

# **REGULATION OF APOPTOSIS BY THE EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEIN LMP1**

**A dissertation submitted for the degree of Ph.D.**

**by**

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June 2001

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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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*I dedicate this thesis to my family for all the love and support  
you have given me*

## ACKNOWLEDGEMENTS

I thank the Almighty for His graces and mercies in getting me this far on life's path

I would like to thank my supervisor Dr Dermot Walls, for giving me the opportunity to carry out my Ph D in his lab group I thank him for the help and encouragement he has given me over the years but especially with the preparation of my thesis

Thanks to Professor Martin Rowe (School of Medicine, University of Wales, Cardiff) and members of his lab (Anja, Paul, Matt and Ceri) for the opportunity of carrying out some of the work presented in this thesis in their lab and providing materials and advice over the years Thanks to Professor Celine Gelinas (School of Medicine and Dentistry, University of New Jersey) for providing the *bfl-1* promoter reporter constructs and providing information on the promoter sequence Special thanks to Dr Katherine Fitzgerald (Department of Biochemistry, Trinity College Dublin) for advice on conducting the bandshift assays and providing reagents

To all my lab friends Olivia, Joanne, Pam M , Pam P and Sinead, thanks for all your help and advice, scientific and otherwise Thanks for the chats, the laughs, keeping me company on my numerous smoke breaks etc etc Also, thanks to all in the Biotechnology Department and in the Research and Development center at D C U (Bioresearch Ireland) who helped with reagents or equipment

Special thanks to my mentors Joy, Joyce, Natasha, Feodor and Elena for helping me with my first steps in science To my close friends over the years John, Brian, Emmett, Hala, Bernie, Patricia, Mel and Martina, thanks for giving me the strength to get this far Thanks to Rita and Elizabeth for the relaxation therapy during the writing of this thesis

Last but by no means least, special thanks to my family and Frank, Su, Norman and Maji I dedicate this thesis to all of you Thanks for your unconditional love and support and making me believe in myself Thanks for always being there for me

## ABSTRACT

The Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that is implicated in the aetiology of African Burkitt's lymphoma (BL) and several other cancers of lymphoid/epithelial origin. *In vitro*, expression of all of the eleven EBV latent proteins or just the oncogenic Latent Membrane Protein 1 (LMP1) can protect BL cells from apoptosis by several stimuli including growth factor deprivation and this is related to the ability to upregulate the expression of several anti-apoptotic *bcl-2*-related genes. In this study, it is shown that EBV latent gene expression and in particular LMP1 can induce the upregulation of expression of the anti-apoptotic gene *bfl-1* in addition to *bcl-2* in BL cells and also that Bfl-1 expression can not only protect BL cells from serum-deprivation-induced apoptosis but also exert a proliferative effect under these culture conditions. Bfl-1 has been shown by others to suppress apoptosis and exhibits proliferative and potent cooperative transforming properties. Both increased mRNA stability and increased promoter activity were found to contribute to the effect of LMP1 on *bfl-1* expression. The transcription factor NF- $\kappa$ B was shown to mediate the effect of LMP1 on *bfl-1* promoter activity in both BL and T cells; however, cell-type-dependent differences in the regions of the promoter targeted by LMP1 were observed. CD40, which engages similar signaling proteins (TRAFs) as LMP1, also stimulates *bfl-1* mRNA expression; however, mechanistic similarities and differences were found between the two TNFR family members in this effect. LMP1 could also be demonstrated to contribute to activation of the *bfl-1* promoter in EBV-immortalised B lymphoblastoid cell lines (LCLs); however, NF- $\kappa$ B activation may not be the only mechanism regulating *bfl-1* promoter activity in different LCLs. A minor but significant finding in this study is that both transcriptional and translational mechanisms are involved in the LMP1-mediated upregulation of *bcl-2* expression, with the latter exerting a stronger effect. The upregulation of *bfl-1* expression by LMP1 represents the first example of regulation of expression of this anti-apoptotic gene by a viral protein and extends the list of anti-apoptotic proteins whose expression is controlled by LMP1. Protection against apoptosis constitutes an important part of the latent phase of the EBV life cycle, in that it provides a mechanism to ensure life-long viral persistence in the host by mimicking the natural process of selection of B cells into the memory compartment. The regulation of expression of *bcl-2*-related genes by LMP1 provides an important link between EBV and its cellular survival and growth transforming properties.

## ABBREVIATIONS

A	Adenosine
aa	Amino acid
Abs	Absorbance
AIDS	Acquired Immune Deficiency Syndrome
amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammonium persulphate
ATP	Adenosine tri-phosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
$\beta$ -gal	$\beta$ -galactosidase
BL	Burkitt's Lymphoma
BSA	Bovine serum albumin
C	Cytosine
CAT	Chloramphenicol acetyl transferase
CD	Cluster of differentiation
cDNA	complementary DNA
CIP	Calf intestinal phosphatase
CMV	Cytomegalovirus
CTAR	Carboxy terminal activation region
CTP	Cytosine tri-phosphate
DEPC	Diethylpyrocarbonate
dH <sub>2</sub> O	Distilled water
dNTP	Deoxy nucleoside tri-phosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EA-D	Early antigen-diffuse
EA-R	Early antigen-restricted
EBER	Epstein-Barr virus encoded RNA

EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic Mobility Shift Assay
FCS	Fetal calf serum
G	Guanosine
GTP	Guanosine tri-phosphate
HA	Haemagglutinin antigen
HEPES	N-[hydroxyethyl]piperazine N'-[2-ethane sulfonic acid]
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
LB	Luria-Bertam Broth
LCL	Lymphoblastoid cell line
LMP	Latent Membrane Protein
LP	Leader Protein
Luc	Luciferase
mAb	monoclonal antibody
MAPK	Mitogen Activated Protein Kinase
MCS	Multiple cloning site
mRNA	messenger RNA
NBT	Nitroblue tetrazolium
NPC	Nasopharyngeal carcinoma
n s	non-specific
OA	Okadaic acid
OD	Optical Density
OHL	Oral Hairy leukoplakia
ori	Origin of replication
p	Plasmid

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating-cell nuclear antigen
PI3-K	Phosphatidylinositol-3-kinase
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
pRB	Retinoblastoma protein
RBP-Jκ	Recombination Signal binding protein-Jκ
RNA	Ribonucleic acid
RNase	Ribonuclease
RPA	Ribonuclease Protection assay
s s	super-shifted complex
SDS	Sodium dodecyl sulphate
SSC	Standard sodium citrate
ssDNA	salmon sperm DNA
SV40	Simian Virus 40
T	Thymidine
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline
TBST	TBS + Tween20
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TESS	Transcription factor element search software
TF	Transcription factor
TNF	Tumour necrosis factor
tRNA	Transfer RNA
TRAF	TNF-receptor-associated factors
upH <sub>2</sub> O	Ultrapure water
U	Uracil
UTP	Uracil tri-phosphate

UV	Ultraviolet
v/v	volume per volume
VA	sodium orthovanadate
w/v	weight per volume

## UNITS

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

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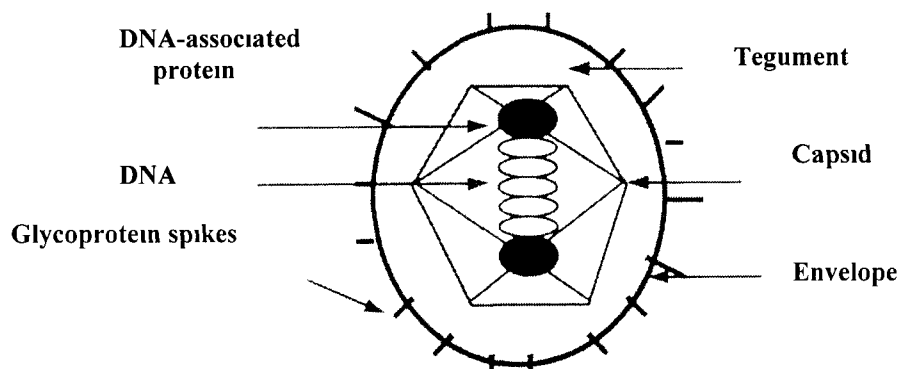
## **CHAPTER 1**

