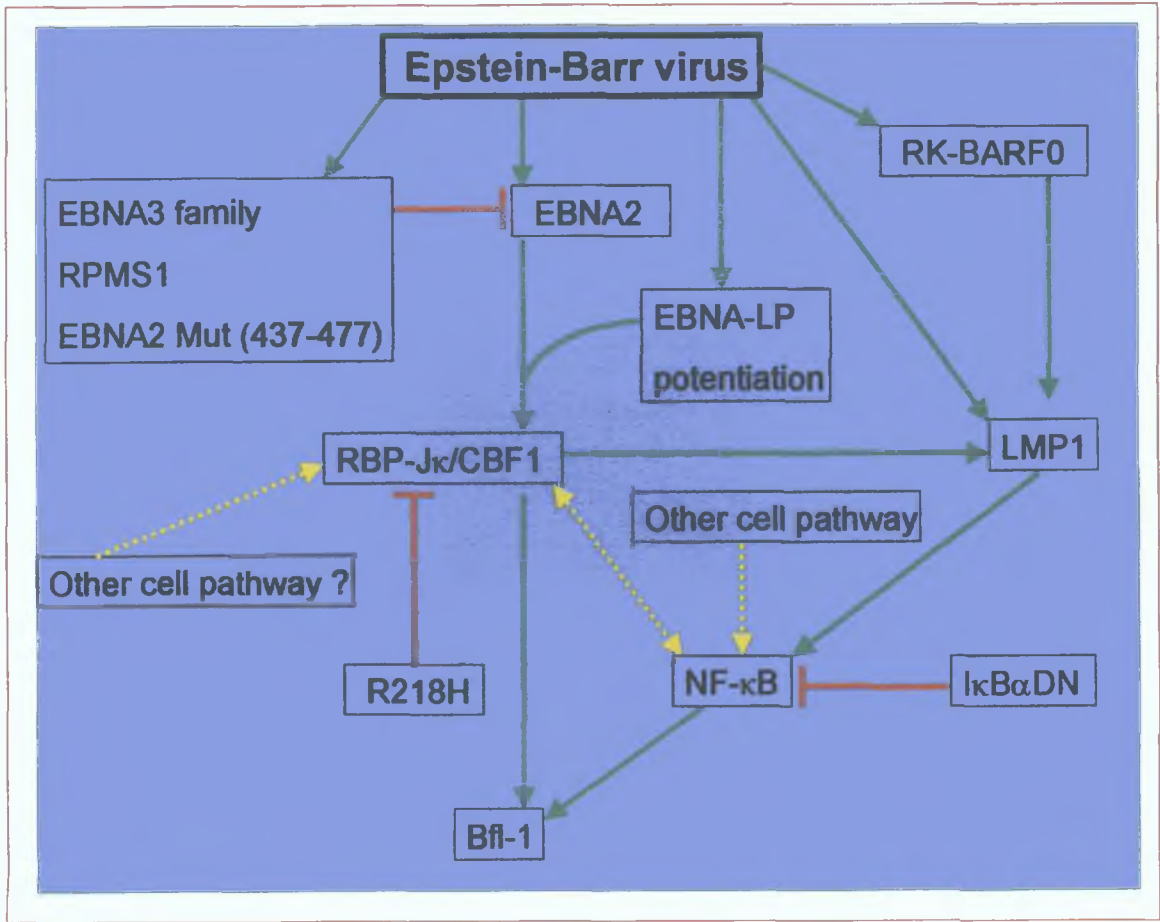


Figure 6.1: Signalling pathways that link EBV to the up-regulation of Bfl-1. It was demonstrated in the current study that EBNA2 trans-activates the *bfl-1* promoter in an RBP-Jκ/CBF1-dependent manner. Co-expression of an RBP-Jκ/CBF1 mutant (pERBOSneo-R218H) that cannot bind DNA was found to inhibit this activity. In addition an EBNA2 mutant with a trans-activation domain deletion [EBNA2 Mut 437-477] was also found to block EBNA2-mediated trans-activation of *bfl-1*. Over-expression of an IκBα super-repressor mutant (pEFCX-IκBαDN) was used to block LMP1-mediated activation of the *bfl-1* promoter. Other EBV proteins that are known to modulate EBNA2 and LMP1 activity are also indicated.