### **INTRODUCTION**

## 1.0 EPSTEIN BARR VIRUS

### 1.1 DISCOVERY AND CLASSIFICATION

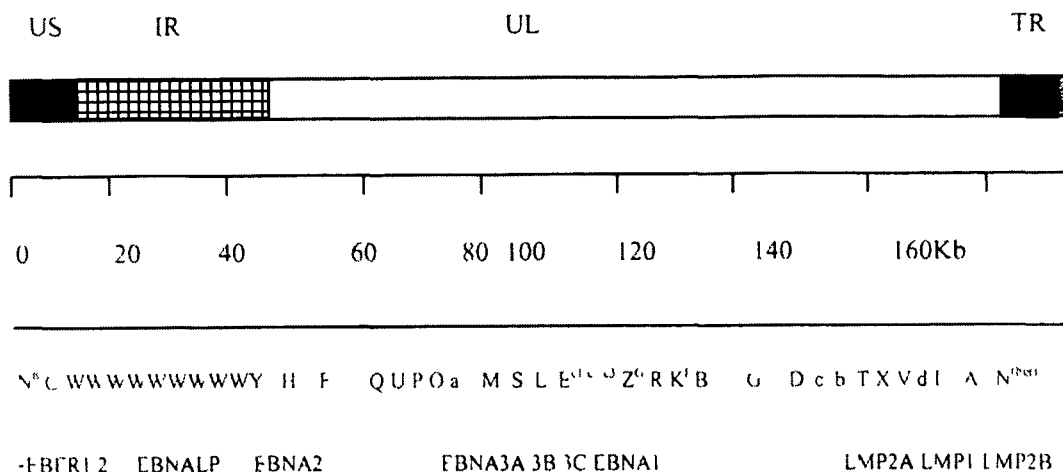
The Epstein-Barr virus (EBV) was discovered in cultured lymphoblasts from samples of African Burkitt's lymphoma by Epstein *et al* , (1964) Since its discovery as the virus associated with African Burkitt's lymphoma, EBV has also been found to be associated with many other malignant diseases including Hodgkin's lymphoma, T-cell lymphomas, nasopharyngeal carcinoma, post-transplant lymphoproliferative diseases, and gastric carcinomas as well as non-malignant diseases such as infectious mononucleosis (reviewed by Klein, 1994, Kieff 1996, Dillner and Kallin, 1988, Chen *et al* , 1993, Imai *et al* , 1994) EBV is a ubiquitous human herpes virus, for which humans are the exclusive natural host, infecting between 90-95% of the population (Kieff, 1996) The herpesviruses are a family of DNA viruses found commonly in humans and animals Herpes viruses are biologically classified into three subfamilies, alpha, beta and gamma with EBV belonging to the gammaherpesvirus subfamily and the genera *Lymphocryptovirus* Viruses of this subfamily are characterised by their tropism for lymphoid cells and their capacity to induce cell proliferation *in vivo* resulting in transient or chronic lymphoproliferative disorders and *in vitro* where they can immortalize the infected cell Taxonomists have renamed EBV as human herpesvirus 4 (HHV4) but EBV is still its commonly used name (IARC Monographs, 1997) Like other herpesviruses, a mature EBV virion has a toroid-shaped protein core that is wrapped with double-stranded DNA and this is surrounded by an icosahedral capsid with 162 capsomers (Figure 1 1) The capsid is surrounded by an amorphous material, the tegument, composed of globular proteins The envelope of herpesviruses have numerous glycoprotein spikes (Kieff, 1996)



**Figure 1 1 Schematic representation of herpes virus structure**

## **1.2 EBV GENOME STRUCTURE**

The EBV genome is a linear, double stranded 172 Kb DNA with a guanine/cytosine content of 60% (Kieff, 1996). The genome encodes an estimated 100 genes, but like many viruses there is complicated differential splicing of RNA transcripts and hence, the number of proteins encoded by the genome may be greater (Kieff, 1996). The EBV genome was completely sequenced from the EBV strain B95-8, initially cloned as a *Bam* H1 fragment library. Therefore, open reading frames (ORF), genes, sites for transcription or RNA processing are frequently referred to as specific *Bam* H1 fragments (Baer *et al* , 1984). For example, the BARF1 ORF is found in the *Bam* H1 fragment (BA) and it is the first ORF (F1) extending in a rightward ® direction. A simplified schematic representation of the EBV genome is outlined in figure 1 2.



**Fig 1.2 A schematic representation of the EBV genome** The genome contains repeat regions, 0.5 Kb terminal direct repeats (TR) and 3.0 Kb internal direct repeats (IR) that divides the genome into short and long largely unique sequence domains (US and UL). The *Bam*HI fragments are represented by the letters (N, C, W etc.). The *Bam*HI fragment location of the EBV latent genes are indicated below the fragments: EBER, EBV encoded RNA, EBNA, EBV nuclear antigen, LMP, latent membrane protein, LP, leader protein. Adapted from IARC Monographs (1997).

Two EBV types circulate in most human populations (Gerber *et al*, 1976, Young *et al*, 1987, Rowe *et al*, 1989). These genomes formerly known as type A and type B are now referred to as type-1 and type-2. The genomes are almost identical except for the genes that encode some of the Epstein-Barr nuclear antigens (EBNAs) such as EBNA2, EBNA3A, 3B, 3C and EBNA1P, in latently infected cells (Nanoyama and Pagano, 1973, Bornkamm *et al*, 1980). The differences in type-1 and type-2 EBV genomes are reflected in type-specific and type-common epitopes for antibodies (Young *et al*, 1987) and T-cell recognition (Moss *et al*, 1988). As type-1 EBV is more common in developed societies, most EBV immune human sera from these countries react preferentially or exclusively with type-1 LMP1, EBNA2, EBNA3A, 3B, 3C and EBNA1P. African sera are almost evenly split in their serological reactivity. However, the recovery of type-2 virus from blood is unusual (Young *et al*, 1987, Rowe *et al*, 1989). In culture, EBV type-2 infected

lymphocytes grow less efficiently *in vitro* than their type-1 infected counterparts (Rickinson *et al* , 1987)

### **1.3 EBV STRATEGY OF INFECTION**

#### **1.3.1 Reservoir of EBV**

Two forms of EBV-cellular infection are recognised latent and replicative (or lytic) The virus exhibits a tropism for lymphocytes and epithelial cells and both cell compartments have been alternately considered the main virus-reservoir (reviewed in Power and Walls, 1993) As long as B-cells were regarded as the only EBV-infectable cells *in vitro*, they or their precursors were regarded as the most probable carriers of the latent virus The regular *in vitro* outgrowth of permanent B-lymphoblastoid cell lines (LCLs) from the peripheral blood or lymphoid tissues of all seropositive persons was often quoted in support of this view (reviewed in Power and Walls, 1993) With the discovery that the proliferative and cytotoxic T-cell (CTL) responses to autologous EBV-transformed LCLs are as strong as the corresponding T-cell responses against allogeneic targets, the hypothesis was put forward that EBV-carrying B-cells are continuously destroyed by the immune response and must be newly generated by repeated infection from the epithelial cell compartment The detection of EBV genomes in oropharyngeal epithelia where lytic infection is observed support this view (reviewed in Farrell, 1995)

The predominant role of the epithelial cell compartment in virus-persistence was challenged by findings in bone marrow transplant recipients (reviewed in Power and Walls, 1993) In one case, where the marrow donor was seropositive, the virus of the recipient was totally replaced by the strain of the donor The eradication of the resident virus by the combined irradiation and drug treatment suggested that the latent virus is harboured in a tissue compartment that has been destroyed by the treatment and replaced by the graft These observations have led to the suggestion that the reservoir responsible for EBV-persistence is in the hematopoietic compartment, and the long-lived memory B-cell pool was suggested as the most likely reservoir for latent EBV genomes Affirmative

proof for the reservoir of EBV infection residing in the B-cell compartment was provided by the finding of the virus in resting memory B cells of the peripheral blood of healthy carriers (Decker *et al.*, 1996; Miyashita *et al.*, 1997).

Entry into the memory pool is normally restricted to a very small proportion of B-cells *in vivo* and depends on positive selection by antigen in germinal centres (GCs) (Liu *et al.*, 1989). The mechanism of positive selection is based on the receipt of signals which protect against apoptotic death, since unselected GC B-cells (centrocytes) die by apoptosis (Liu *et al.*, 1991). EBV is thought to be able to ensure persistence in the host by protecting infected B-cells from apoptotic death in the GC, thereby mimicking/bypassing the selection mechanism and ensuring entry of the infected cell into the memory pool.

### **1.3.2 Early events in EBV-infection of B-lymphocytes**

EBV infection of primary B-lymphocytes *in vitro* involves binding of CD21 on the B-lymphocyte plasma membrane which is expressed by approximately 10% of all B cells (Kieff, 1996). CD21 (also known as CR2) is the receptor for the C3d component of complement. After binding, aggregation of CD21 in the plasma membrane, the co-aggregation of surface immunoglobulins (sIg) and internalization of EBV into cytoplasmic vesicles occur (Nemerow and Cooper, 1984; Carel *et al.*, 1990). The virus envelope then fuses with the vesicle membrane, releasing the nucleocapsid and tegument into the cytoplasm. Penetration is usually complete within 1-2h. Superinfection of established BL cell lines is somewhat different in that EBV binding does not result in as significant a patching of CD21 and sIg and the envelope fuses with the plasma membrane, releasing the nucleocapsid and tegument into the plasma membrane. The observed differences in mode of infection between primary B-lymphocytes and BL cells are likely to be due to the cytoskeletal abnormalities of the tumour cells (Kieff, 1996). The EBV outer envelope glycoproteins gp350 and gp220 constitute the CD21 ligand. The interaction of CD21, gp350 and gp220 mediates EBV absorption (Kieff, 1996; Nemerow *et al.*, 1987; 1989). Another EBV glycoprotein, gp85, has also been implicated

in the fusion of the EBV envelope with the vesicle membrane (Miller and Fletcher, 1988) Little is known about EBV capsid dissolution, genome transport to the cell nucleus or DNA circularisation

Circularisation (as a result of fusion of the terminal repeats) of the viral genome appears to be required for successful immortalisation of B-cells and is associated with latent infection *in vivo* and *in vitro* The circular or episomal configuration of the viral DNA is generally retained following immortalisation, with subsequent increases in rates of replication generating multiple episome copies (reviewed in Kieff, 1996, Joske and Knecht, 1993) Replicative infection by EBV is associated with linearised viral genome and is thought to require cellular RAG gene expression (RAG gene products are a part of the cellular immunoglobulin recombination machinery and is upregulated by EBV infection) in addition to viral gene expression A homolog of the RAG protein, BALF-2, encoded by the viral genome has been identified and is also implicated in the process of the interconversion of the genome (Dreyfus *et al* , 1996)

Much less is known about epithelial cell factors that facilitate viral entry, though viral entry is not likely to involve CD21 since this molecule is either not expressed or expressed at very low levels by epithelial cells One *in vitro* epithelial cell system developed has been based on the usage of antibody to EBV to assist viral entry (reviewed in Sugden, 1992) In this system, addition of specific anti-EBV gp350 polymeric IgA (pIgA) molecules in culture was shown to assist entry of EBV into a human epithelial cell line that expresses the receptor for pIgA Moreover, this study revealed that the infected cells expressed viral genes characteristic of the lytic phase More recently, an efficient system of infection based on coculturing epithelial cells with an EBV producing BL cell line was developed and the requirement of cell-cell contact for efficient infection was demonstrated (Imai *et al* , 1998) In this system, the EBV-infected epithelial cell lines exhibited the pattern of latent gene expression characteristic of EBV-associated gastric cancers or LMP-1-negative NPC (table 1 3) The epithelial cell factor that is responsible for facilitating virus entry in this system is still unknown

#### 1.4 LYMPHOBLASTOID CELL LINES AS A MODEL FOR STUDYING LATENT INFECTION OF B-LYMPHOCYTES BY EBV

Most human peripheral blood B-lymphocytes are susceptible to EBV infection and infection leads to the establishment of immortalised cell lines called lymphoblastoid cell lines (LCLs) (reviewed in Farrell, 1995). The virus does not usually replicate in recently infected B-lymphocytes, instead they become stably latently infected. However 2-3% of the cells in cultures of LCLs exhibit lytic infection. In latently infected B-lymphocytes, EBV expresses six different nuclear proteins or EBNA (EBNA-1, -2, -3A, -3B, -3C, -LP), three integral membrane proteins or LMPs (LMP-1, -2A, 2B) and two small non polyadenalated RNAs or EBERs (EBER -1, -2). These viral gene products maintain the latent infection and cause the previously resting B-lymphocytes to continuously proliferate (Mark and Sugden, 1982). Recombinant genetic analyses of the EBV genome have demonstrated that of the 11 latent gene products, five are absolutely required for B cell immortalisation. EBNA-1, 2, 3a, 3c and LMP-1, while EBNA-LP and LMP-2A appear to improve the efficiency of immortalisation (Hammerschmidt and Sugden, 1989, Cohen *et al* , 1989, Kaye *et al* , 1993, Tomkinson *et al* , 1993, Kieff, 1996). The EBV infected proliferating B-lymphocytes are similar to activated B-lymphocytes in their secretion of immunoglobulin and their adherence to each other (Klein, 1987, Zhang *et al* , 1991). Approximately 1 in every  $10^5$ - $10^6$  of the B-lymphocytes purified from the peripheral blood of previously infected people are latently infected with EBV (Sixbey and Pagano, 1985).

While the growth transformation of human B-cells from the resting state to the continuously proliferating LCL is always associated with the expression of the full spectrum of 11 EBV latent proteins, the mechanisms through which the virus subverts normal B-cell growth regulation are not fully understood. A key issue in this regard is the observation that all LCLs display a consistent array of activation/differentiation antigens on their surface (reviewed in Gregory, 1995) irrespective of the B-cell maturation stage at the moment of EBV infection (Table 1.1). These antigens, whose functions include those of autocrine growth and survival signaling (e.g., CD23 or CD40)

as well as intercellular adhesion [e g , leukocyte function antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1) or LFA-3], are typically induced or up-regulated transiently on the B-cell surface following physiological activation by cognate antigen and T-cell factors Their strong expression by all LCLs strongly suggests that at least some of them are required to mediate the growth-transforming properties of the virus

**TABLE 1 1   Cell surface molecules modulated following EBV infection of B-lymphocytes**  
*(adapted from Gregory, 1995)*

Molecule	Structure/Function	EBV-
		Induced change (↑ or ↓)
CD10	Neutral endopeptidase	↓
CD11a/CD18 (LFA-1)	Intercellular adhesion	↑
CD21	C3d/EBV receptor, CD23-hgand	↑
CD23	Autocrine growth, adhesion	↑
CD30	Neuronal growth factor receptor family member	↑
CD39	Function unknown	↑
CD40	Growth signaling, inhibition of apoptosis	↑
CD44	Intercellular adhesion	↑
CD54 (ICAM-1)	Intercellular adhesion	↑
CD58 (LFA-3)	Intercellular adhesion	↑
CD70	CD27-ligand	↑
CD77	Neutral glycosphmgolipid	↓
CD95 (Fas)	Induction of apoptosis	↑

## 1.5 BURKITT'S LYMPHOMA AS A MODEL FOR STUDYING LATENT INFECTION OF B LYMPHOCYTES BY EBV

Most non-EBV infected continuous B-lymphocyte cell lines are derived from sporadically occurring EBV-negative Burkitt's lymphomas (BL) and many can be infected with EBV *in vitro* (Calendar *et al.*, 1987; 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 in chromosome 1 (Berger and Bernheim, 1985). A fundamental feature of BL tumour lines which has imposed restrictions on models of BL pathogenesis is the marked phenotypic heterogeneity that is observed between different lines (reviewed in Gregory, 1995). However, a limited rationale for some of the phenotypes assumed by BL cells, at least in cases of EBV-positive tumours, has been achieved.

Broadly speaking, successful discrimination of BL lines into phenotypic groups has been based upon the expression of two categories of antigens which may be loosely termed (1) "BL specific" antigens and (2) "activation" antigens (reviewed by Gregory, 1995). The first category constitutes CD77 (a glycolipid antigen, also called BL-associated antigen) and CD10 (CALLA-common acute lymphoblastic leukemia-associated antigen); the second category includes cell-surface molecules such as CD23, CD30, CD39 and CD70 that are found to be constitutively up-regulated on EBV-LCLs. EBV-positive BL lines have been categorised into four groups: group I cell populations coexpressed only CD10 and CD77 of the panel of markers tested; group I/II cells additionally expressed CD70; group II populations expressed the surface markers, CD10, CD77, CD23, CD30, CD39, and CD70 and group III cells expressed CD23, CD30, CD39 and CD70 but not CD10 or CD77 (Rowe *et al.*, 1987). The importance of the group I to III categorisation scheme lay in its ability not only to identify heterogeneity among BL lines, but also to pick up changes that occurred within individual EBV-positive BL lines shortly after their establishment *in vitro* (Table 1.2). In particular, in studies of freshly isolated BL tumour populations and cell lines from cases of EBV-positive African BL, the tumour biopsy

cells were found, in all cases, to display the group I phenotype. In some lines following their establishment *in vitro* the group I phenotype was either retained or the cell populations fluctuated between group I and its subcategory group I/II, during continuous culture for several months. Other lines, however, underwent a rapid process of phenotypic drift from group I, through the transient group II stage to group III. This propensity for phenotypic change was found to be a feature of EBV-positive, rather than of EBV-negative, cases of BL, whether of sporadic or endemic origin (reviewed by Gregory, 1995).

TABLE 1 2 Phenotypic grouping of Burkitt's Lymphoma cell lines  
(adapted from Gregory, 1995)

Phenotype	Group I	GroupIII
Cell growth pattern	Dispersed single cells	Multicellular clumps
Cell surface antigens	CD10 <sup>+</sup> , CD77 <sup>+</sup> , CD23 <sup>-</sup> , CD39 <sup>-</sup> ,CD30 <sup>-</sup> ,CD70 <sup>-</sup> , LFA-1 <sup>LO</sup> ,ICAM-1 <sup>-</sup> , LFA-3 <sup>-</sup>	CD10 <sup>-</sup> ,CD77 <sup>-</sup> ,CD23 <sup>+</sup> , CD39 <sup>+</sup> ,CD30 <sup>+</sup> ,CD70 <sup>+</sup> , LFA-1 <sup>HI</sup> , ICAM <sup>+</sup> LFA-3 <sup>+</sup>
EBV latent genes	EBNA-1	EBNA-1, -2, -3A,B,C, -LP,LMP-1,-2A, -2B, EBERs
Bcl-2 protein expression	Negative or low	High
Apoptosis rate	High	Low
Overall phenotype	Resembles parental tumour	Resembles EBV-transformed LCL

The cell surface changes that characterise the groupI → group III phenotypic drift are accompanied by changes in morphology (Rowe *et al* , 1987,Gregory *et al* , 1990). Thus, whereas group I lines grow as a dispersed, single-cell suspension, the cells of group III

lines acquire a motile morphological appearance and cluster together in large multicellular aggregates, reminiscent of the constituent cells of normal EBV-LCLs. The molecular basis for the observed homotypic adhesion of group III cells was found to be via LFA-1/ICAM-1 interactions on closely apposed cells. In terms of cell surface expression, LFA-1 was found to be present at low levels on group I cells, whereas ICAM-1 was absent; on group III cells, both antigens are present, as on EBV-LCL, expressed at high levels (Gregory *et al* , 1990).

Analysis of changes in EBV latent protein expression during the group I → group III phenotypic drift led to the suggestion that this drift is EBV-driven (Gregory *et al* , 1990). Thus, whereas group I BL cells express only EBNA-1 and the EBERs (Rymo, 1979, Howe and Shue, 1989), cells in latency II resemble latency I cells as they express EBNA1 and the EBER RNAs but also express LMP1, LMP2A and LMP2B (Rowe *et al* , 1987). The classic features of latency I are exhibited in endemic BL biopsies and in early passage cell lines derived from these tumours (Rowe *et al* , 1987). Two EBV-related clinical conditions exhibit the latency II program: nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD) (Table 1.3). Group III cells were found to resemble EBV-LCLs in that they express the full complement of EBV latent proteins - all six EBNAs, the three LMPs and the two EBERs (Gregory *et al* , 1990). Two EBV-associated diseases best exemplify the latency III program, infectious mononucleosis and posttransplantation lymphoproliferative disorder (PTLD) which is a potentially fatal immunoblastic lymphoma in transplant patients. The EBV-specific cytotoxic T-cell response is considered to be the most important factor in controlling EBV-induced B-cell proliferation *in vivo*. All of the latent proteins except for EBNA1 elicit a strong immunogenic response and this explains why expression of latent proteins in EBV-associated BL is restricted to EBNA1 and also why EBV-associated B-lymphocyte malignancies (such as PTLD) in immuno-suppressed patients exhibit a wider panel of latent gene expression (Liebowitz, 1994).