A role for both LMP1 and EBNA2 was demonstrated in the context of the EBV-infected B lymphocyte. While blocking either LMP1- or EBNA2-mediated signalling alone did not lead to a decrease in *bfl-1* promoter activity, blocking signalling from both these proteins simultaneously led to significant inhibition in *bfl-1* activity. A panel of cell lines (ERE2.5 and its derivatives), in which functional EBNA2 could be expressed conditionally, allowed dissection of the individual roles of EBNA2 and LMP1 with regard to *bfl-1* regulation. Induction of EBNA2, and the resulting LMP1 expression, led to a transient increase in the level of *bfl-1* mRNA. In the absence of

functional EBNA2 and LMP1, *bfl-1* mRNA levels were seen to significantly decrease in the panel of EREB cell lines. LMP1 maintained elevated *bfl-1* mRNA levels in the absence of functional EBNA2. Induction of functional EBNA2 alone in the panel of EREB cell lines led to an increase in the level of *bfl-1* mRNA. These data indicate that both LMP1 and EBNA2 play a role in the regulation of the *bfl-1* gene. The up-regulation of *bfl-1* by these two EBV latent proteins may be a key event in the rescue of EBV-infected B cells from apoptosis and an important route by which EBV gains access to the memory B cell pool and contributes to oncogenesis.

In healthy EBV-carriers, all of the EBV latent proteins, including EBNA2 and LMP1 have been found in naïve IgD⁺ tonsillar B cells (Joseph *et al.*, 2000). These latently infected naïve B cells are present in the tonsil only when viral replication has been detected, suggesting that they represent newly infected cells (Joseph *et al.*, 2000). EBNA2 induction, by addition of estrogen to the culture medium of EREB cell lines, represents an established regulatable model for the growth transformation of resting B cells by EBV. Induction of functional EBNA2, and the resulting LMP1 expression, was demonstrated to lead to a transient increase in the level of *bfl-1* mRNA after 6 hours in all of the EREB cell lines tested. The maximum induction of the anti-apoptotic *bcl-2* by LMP1 peaks at 48-72 hours (Rowe *et al.*, 1994). In contrast to *bcl-2*, the induction of *bfl-1* mRNA appears to be an immediate and direct effect of EBV infection, as is the case with *A20* (Laherty *et al.*, 1992). These findings support the hypothesis that *bfl-1* possibly functions as a rapidly inducible, short-term effector of cell viability. Elevated levels of several anti-apoptotic proteins would increase the range of apoptotic stimuli against which the host cell can protect itself. The anti-apoptotic effects of Bfl-1 have been demonstrated, and it has been shown to suppress apoptosis induced by serum depletion (D'Souza *et al.*, 2000), the pro-inflammatory cytokine TNF α (Karsan *et al.*, 1996b; Zong *et al.*, 1999; Duriez *et al.*, 2000), tumour suppressor p53 (D'Sa-Eipper *et al.*, 1996), B cell receptor aggregation (Kuss *et al.*, 1999; Grumont *et al.*, 1999; Craxton *et al.*, 2000), the pro-apoptotic factors Bax and Bad (Zhang *et al.*, 2000; Holmgreen *et al.*, 1999). It has also been found to suppress chemotherapy-induced apoptosis (Wang *et al.*, 1999; Cheng *et al.*, 2000; Kim *et al.*, 2004). The up-regulation of *bfl-1* by LMP1 and/or EBNA2 during primary EBV infection is likely to be a crucial immediate early response that enables cells to survive, or in any case, survive until such time that Bcl-2 is up-regulated.