**TABLE 1 3   Pattern of EBV latent gene expression**  
*adapted in part from the IARC monograph ,1997*

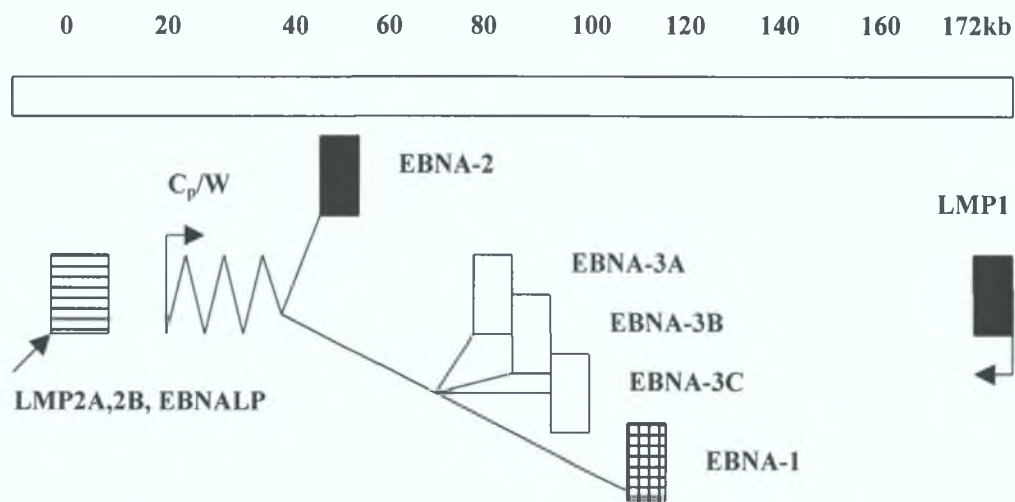
Type of Latency	Gene Product	Examples	Reference
I	EBERs, EBNA1	Burkitt's lymphoma Gastric Carcinoma	Rowe <i>et al</i> , (1987) Imai <i>et al</i> , (1994)
II	EBERs, EBNA1, LMP1, 2A, 2B, BARFO	Hodgkin's disease Nasopharyngeal carcinoma	Deacon <i>et al</i> , (1993) Hitt <i>et al</i> , (1989) Brooks <i>et al</i> , (1992)
III	All EBV latent genes	PTLD, IM	Young <i>et al</i> , (1989) Tirney <i>et al</i> , (1994)
Other	EBNA-1,-2, LMP-1	T-cell lymphomas	Harabuchi <i>et al</i> , (1990)
Other	EBERs, EBNA-1, -2	Smooth muscle tumours	Lee <i>et al</i> , (1995)
Other	EBNA-1,-2,-LP ,LMP1	Oral hairy leukoplakia	Webster-Cyriaque <i>et al</i> , (2000)

PTLD Post-transplant lymphoproliferative disorder, IM infectious mononucleosis

**1.6 EBV LATENT GENES**

The binding of EBV to CD21 and the subsequent infection of the cell triggers a cascade of events leading ultimately to the immortalization of the cell. The immortalization of the primary B cell requires the expression of several viral genes (Allday *et al* , 1989, Rooney *et al* , 1989, Alfieri, *et al* , 1991). The first viral proteins to be expressed in B

cells upon EBV infection are EBNA2 and EBNA-LP and are detected by 12 h of infection. By 48 h post-infection, expression of EBNA-1, EBNA3s and LMP-1 is induced and is later followed by the expression of EBERs and LMP-2 (Alfieri *et al.*, 1991). Transcription of nuclear protein is initiated at RNA polymerase II-dependent promoters in the *Bam* H1 C (Cp) and *Bam* H1 W (Wp) regions of the viral genome (Rogers *et al.*, 1992) (Figure 1.3). EBNA2 and EBNA-LP are initially transcribed from the very strong promoter Wp, which is present in multiple copies in the major internal repeat. Once immortalization is established, Wp activity declines and transcription of the EBNA genes switches to using the Cp promoter (Woisetschlaeger *et al.*, 1990). All EBNA coding mRNAs are derived from the same transcriptional unit by alternative splicing and alternative polyadenylation (Figure 1.3). The coding exons for most of the EBNAs are towards the 3' end of the mRNAs and are preceded by the highly spliced leader exons, which are encoded within the major internal repeat of the genome (Farrell, 1995).



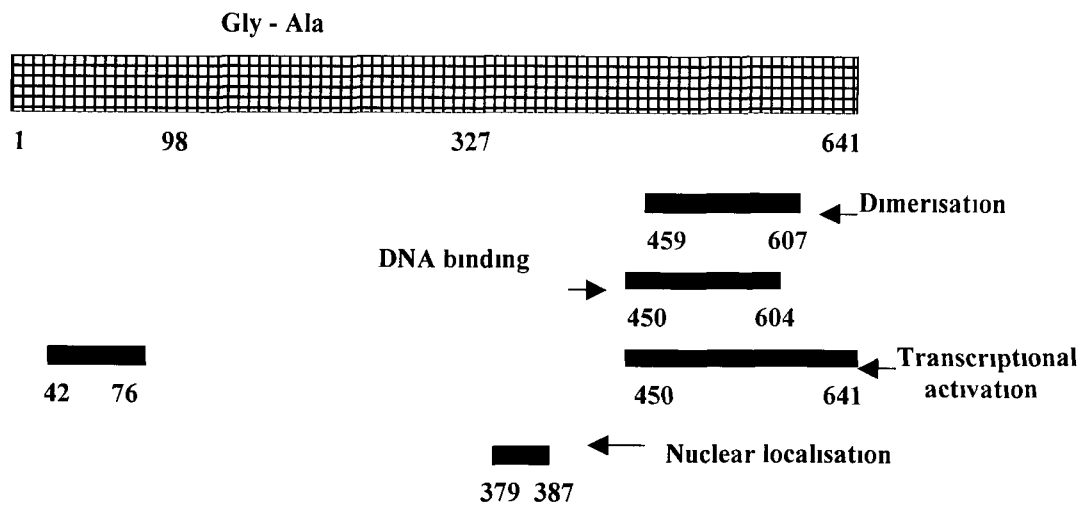
**Figure 1.3** A simplified outline of the splicing of the EBV nuclear antigen coding mRNAs. Transcription initiation is shown to arise from the Cp promoter. The EBNA mRNAs all derive from the same transcription unit by alternative splicing and alternative polyadenylation.

### 1.6.1 EBNA1

EBV nuclear antigen 1 is the only EBV latent gene that is detectable in all EBV infected cells. The 73 kDa protein consists of a short amino-terminal region, a 20-40 kDa glycine-alanine repetitive sequence flanked by arginine-rich sequences and a highly

charged acidic carboxy terminal sequence (Hennessy and Kieff, 1983) (figure 1 4) The primary role of EBNA-1 is in the maintenance of viral replication During latent infection of human host cells, EBV genomes are maintained as double-stranded DNA episomes that replicate once every cell cycle (Adams, 1987, Yates and Guan, 1991) The carboxy terminus of EBNA1 determines its nuclear localisation by interacting with a specific protein that is homogeneously distributed on chromosomes (Harris *et al* , 1985, Petti *et al* , 1990) This property is likely to be important for segregation of episomes into progeny nuclei during mitosis EBNA1 is the only EBNA that continues to be made during lytic infection (IARC Monograph, 1997)

EBNA1 is a sequence-specific DNA binding protein that binds as a dimer to the sequence TGGATAGCATATGCTATCCA present in the latent cycle origin of replication *ori P* (Ring, 1994) Regions of the protein important for DNA binding and transactivation of *ori P* are located in the carboxy-terminal third of the protein (Ambinder *et al* , 1991) (Figure 1 4) Furthermore, *ori P* acts as an EBNA1-dependent enhancer and plays a crucial role in the regulation of viral transcription from both the C and the LMP1 promoter in growth-transformed cells (Sugden and Warren, 1989, Gahn and Sugden 1995)



**Figure 1 4 Functional domains of EBV nuclear antigen 1 (EBNA1)** The Gly-Ala box is a repetitive region composed entirely of glycine and alanine, it varies in length between viral strains *Adapted from Farrell, 1995*

The promoter from which the EBNA1 gene is transcribed differs between cell types. In EBV transformed LCLs, all the EBNA genes are derived from a highly spliced transcript that is generated by transcription from the C or W promoters located on the *Bam* H1 C and W fragments respectively (Middleton *et al.*, 1991). In the absence of a stimulus to induce virus replication, RNA transcripts from latently infected early passage type-I BL cells, is initiated from a promoter located in the *Bam* H I-Q fragment (Nonkwelo *et al.*, 1995). This promoter is designated Qp. Following the switch from latent to lytic infection, EBNA1 transcription is controlled by Fp upstream of Q (Lear *et al.*, 1992).

EBNA1 can bind RNA *in vitro* through arginine/glycine motifs (Snudden *et al.*, 1994). EBNA1 also activates expression of the lymphoid recombinase genes (RAGs) through an as yet unidentified mechanism (Srinivas and Sixbey, 1995). Activation of the RAGs could promote chromosomal rearrangements and translocations and possibly also facilitate viral integration. Expression of EBNA1 in EBV negative cell lines has no obvious effect upon cellular growth characteristics. However, the expression of EBNA1 in the B cells of transgenic mice has been shown to be associated with the development of lymphocytic lymphoma and leukemia suggesting that EBNA1 predisposes the mouse lymphocytes to oncogenic change (Wilson and Levine, 1992).

### 1.6.2 EBNA2

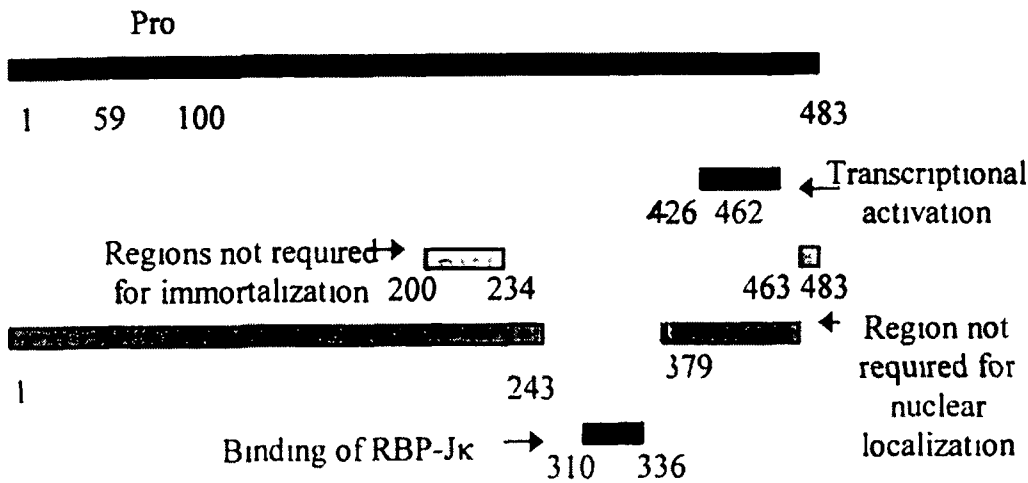
The EBV nuclear antigen 2 (EBNA2) is one of the first EBV-encoded proteins expressed after primary infection of B-lymphocytes. EBNA2 differs extensively between type-1 and type-2 EBV isolates (Aitken *et al.*, 1994) and is the primary determinant of the biological difference that enables the type-1 strains to transform B lymphocytes with greater efficiency (Rickinson *et al.*, 1987). In nuclear fractions, EBNA2 is associated with nucleoplasmic chromatin and nuclear matrix fraction (Petti *et al.*, 1993). EBNA2 is an 83 kDa protein containing a polyproline region, a glycine arginine repeat and a highly acidic carboxy terminus and is phosphorylated on serine and threonine residues (Dambaugh *et al.*, 1984). EBNA2 is a trans-activator of cellular and viral gene expression including *CD21*, *CD23*, the *c-fgr* oncogene, *cyclin D2*, *TNF- $\alpha$*  and viral genes including

*LMP1* and *LMP2* (Knutson, 1990; Abbot *et al.*, 1990; Ghosh and Keiff, 1990; Peng and Lundgren, 1992; Wang *et al.*, 1987; 1990a;1990b; Tsang *et al.*, 1991; Fahraeus *et al.*, 1993; Spender *et al.*, 2001). EBNA2 has also been shown to play an important role in the maintenance of proliferation of LCLs and this effect is mediated by its ability to regulate the expression of the cell cycle-related proteins cyclin D2, cdk 4 (drive cell cycle progression in early G1) and c-Myc (Kempkes *et al.*, 1995; Zimmer-Strobl *et al.*, 1996, Kaiser *et al.*, 1999). However, in the context of BL cells, expression of EBNA2 exerts growth-inhibitory effects most likely due to a downregulation of c-Myc expression (Jochner *et al.*, 1996).

Three regions within EBNA-2 have been identified which appear to be stringently required for transformation and the *trans*-activating activity of EBNA2 and these are located between amino acid residues 95 and 110, 280 and 337 and 425 and 462 (Figure 1.5). The exact role of the 95-110 region is unclear, the 425-462 region is essential due to its acidic *trans*-activating properties and the 280-337 region mediates the interaction with sequence-specific DNA binding proteins that bring EBNA2 to its responsive elements as EBNA2 does not interact directly with DNA (IARC Monographs, 1997). Many of the promoters activated by EBNA2 share the core sequence GTGGGAA (Waltzer *et al.*, 1994, Tun *et al.*, 1994). Through studies of its responsive elements a 28 amino acid polypeptide corresponding to residues 310-336 of EBNA2 (Figure 1.5) was used to purify a nuclear protein p63. The sequence of p63 revealed it to be the previously characterised recombination signal sequence binding protein RBP-J $\kappa$  or CBF-1, a widely expressed and highly conserved protein that acts as a key adapter for transcription of regulatory factors of cellular genes. CBF-1 acts as a transcription repressor and repression is exerted in part by tethering a histone deacetylase (HDAC) corepressor complex to the promoter (Henkel *et al.*, 1999; Hsieh and Hayward, 1995; Waltzer *et al.*, 1995; Kao *et al.*, 1998). Repression is believed to be a consequence of histone deacetylation which leads to chromatin remodeling and loss of transcription factor access to the nucleosome-associated promoter sequences. EBNA2 activates transcription by binding to the repression domain of CBF1 to relieve repression and bringing a transcriptional activation/acidic domain to the promoter (Hsieh *et al.*, 1995). The acidic

domain of EBNA2 interacts with the basal transcriptional machinery including TFIIB, TAF-40 and RPA70 (Tong *et al* , 1995a, 1995b) Recently, interaction with both CBF-1 and SKIP (Ski-interacting protein) were shown to be required for transactivation by EBNA-2 (Zhou *et al* , 2000)

**Fig 1 5 Functional domains of EBV nuclear antigen 2 (EBNA2)** The pro box is a region composed



entirely of proline, it varies in length between viral strains Adapted from Farrell 1995

EBNA2 regulates expression of the EBV latency Cp promoter that drives expression of the EBNA family of nuclear proteins in type III latency and also contributes to the positive regulation of the *LMP2A*, *LMP2B*, and *LMP1* promoters (Jin and Speck, 1992, Sung *et al* , 1991, Tsang *et al* , 1991, Zimber-Strobl *et al* , 1993) Targeting through Pu 1 (Sp1) has been described for the *LMP1* promoter (Johanssen *et al* , 1995, Laux *et al* , 1994), while CBF-1 has been identified as the targeting partner for the viral Cp, *LMP2A*, and divergent *LMP1* and *LMP2B* promoters (Grossman *et al* , 1994, Henkel *et al* , 1994, Waltzer *et al* , 1995, Zimber-Strobl *et al* , 1994) EBNA2 regulates its own expression, and deletion of the region containing the CBF1 binding site from Cp biases promoter usage toward Wp (Yoo *et al* , 1997) Although the regulation of many of the EBNA-2-target genes such as those encoding CD23, interleukins and beta interferon have been shown to be mediated by CBF1 (Kanda *et al* , 1999, Krauer *et al* , 1998, Ling *et al* , 1994; Wang *et al* , 1991), there are some responsive genes, such as the *cyclin D2* gene

(Kempkes *et al.*, 1995; Sinclair *et al.*, 1994) which lack CBF-1 binding sites in their promoter regions (Spender *et al.*, 2001). These genes may be activated as part of a downstream response cascade, or additional EBNA2 targeting mechanisms may exist.

The EBNA2-responsive elements in the promoters of several EBNA-2-regulated genes have a large complex structure and although the CBF-1 binding sites are essential, optimal activation by EBNA2 also requires cooperation with other transcription factors. The Cp EBNA2-responsive element contains a CBF2 binding site adjacent to the CBF1 binding site, and the CBF2 site contributes to EBNA2 responsiveness (Fuentes-Panana and Ling, 1998; Jin and Speck, 1992, Ling *et al.*, 1993a, Robertson *et al.*, 1995). The LMP1 promoter is complexly regulated with both CBF1 and PU.1 targeting sites and multiple transcription factor binding sites that enhance responsiveness (Johannsen *et al.*, 1995; Laux *et al.*, 1994, Sjoblom *et al.*, 1998).

The effects of EBNA-2 on CBF-1-mediated transactivation of its target genes can be modulated by EBV latent proteins including EBNA3s and the *BamHI*-A rightward transcripts (BARTs) (Waltzer *et al.*, 1996, Smith *et al.*, 2000; Zhang *et al.*, 2001). EBNA-3A and EBNA-3C have been reported to stably interact with RBP-Jk (Bain *et al.*, 1996; Robertson *et al.*, 1995; Zhao *et al.*, 1996) and repress EBNA-2-activated transcription by destabilising the binding of RBP-Jk to DNA (Johannsen *et al.*, 1996; Waltzer *et al.*, 1996). One of the BART family transcripts contains an open reading frame, RPMS1, that encodes a nuclear protein called RPMS. RPMS interacts with both CBF-1 and components of the CBF1-associated corepressor complex to negatively regulate the activity of EBNA2 (Zhang *et al.*, 2001; Smith *et al.*, 2000).