In lymphoid follicles, the *bfl-1* transcript has been detected in the germinal centres (Jung-Ha *et al.*, 1998), which are the sites where an activated naïve B cell, which is responding to foreign antigen during an immune response, undergoes the transition into a long-lived memory B cell (Liu and Arpin, 1997; MacLennan *et al.*, 1988). CD40 activation is necessary for rescuing germinal centre B cells from spontaneous apoptosis, and long-lived B cells are distinguished by elevated expression of *bfl-1* (Tomakayo *et al.*, 1998). 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* by pro-inflammatory cytokines in endothelial, leukemic, and hemopoietic cells is in keeping with a role for protecting them from apoptotic extinction (Choi *et al.*, 1995; Karsan *et al.*, 1996a; Karsan *et al.*, 1996b; Lin *et al.*, 1996; Moreb and Scheweber, 1997). Consistent with a role for NF- κ B in this process is the reduction in constitutive *bfl-1* mRNA levels in B cell lines derived from c-Rel^{-/-} mice (Grumont *et al.*, 1999). The ability of LMP1 to mimic the cellular growth and cell survival signals, that normally result from the binding of CD40 ligand, by up-regulating the expression of anti-apoptotic members of the Bcl-2 family of proteins, including *bfl-1*, is very significant from the standpoint of the mechanism of persistence of EBV in the memory B cell in the peripheral blood. *In vivo*, EBV-positive tonsillar memory B cells express a restricted pattern of latent gene expression, where only EBNA1, LMP1 and LMP2A are expressed (Babcock and Thorley-Lawson, 2000). The actions of LMP1 and LMP2A, provide surrogate T-cell help and B-cell receptor signals, in a ligand-independent manner, that memory B cells require for long-term survival (Caldwell *et al.*, 1998). Since BL cells, such as DG75, exhibit phenotypic features reminiscent of germinal centre B cells (MacLennan *et al.*, 1988), the NF- κ B-dependent activation of *bfl-1* by LMP1 may be a key event in the rescue of EBV-infected B cells from apoptosis and an important route by which EBV gains access to the memory B cell pool, in order to establish a life-long infection in the host.

Experiments carried out using the panel of EREB cell lines, demonstrated that LMP1 maintains elevated *bfl-1* mRNA levels in the absence of functional EBNA2. This finding implies a role for LMP1 in the up-regulation of *bfl-1* in cells that do not express EBNA2. Indeed LMP1 has been detected in the absence of EBNA2 in the majority of EBV-associated tumours such as NPC, HD and T- and NK-cell lymphomas. Although EBNA2 is the major viral trans-activator of LMP1 expression, recent studies have implicated the EBV BART RK-BARF0 in the activation of LMP1 expression (indicated

in Figure 6.1). It has been demonstrated using yeast two-hybrid screening, co-immunoprecipitation and confocal microscopy that RK-BARF0 interacts with the Notch4 ligand binding domain, inducing translocation of a portion of the full-length, un-processed Notch4 to the nucleus by using the Notch4 NLS (Kusano *et al.*, 2001). In EBV-infected, EBNA2-negative cells, RK-BARF0 induced expression of LMP1 and this induction was found to be dependent on the RK-BARF0/Notch4 interaction domain (Kusano *et al.*, 2001). Furthermore, functional binding sites for members of the STAT family of transcription factors have been identified in the promoter for *LMP1* gene, which was found to be responsive to JAK/STAT signalling in reporter assays (Chen *et al.*, 2001). The activation of LMP1 expression by RK-BARF0 or JAK/STAT signalling may be responsible for the expression of LMP1 in EBV-associated tumours, in which EBNA2 is not expressed. The question remains, however, as to whether LMP1 is the only EBV latent protein that maintains *bfl-1* in these EBNA2-negative tumours.

The trans-activation of *bfl-1* by EBNA2 demonstrates for the first time that EBNA2 directly targets the transcriptional regulation of an anti-apoptotic protein. The identification of an anti-apoptotic function for EBNA2 implies that, in addition to LMP1, EBNA2 contributes to cell survival, as well as to the proliferative response that occurs during initial EBV infection. A protective role for EBNA2 may both complement the LMP1-induced survival signals, and provide initial protection before LMP1 is expressed during primary EBV infection of B cells. During experiments carried out using the EREB cell lines, LMP1 expression was detected at one hour post-induction of functional EBNA2. As a result, it was difficult to assess which protein was responsible for the initial up-regulation in the level of *bfl-1* mRNA. The majority of EBV-associated tumours do not express EBNA2, presumably because the EBNA2 and co-ordinately expressed EBNA3 latency proteins are highly immunogenic. However, EBNA2 is expressed in EBV-associated tumours arising in immuno-compromised patients, for example in post-transplant lymphoproliferative disease (PTLD) and in primary central nervous system lymphomas in AIDS patients (Auperin *et al.*, 1994; Cen *et al.*, 1993; Delecluse *et al.*, 1995), and the anti-apoptotic effect of EBNA2 may be a factor in the development of these malignancies. Furthermore, there is increasing evidence to suggest an anti-apoptotic role for EBNA2 independently of LMP1. EBNA2 has been detected in LMP1-negative smooth muscle tumours in PTLD patients (Lee *et al.*, 1995), providing a possible mechanism for *bfl-1* up-regulation in the absence of LMP1. Additionally, EBNA2 interacts with Nur77, an orphan member of the nuclear