#### **1.6.2.1 EBNA2 and the Notch signaling pathway**

CBF-1/RBP-Jk is highly conserved in evolution from nematodes to humans (Artavanis-Tsakonas, 1995). CBF-1 acts downstream of the receptor Notch. Activation of the Notch receptor by binding of its ligand Delta, Jagged or Serrate (in *Drosophila*) leads to proteolytic cleavage of the receptor at the inner side of the membrane (Schroeter *et al.*,

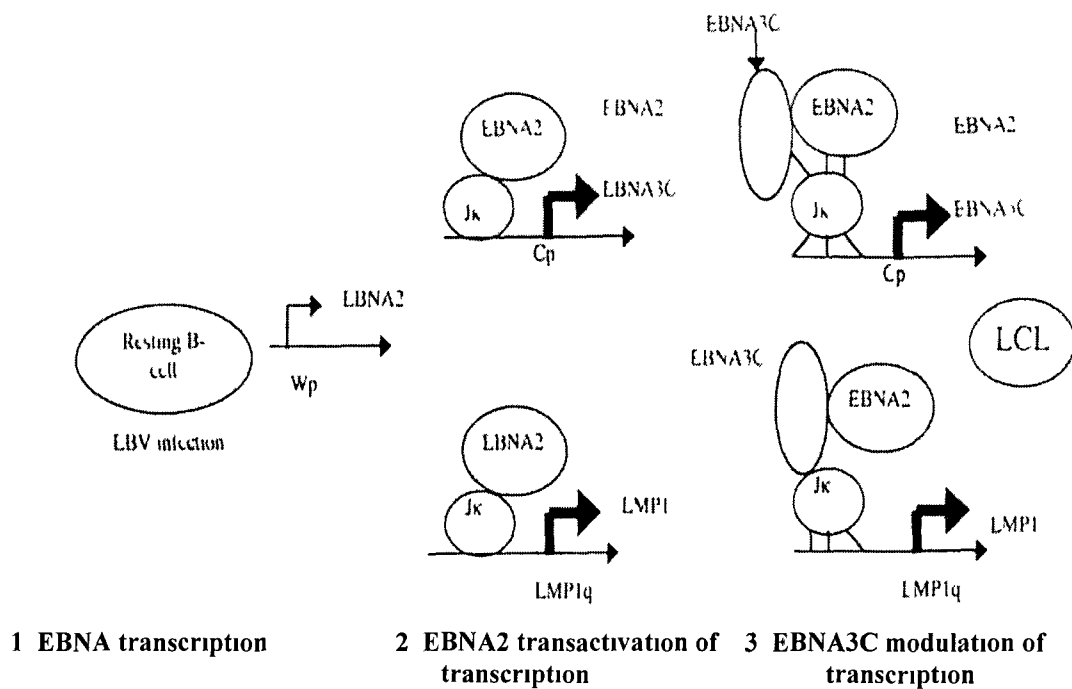
1998) The intracellular domain (Notch-IC) is then translocated to the nucleus where it activates genes by interacting with CBF-1 (Struhl and Adachi, 1998, Lecourtois and Schweisguth, 1995) Four Notch receptors have been cloned in humans and mice Notch1 (Tan1), Notch2, Notch3 and Notch4 (int3) In vertebrates, the Notch signal transduction pathway has an essential function during embryogenesis and is involved in the differentiation processes of neuronal precursors, myoblasts and Malpighian tubules (Artavanis-Tsakonas *et al* , 1995, Kopan *et al* , 1994, Nye *et al* , 1994) Notch signalling is also involved in the renewal and differentiation of hematopoietic cells For example, Notch influences the choice between CD4 and CD8 as well as the choice between the alpha-beta versus gamma-delta T-cell lineage (Robey *et al* , 1996, Washburn *et al* , 1997) Also, constitutive Notch activation is a characteristic feature of a subset of T-cell leukemias and lymphomas in humans (Ellisen *et al* , 1991) EBNA-2 has been regarded as a functional homolog of the activated Notch protein by virtue of the association of both of these proteins with the CBF-1 repression domain to relieve repression and consequently stimulate activation of genes via an endogenous transactivation domain (Hsieh *et al* , 1996, 1997) Consequently, both proteins show a certain degree of overlap in modulation of expression of genes including *CD21*, *Igμ* , *LMP-1* and *LMP-2A* (Strobl *et al* , 2000, Hofelmayer *et al* , 1999, Sakai *et al* , 1998, Strobl *et al* , 1997) These observations have raised the question as to whether Notch may play a physiological role during B-cell activation CD23, whose expression is upregulated by EBNA-2 in BL cells, is not activated by murine Notch1- IC, suggesting that the functions encompassed by EBNA-2 may extend beyond that of Notch-IC Activated Notch 1 can also transiently substitute for EBNA2 in the maintenance of proliferation of LMP-1-expressing immortalised B cells (Hofelmayer *et al* , 2001) However, in this study it was shown that, unlike EBNA2, murine Notch1-IC is unable to upregulate the expression of *c-myc* in these cells The commonality of the EBNA2 and NotchIC interactions with CBF1 suggests that the early steps in EBV immortalization of B cells may mimic an aspect of Notch signaling Furthermore, the similarity in their functions may provide insights into the role of EBNA-2 in tumours with which it is known to be associated such as smooth muscle tumours (Lee *et al* , 1995) In this regard it is interesting to note that both EBNA-

2 and Notch-IC have been shown to block muscle cell differentiation (Sakai *et al* , 1998, Hofelmayr *et al* , 1999)

### 1.6.3 EBNA3A, 3B, 3C.

EBNA3A, 3B and 3C are encoded by 3 genes located in tandem in the EBV genome. Each is encoded by a short and a long exon (Hennessy *et al* , 1985, Kallin *et al* , 1986, Pettì and Kieff, 1988, Rickstein *et al* , 1988). The mRNAs that encode these proteins are the least abundant EBNA mRNAs, with only a few molecules occurring in each latently infected cell. The proteins are located in large nuclear clumps in the nuclear matrix, chromatin and nucleoplasmic fractions but not in the nucleolus (Pettì *et al* , 1990). Type-1 and 2 strains of EBV exhibit 84%, 80% and 72% amino acid identity in their EBNA3A, 3B and 3C sequences respectively (Sample and Kieff, 1990). EBNA3A and 3C are required for B-cell transformation whereas EBNA3B is not (Tomkinson and Kieff, 1992a, Tomkinson *et al* , 1993). EBNA3A, 3B and 3C proteins have been shown to inhibit the transcriptional activation of several EBNA2-responsive promoters (such as TP1 and LMP1 promoters) by destabilizing RBP-J $\kappa$  and RBP-J $\kappa$  complexes from binding to their cognate RBP-J $\kappa$  binding sites (Le Roux *et al* , 1994) (Figure 1.6). EBNA3 proteins are thus believed to counter balance and finely tune the action of EBNA2 (Waltzer *et al* , 1996, Robertson *et al* , 1995, 1996, Zhao *et al* , 1996). Also, by inhibiting EBNA2-mediated transactivation of the Cp promoter, a feedback loop of inhibition by EBNA 3 proteins of their own synthesis is created (Waltzer *et al* , 1996, Robertson *et al* , 1995) (Figure 1.6).

There is increasing evidence that EBNA3C, like EBNA2, functions as a *trans*-activator of both cellular and viral genes. Transfection of an EBNA3C expression construct into an EBV negative BL cell has been shown to result in the upregulation of the EBV receptor CD21 (Wang *et al* , 1990). Furthermore, expression of EBNA3C in the Raji cell line (in which the EBV genome is deleted for most of the EBNA3C open reading frame) induces an up-regulation of LMP1 and the cellular proteins CD23 and vimentin (Allday *et al* , 1993, Ring, 1994).



**Figure 1 6 A schematic model of the mechanism by which EBNA3C counteracts EBNA2-mediated activation** EBNA3C destabilises the interaction of RBP-J $\kappa$  and RBP-J $\kappa$ /EBNA2 complexes binding DNA  
*Adapted from Robertson et al , 1995*

Recently, EBNA3C but not EBNA3A or EBNA3B was demonstrated to possess transactivation properties independent of CBF-1 (Zhao and Sample, 2000) In this study EBNA3C was shown to activate the LMP-1 promoter in conjunction with EBNA2, and this effect was mediated by a Sp1-1/Sp1-B binding site in the promoter Like EBNA2, EBNA3C could also bind directly to Sp1-1 and Sp1-B *in vitro* Binding to the Sp1-binding site by both EBNA2 and EBNA3C via the Sp1 proteins is thought to form a stable complex that can effectively bring about transcription from the LMP-1 promoter

#### 1.6.4 EBNA LP

EBNA leader protein (EBNA LP) is so designated because it is encoded by the 5' leader sequence of bicistronic mRNAs specifying the other EBNAs. The translation initiation codon for EBNA LP is created by a splicing event that occurs near the 5' end of the message (Sample *et al* , 1986, Speck *et al* , 1986, Rogers *et al* , 1990). The EBNA-LP protein is composed of amino-terminal repetitive segments followed by a unique carboxy-terminal sequence. EBNA-LP is frequently observed as a ladder of proteins in gel electrophoresis which differ in the number of amino-terminal repeats due to the fact that the repetitive part of the protein is derived from exons in the major internal repeat of the virus, which varies in copy number in any EBV population (Hammerschmidt and Sugden, 1989). The protein is strongly associated with the nuclear matrix (Petti *et al* , 1990, Jiang *et al* , 1991). Association of EBNA LP with pRb and p53 has been suggested based on *in vitro* biochemical interaction and colocalisation of EBNA LP with pRb in cells, the *in vivo* relevance of this is unknown (Jiang *et al* , 1991, Szekely *et al* , 1993). EBNA LP localises to PML (promyelocytic leukemia) nuclear bodies and associates with hsp (heat-shock protein)-70 (Szekely *et al* , 1996, Kitay and Rowe, 1996). Transient transfection of EBNA-LP and EBNA2 into primary B-lymphocytes co-stimulated with gp350 demonstrated that the two proteins co-operate in the induction of G<sub>0</sub> to G<sub>1</sub> transition as marked by an increase of *cyclin D2* transcription, however, the mechanism of action remains unclear (Sinclair *et al* , 1994). EBNA-LP stimulates EBNA-2-mediated activation of the LMP-1 promoter and of the LMP1/LMP2B bidirectional transcriptional regulatory element (Harada and Kieff, 1997). EBNA-LP alone exerts a negative effect. Like EBNA2, EBNA LP can also bind CBF-1. However, coactivation by EBNA-LP requires only the EBNA-2 acidic domain to be localised near a promoter as evidenced by transient transfection studies with a fusion protein of the EBNA2 acidic domain and Gal4 DNA binding domain and a reporter plasmid having Gal4 DNA binding sites upstream of a minimal promoter (Harada and Kieff, 1997). The coactivation function of EBNA LP was shown in this study to reside in the repeating domains while the carboxy terminal unique domain has a regulatory role on this coactivation function. The acidic domain of EBNA2 associates with the nuclear protein p100 and can interact with basal and activated

transcription factors (Tong *et al.*, 1995a; 1995b). EBNA1P is thought to act by enhancing these interactions of the EBNA2 acidic domain which compensates for the relative weakness of the acidic domain in transactivation.