hormone receptor super-family, to protect against Nur77-mediated apoptosis (Lee *et al.*, 2002). As LMP1 cannot protect against Nur77-mediated apoptosis, EBNA2 has the ability to protect B cells against specific apoptotic agents, against which LMP1 is not effective (Lee *et al.*, 2004). Since a tumourigenic cell phenotype is associated not only with increased proliferation, but also with increased resistance to apoptosis, EBNA2 may provide such resistance by both blocking the effects of stimuli that elicit Nur77-mediated apoptosis and simultaneously up-regulating expression of the *bfl-1* gene.

The crucial role of the DNA binding protein RBP-J κ /CBF1 in EBNA2-mediated trans-activation was demonstrated during the study, when it was shown that over-expression of a non-DNA binding RBP-J κ /CBF1 mutant dramatically inhibited EBNA2-mediated trans-activation of the *bfl-1* and *LMP1* promoters (Figure 6.1). RBP-J κ /CBF1 also plays an important role in the Notch signalling pathway, which regulates cell fate decisions in many different organisms. It is of interest that EBNA2 interacts with components of a cellular signalling pathway to modulate gene expression. Expression of EBNA2 is restricted to naïve IgD⁺ B cells in secondary lymphoid tissue in healthy EBV-positive individuals (Joseph *et al.*, 2000; Babcock and Thorley-Lawson, 2000). IgD⁺ B cells are concentrated in the marginal zone of germinal centres. Studies using transgenic mice that are conditionally deficient for RBP-J κ /CBF1 in B cells show a selective loss of marginal zone B cells, implicating Notch signalling in the development of this cell type (Tanigaki *et al.*, 2002). Reinforcing this observation, transfer of MINT-deficient splenic B cells into recipient mice led to preferential differentiation into marginal zone B cells (Kuroda *et al.*, 2003). MINT/SHARP, which interacts with RBP-J κ /CBF1 and antagonizes Notch activity, is highly expressed in mouse follicular B cells and has low expression in marginal zone B cells. It has been postulated that marginal zone B cells express high levels of Notch2 (Witt *et al.*, 2003) and furthermore, conditional deletion of Notch2 in mice resulted in a deficiency in marginal zone B cells (Saito *et al.*, 2003). The fact that EBNA2 is selectively expressed in the same compartment of the germinal centre where Notch2 expression is essential for B cell development suggests that EBV is using EBNA2 as a virally controlled, constitutive Notch substitute (Hayward, 2004).

CHAPTER 7

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APPENDIX

SOLUTIONS FOR DNA MANIPULATION

STORAGE OF DNA

0.5 M EDTA (pH 8.0)

186.1 g	EDTA
800 ml	dH ₂ O

The pH was adjusted to 8.0 by addition of NaOH pellets and the volume adjusted to 1 L with dH₂O. The solution was sterilised by autoclaving and stored at RT.

TE buffer (pH 8.0)

10 mM	Tris-Cl (pH 8.0)
1 mM	EDTA (pH 8.0)

BACTERIAL GROWTH MEDIA

Ampicillin stock solution

A stock solution of ampicillin was made up to a concentration of 100 mg/ml of dH₂O. The stock solution was filter sterilised and stored at -20°C.

LB broth

5 g	tryptone
2.5 g	yeast extract
5g	NaCl

The volume was adjusted to 500 ml, followed by autoclaving for 15 minutes at 15 lb/sq. and storage at 4°C.

LB broth with ampicillin

Ampicillin was added to autoclaved LB broth to a final concentration of 100 µg/ml and stored at 4°C.

LB agar

5 g	tryptone
2.5 g	yeast extract
5 g	NaCl
7.5 g	agar

The volume was adjusted to 500 ml with dH₂O, followed by autoclaving and agar plates were stored at 4°C.

LB agar with ampicillin

Ampicillin was added to autoclaved LB agar to a final concentration of 100 µg/ml (after cooling the LB agar to ~50°C). Plates were stored at 4°C.

SOB medium

10 g	tryptone
2.5 g	yeast extract
0.025 g	NaCl
5 ml	KCl (250 mM)

The pH was adjusted to 7.0 with 5 M NaOH.

The volume was adjusted to 500 ml with dH₂O and the medium autoclaved.

2.5 ml of 2 M MgCl₂ was added after cooling the broth to 5°C and the medium was stored at 4°C.

SOC medium

98 ml	SOB medium
2 ml	1 M glucose (filter sterilised)

Stored at 4°C.

SOLUTIONS FOR PREPARATION OF COMPETENT CELLS

TFB1

30 mM	potassium acetate
10 mM	CaCl ₂
50 mM	MnCl ₂
100 mM	RbCl
15 %	glycerol

The pH was adjusted to 5.8 with 1 M acetic acid, and the solution filter sterilised and stored at RT.

TFB2

100 mM	MOPS (pH 6.5)
75 mM	CaCl ₂
10 mM	RbCl
15 %	glycerol

The pH was adjusted to 6.5 with 1 M KOH, and the solution filter sterilised and stored at RT.

SOLUTIONS FOR MINIPREPARATIONS OF DNA

Solution I

50 mM	glucose
25 mM	Tris-Cl (pH 8.0)
10 mM	EDTA (pH 8.0)

Stored at RT

Solution II (prepared on day of use)

0.2 M	NaOH
1 % (w/v)	SDS

Solution III (3 M potassium acetate)

29.6 g	potassium acetate
50 ml	dH ₂ O
11.5 ml	glacial acetic acid

Adjust volume to 100 ml with dH₂O.

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

Stored at RT.

DNase-free RNase

RNase A (1 mg/ml) in upH₂O.

Heated to 100°C for 30 minutes.

Cooled slowly and stored at -20°C.

50 % (v/v) Glycerol

25 ml	glycerol
25 ml	dH ₂ O

The solution was autoclaved and stored at RT.

SOLUTIONS FOR MAXIPREPARATIONS OF DNA (QIAGEN® BUFFERS)

Buffer P1 (Re-suspension buffer)

50 mM	Tris-Cl (pH 8.0)
10 mM	EDTA (pH8.0)
100 µg/ml	RNase A

Stored at 4°C following addition of RNase A.

Buffer P2 (Lysis buffer)

200 mM	NaOH
1 % (w/v)	SDS

Stored at RT.

Buffer P3 (Neutralization buffer)

3 M	potassium acetate (pH 5.5)
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Stored at RT.

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 RT.

Buffer QC (Wash buffer)

1 M	NaCl
50 mM	MOPS (pH 7.0)
15 % (v/v)	isopropanol

Stored at RT.

Buffer QF (Elution buffer)

1.25 M	NaCl
50 mM	Tris-Cl (pH 8.5)
15 %	isopropanol

Stored at RT.

SOLUTIONS FOR AGAROSE GEL ELECTROPHORESIS**50 X TAE (Tris-acetate/EDTA electrophoresis buffer)**

242 g	Tris base
57.1 ml	glacial acetic acid
100 ml	0.5 M EDTA (pH 8.0)

The volume was adjusted to 1 L with dH₂O and the buffer was stored at RT.

1 X TAE (Working solution)

20 ml	50 X TAE
980 ml	dH ₂ O

Stored at RT.

Agarose gel loading dye

40 % (w/v)	sucrose
0.25 % (w/v)	bromophenol blue

Stored at RT.

Ethidium bromide

10 mg	ethidium bromide
1 ml	dH ₂ O

The solution was stored in the dark at RT.

SOLUTIONS FOR CELL CULTURE

MEDIA & SUPPLEMENTS

Supplemented RPMI 1640 (200 ml)

176 ml	RPMI 1640
20 ml	foetal bovine serum (decomplemented at 50°C for 30 minutes)
2 ml	200 mM L-glutamine
2 ml	penicillin/streptomycin (10 mg/ml)

Estrogen (β -Estradiol)

A 20 mM solution was prepared in 100 % ethanol and stored at -20°C .