### 1.6.5 LMP1

Latent membrane protein 1 (LMP1) mRNA is the second abundant viral transcript in latently infected cells (Fennewald *et al.*, 1984; Sample and Kieff, 1990). The product, encoded by three exons, is an integral membrane protein consisting of a short 23 amino acid N-terminal cytoplasmic domain, six hydrophobic transmembrane domains separated by short reverse turns and a 200 aminoacid carboxy-terminal cytoplasmic domain (Figure 1.7). LMP1 forms patches in the cell membrane, to which the intermediate filament protein vimentin colocalizes, however, patch formation does not require vimentin or other EBV proteins (Liebowitz *et al.*, 1986; Moorthy and Thorley-Lawson, 1990, 1993a). LMP1 oligomerises in the plasma membrane in the absence of ligand binding and oligomerisation is crucial to its activity (Floettmann and Rowe, 1997; Gires *et al.*, 1997). LMP1 is palmitoylated and the transmembrane domains facilitate its association with lipid rafts (Higuchi *et al.*, 2001). *LMP1* is the only known EBV gene that can independently transform rodent fibroblasts and render them tumorigenic (Wang *et al.*, 1985). Transgenic mice carrying an Ig heavy chain promoter/enhancer-driven LMP-1 gene develop lymphomas with high incidence *in vivo* (Kulwichit *et al.*, 1998), demonstrating that LMP1 alone is a transforming gene even in situations lacking an EBV background. LMP1 is highly toxic if expressed at high levels (Hammerschmidt *et al.*, 1989).

At least half of the LMP1 protein expressed is associated with the cytoskeleton as defined by resistance to extraction with non-ionic detergents and co-localization with vimentin. In various cell types, nascent, non-ionic detergent-soluble LMP1 has a half-life of less than 2 hours and is converted to an insoluble, cytoskeleton associated form, with a half-life of 2-15h, that is phosphorylated on serine and threonine residues in the carboxy terminal domain (Baichwal and Sugden, 1987; Martin and Sugden, 1991; Mann and

Thorley-Lawson, 1987, Moorthy and Thorley-Lawson, 1990, 1993a) Phosphorylation appears to be important for the function of LMP1. In LCLs, LMP1 is made at a steady rate and within 30 minutes of being made localises to the plasma membrane in the phosphorylated form (Baichwal and Sugden, 1987, Martin and Sugden, 1991, Mann and Thorley-Lawson, 1987, Moorthy and Thorley-Lawson, 1993). This fraction of LMP1 is associated with the cytoskeleton and forms patches in the membrane (Liebowitz *et al* , 1986, Mann and Thorley-Lawson, 1987). LMP1 is then turned over and inactivated by a specific cleavage with a half-life of less than 2 hours (Moorthy and Thorley-Lawson, 1990, 1993a). LMP1 migrates on SDS-PAGE between 58 and 63 kDa depending on the EBV strain (Liebowitz *et al* , 1986, Thorley-Lawson and Israelsohn, 1987). An amino-terminal truncated form of LMP-1 is expressed during lytic infection and migrates with a molecular weight of 45 kDa in SDS -PAGE (Baichwal and Sugden, 1997, Hudson *et al* , 1985). The lytic form of LMP-1, lyLMP-1, which is also found associated with the EBV virion can negatively modulate full-length LMP-1 function but not by affecting LMP1 oligomerisation (Erickson and Martin, 2000).

LMP1 also alters the growth of EBV-negative BL lines when expressed stably or by transient transfection at an appropriate level. In fact, LMP1 induces many of the changes usually associated with EBV infection of primary B-lymphocytes or with antigen activation of primary B-lymphocytes, including cell clumping, increased cell size, increased villous projections and increased vimentin expression (Gregory, 1995). Furthermore, such LMP-1 expressing cells exhibit increased cell surface expression of CD23, CD39, CD40, CD44, LFA-1, ICAM-1 and LFA-3 and class II major histocompatibility complex (MHC) and decreased expression of CD10 (Wang *et al* , 1988b, Birkenbach *et al* , 1989, Wang *et al* , 1990a, Liebowitz *et al* , 1992, Peng and Lundgren, 1992, Zhang *et al* , 1994a, Kieff 1996). LMP1 was also shown to increase certain cytokines with B-cell promoting activity, such as IL-10 (Nakagomi *et al* , 1994). Another important biological consequence of LMP-1 expression is its ability to protect B-lymphocytes from apoptosis by inducing the expression of anti-apoptotic proteins including Bcl-2, Mcl-1 and A20 (Henderson *et al* , 1991, Rowe *et al* , 1994, Wang *et al* , 1996, Martin *et al* , 1993, Laherty *et al* , 1992). Raised steady-state levels of intracellular

calcium, but not inositol triphosphate, have been reported in LMP-1 transfectants of a B lymphoma cell line and LMP-1 has also been shown to induce a calcium/calmodulin-dependent kinase in the same line (Wang *et al.*, 1988b; Mosialos *et al.*, 1994). LMP-1 has also been shown to upregulate the expression of matrix metalloproteinase 9 (MMP-9) and CD44, two proteins which are associated with increased invasiveness/ metastasis of tumours (Yoshizaki *et al.*, 1998; Gregory, 1995; Sneath and Mangham, 1998).

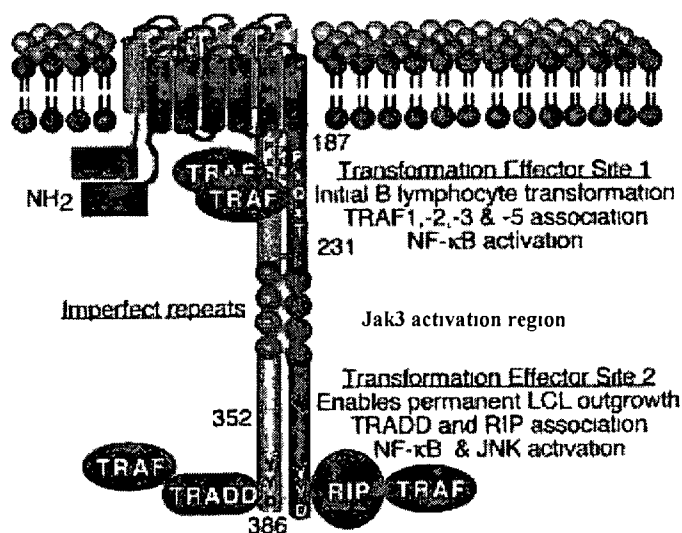
Constitutive LMP1 expression in EBV-immortalised B cells carrying a conditional (estrogen-regulatable) EBNA-2 gene has provided evidence that LMP1 in the absence of functional EBNA2 was incapable of sustaining B-cell proliferation (at levels of expression similar to that observed in LCLs) but induced a phenotype consistent with prolonged cell viability (Zimber-Strobl *et al.*, 1996). Proliferation of these transfectants resumed only in the presence of functional EBNA-2. Studies involving conditional expression of LMP-1 in EBV-immortalised B cells (expressing all of the other latent genes) have revealed that LMP-1 expression was required for continuous proliferation. In the absence of LMP-1 expression, these cells remained in a resting state (Kilger *et al.*, 1998). Although expression of LMP-1 as the sole EBV gene can induce DNA synthesis in primary B-cells (Peng and Lundgren, 1992, Zimber-Strobl *et al.*, 1996), the inability to sustain proliferation was shown to be due to the inability of the cells to progress through S phase (Zimber-Strobl *et al.*, 1996). In studies with established EBV-negative BL cell lines in which expression of LMP-1 was regulated by tetracycline, induction of LMP-1 expression resulted in a transient cytostatic effect due to an accumulation of cells at the G2/M phase of the cell cycle and a decrease in c-Myc expression (Floettmann *et al.*, 1996).

Transfection studies with LMP-1 deletion mutants indicate that the cytoplasmic amino terminus is not responsible for the activating effects, the transmembrane domains are critical, due to their importance for LMP1 aggregation in the plasma membrane, and the carboxy-terminal domain is essential (Wang *et al.*, 1988b; Martin and Sugden, 1991; Moorthy and Thorley-Lawson, 1993a; 1993b). As expected, EBV recombinants lacking LMP1 were unable to induce growth transformation of primary B cells (Kaye *et al.*,

1993). Further experiments with EBV recombinants carrying deletion mutants of LMP-1 have shown that the transmembrane domain and the membrane proximal carboxy-terminal region are the principal LMP-1 components that mediate primary B-lymphocyte growth transformation while the distal region (last 155 amino acids) of the carboxy terminal region is critical for LCL outgrowth (Izumi *et al.*, 1994; 1997; Izumi and Kieff, 1997; Kaye *et al.*, 1993; 1995; 1999).