Tetracycline

A 5 mg/ml stock was prepared in 100 % ethanol and stored at -20°C .

Phosphate buffered saline (PBS)

5 tablets were dissolved in 500 ml dH_2O to give a 1 X working concentration of PBS, and the solution was sterilised by autoclaving.

SOLUTIONS FOR DEAE-DEXTRAN TRANSFECTIONS PROTOCOL

Tris buffered saline (TBS)

25 mM	Tris-Cl (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

To make 200 ml TBS:

5 ml	1 M Tris-Cl (pH 7.4)
5.48 ml	5 M NaCl
0.5 ml	2 M KCl
1.4 ml	100 mM CaCl_2
0.1 ml	1 M MgCl_2
0.2 ml	0.6 M Na_2HPO_4
187.32 ml	upH_2O

TBS was prepared from autoclaved stocks, aliquoted and filter sterilised before use.

DEAE dextran

DEAE dextran was prepared at a concentration of 1 mg/ml in TBS on the day of use and filter sterilised.

β -GALACTOSIDASE ASSAY

100 X Mg solution

0.1 M	MgCl ₂
4.5 M	2-mercaptoethanol

Stored at -20°C.

0.1 M sodium phosphate buffer

41 ml	0.2 M Na ₂ HPO ₄ ·2H ₂ O
9 ml	0.2 M NaH ₂ PO ₄ ·2H ₂ O
50 ml	dH ₂ O

1 X ONPG substrate

ONPG was prepared at a concentration of 4 mg/ml in 0.1 M sodium phosphate buffer (pH 7.5) and stored at -20°C.

SOLUTIONS FOR RNA ANALYSIS

DEPC-treated H₂O

1 ml	DEPC
1000 ml	dH ₂ O

The mixture was left in a fume cupboard overnight, followed by autoclaving.

RNA loading buffer

50 %	glycerol
1 mM	EDTA (pH 8.0)
0.25 %	bromophenol blue
0.25 %	xylene cyanol FF
1 µg/ul	ethidium bromide

SOLUTIONS FOR PROTEIN ANALYSIS

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	leupeptin
1 µg/ml	aprotinin
100 µg/ml	PMSF

Stored at 4°C

Leupeptin

A stock solution of leupeptin was made to a concentration 2 mg/ml in dH₂O and stored at -20°C.

Aprotinin

0.1 M stock solution of aprotinin was made up in dH₂O and stored at -20°C.

PMSF

A stock solution of PMSF was made up in isopropanol and stored at -20°C in the dark.

2 X SDS loading buffer

100 mM	Tris-Cl (pH 7.6)
4 % (w/v)	SDS
20 % (w/v)	glycerol
10 % (v/v)	2-mercaptoethanol
0.2 %	bromophenol blue

Stored at RT.

SOLUTIONS FOR SDS PAGE/WESTERN BLOTTING

5 X Tris-glycine running buffer

15.1 g	Tris base
95.4 g	glycine(pH 8.3)
50 ml	10 % (w/v) SDS

Made up to 1 L with dH₂O and stored at RT.

1 X Tris-glycine running buffer

200 ml	5 X Tris-glycine running buffer
800 ml	dH ₂ O

Destain

450 ml	methanol
450 ml	dH ₂ O
100 ml	glacial acetic acid

Store at RT.

Coomassie blue stain

0.25 g	Coomassie Brilliant Blue R250
100 ml	destain

Store at RT.

Transfer buffer

750 ml	dH ₂ O
2.9 g	glycine
5.8 g	Tris base
3.7 ml	10 % (w/v) SDS
200 ml	methanol

Adjusted volume to 1 L with dH₂O and stored at 4°C.

1X Tris buffered saline (TBS)

6.1 g	Tris base
8.8 g	NaCl
800 ml	dH ₂ O

The pH was adjusted to 7.5 with HCl and the volume adjusted to 1 L. Stored at RT.

TBS-T

1 L	1 X TBS
1 ml	Tween 20

Stored at RT.

Blocking Buffer

5 g	non fat dry milk powder
100 ml	TBS-T

Stored at 4°C.