Genetic and biochemical data indicate that LMP-1 is equivalent to a constitutively activated receptor of the tumour necrosis Factor receptor (TNFR) family requiring oligomerisation for its activity in the absence of ligand (Floettmann and Rowe, 1997; Hatzivassiliou *et al.*, 1998; Izumi and Kieff, 1997; Kilger *et al.*, 1998; Mosialos *et al.*, 1995; Gires *et al.*, 1997). Members of the TNFR superfamily including TNFR, IL-1R, CD40, CD30, LMP-1 and lymphotoxin- $\beta$  receptor exert a number of effects including regulation of apoptosis and stress responses via signaling pathways that involve recruitment of adapter proteins called TNF receptor associated factors or TRAFs (Arch *et al.*, 1998; Baker and Reddy, 1996). Mutational analysis has identified two functional domains in the carboxy terminus of LMP-1: CTAR (carboxy-terminal activation region)-1 or TES (Transformation effector site)-1 (residues 187-231) and CTAR-2/TES-2 (residues 351-386) (Huen *et al.*, 1995; Mitchell and Sugden, 1995) (Figure 1.7). CTAR-1 engages the tumour necrosis factor receptor family-associated factors (TRAFs) through a TRAF interaction domain located within this region (Kaye *et al.*, 1996; Devergne *et al.*, 1996; 1998; Eliopoulos *et al.*, 1997; Miller *et al.*, 1997; 1998; Brodeur *et al.*, 1997; Sandberg *et al.*, 1997). In EBV-transformed B cells or transiently transfected BL cells, TRAF-1, -2, -3, and -5 are associated with LMP-1 with engagement of TRAF-2 occurring largely via TRAF1 as TRAF1-TRAF2 heterodimers. However, in epithelial cells, very little TRAF-1 is expressed and only TRAF-2, -3 and -5 are significantly complexed with LMP-1. Critical to the association of TRAFs with LMP1 is a  $P^{204}xQ^{206}xT^{208}$  motif located within CTAR-1. Unlike CTAR-1, CTAR-2 does not directly interact with TRAFs. However, LMP-1 associates with the tumour necrosis factor receptor adaptor protein TRADD (Izumi and Kieff, 1997) as well as receptor interacting protein RIP (Izumi *et al.*, 1999) and it is via these adaptor proteins that

CTAR-2 is thought to recruit TRAF proteins. In contrast to TNFR1, LMP1 does not associate with the death domain (carboxy-terminus) of TRADD but with the N-terminus (Kieser *et al* , 1999)

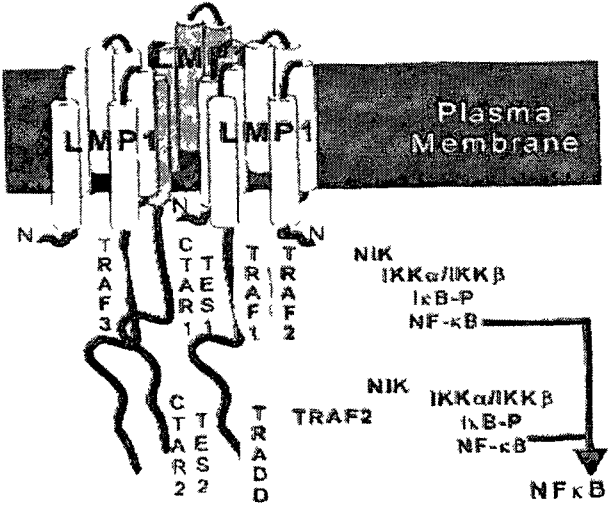


**Figure 1 7 Schematic diagram of LMP-1** LMP1 consists of a 24-aminoacid N-terminal cytoplasmic domain, six transmembrane domains and a 200 amino acid C-terminal cytoplasmic tail. LMP-1 constitutively aggregates in the plasma membrane and associates with TRAFs via the  $P^{204}XQ^{206}XT^{208}$  motif in TES (Transformation Effector Site)-1 or CTAR (Carboxy terminal activation region)-1 (residues 187 – 231) and with TRADD and RIP via the  $Y^{384}Y^{385}D^{386}$  motif in TES-2 or CTAR-2 (residues 352 – 386). The region between aminoacids 275 and 307 of LMP-1 is responsible for activation of JAK3. Adapted from Izumi *et al* , 1999

One of the first signaling functions assigned to LMP-1 is activation of the transcription factor, NF-κB and this function could be demonstrated in both B and T-lymphocytes and epithelial cells (Hammaraskjold and Simurda, 1992, Huen *et al* , 1995, Mitchell and Sugden, 1995). With the discovery that LMP-1 associates with the TRAF signaling molecules and exhibits similar functions to members of the TNF-superfamily, deeper insight into the mechanism of activation of NF-κB by this EBV protein have been realised. Thus, CTAR1 was demonstrated to mediate 20-40% of the total activation of NF-κB by LMP-1 while CTAR-2 makes a greater contribution (60-80%) in this effect in different cell types. Furthermore, TRAF-2 has been shown to be an important mediator of NF-κB activation by both domains. TRAF-3 on the other hand has been shown to act

as a negative modulator of NF- $\kappa$ B activation by LMP-1 by displacing TRAF-1 and TRAF-2 (Kaye *et al* , 1996, Devergne *et al* , 1996) Although the association of CTAR-2 with TRADD is required for NF- $\kappa$ B activation by this region, the association with RIP is not (Izumi *et al* , 1999) The residues between positions 384 to 386 (YYD) of LMP-1 are critical to the association of TRADD and RIP to CTAR2 as well as NF- $\kappa$ B activation and LCL outgrowth (Izumi *et al* , 1997, 1999) Constitutive association of TRAFs and TRADD with the carboxy-terminal region of LMP-1 and NF- $\kappa$ B activation is dependent on ligand-independent aggregation of LMP-1 (Gires *et al* , 1997, Devergne *et al* , 1996, Izumi and Kieff, 1997) TRAF3 has been shown to mediate the effect of LMP-1 on growth inhibition of epithelial cells (Eliopoulos *et al* , 1996) and is implicated in association of LMP1 with the cytoskeleton (Higuchi *et al* , 2001)

The pathway downstream of TRAF-binding to CTAR-1 and TRADD binding to CTAR-2 leading to NF- $\kappa$ B activation has been partially deciphered and is similar to the TNFR signaling pathway (Sylla *et al* , 1998) (Figure 1 8) Aggregation of TRAF-2 recruited to activated LMP-1 somehow leads to activation of the NF- $\kappa$ B-inducing kinase (NIK) which in turn activates IKK (I $\kappa$ B $\alpha$ -kinase)-  $\alpha$  and - $\beta$  IKK $\alpha$  and IKK $\beta$  then phosphorylate I $\kappa$ B $\alpha$  which is then ubiquitinated and degraded releasing active NF- $\kappa$ B The NIK-IKK $\alpha$  -IKK - $\beta$  association is thought to occur in a complex separate but neighbouring to that of LMP-1-TRAF-TRADD (Sylla *et al* , 1998)



**Figure 1 8 Schematic diagram of aggregated LMP1 in the cell plasma membrane showing the pathway involved in activation of NF- $\kappa$ B downstream of TRAF2** TRAF1, TRAF2 and TRAF3 constitutively associate with the LMP-1-proximal NF- $\kappa$ B-activating region, CTAR-1 TRADD constitutively associates with the LMP-1 distal and major NF- $\kappa$ B-activating region, CTAR2 Aggregation of TRAF2 most likely activates NIK which in turn phosphorylates and activates IKK $\alpha$  and IKK $\beta$  IKK $\alpha$  and IKK $\beta$  then phosphorylate I $\kappa$ B $\alpha$ , leading to its ubiquitination and degradation and NF- $\kappa$ B activation  
*Adapted from Sylla et al , 1998*

CTAR-2 but not CTAR-1 is also responsible for the activation of the JNK (c-jun N-terminal kinase)-1 pathway by LMP-1 which leads to activation of the c-Jun-N-terminus component of the transcription factor AP-1 (Kieser *et al* , 1997, 1999, Eliopoulos and Young, 1998) While SEK (stress enhanced kinase)-1 has been shown to mediate activation of JNK1 by LMP-1 (Kieser *et al* , 1997, Eliopoulos and Young, 1998), the components of the pathway upstream of SEK1 are unknown and does not include MEK kinase (a mitogen activated protein kinase kinase kinase) (Kieser *et al* , 1997) However, there is controversy over the involvement of TRADD/TRAF2 in the signaling pathway from the CTAR2 domain of LMP1 to JNK1 Thus, while Eliopoulos *et al* (1999), have demonstrated the involvement of TRADD/TRAF2 in LMP1-mediated signaling to the JNK pathway, the study of Kieser *et al* (1999) demonstrates otherwise It is likely that this discrepancy is due to the differences in the methodology used (different TRAF2 dominant negative mutants and usage of a TRADD mutant in one case and not in the other) The activation of AP-1 has been shown to also be required for the effect of LMP-1 expression on upregulating MMP-9 (Takeshita *et al* , 1999) LMP-1 can also activate the p38 MAPK (mitogen-activated protein kinase) pathway and both CTAR-1 and CTAR-2 are responsible for mediating this effect in a TRAF2-dependent manner (Eliopoulos *et al* , 1999) This pathway is responsible for the upregulation of IL-6 and IL-8 production by LMP-1 in epithelial cells A third functional domain of LMP-1, CTAR3, has recently been identified which localises to a region of the carboxy terminus of LMP-1 (aa 275-307) between CTAR-1 and CTAR-2 (Figure 1 7) This domain is responsible for the interaction of LMP-1 with Janus kinase (JAK)-3 which in turn activates STAT (signal transducers and activators of transcription) proteins (Gires *et al* , 1999) However,

activation of JAK 3 may also require interaction between CTAR1 or CTAR2 and CTAR3 (Brennan *et al* , 2001) Activation of the p21-Rho-like GTP-ase cdc-42 in fibroblasts has been identified as yet another signaling function of LMP-1, however, this function maps to the transmembrane domains of LMP-1 and unlike the TNFR1 signaling pathway is not responsible for mediating LMP-1 signaling to JNK (Gires *et al* , 1999) LMP-1 has also been shown to mediate activation of a Ras-MAPK-dependent pathway and this activity is required for cellular transformation (Roberts and Cooper, 1998)

There appear to be cell-type-specific differences in the effects of induction of LMP-1 For instance, upregulation of Bcl-2 expression by LMP-1 appears to be a B-cell dependent effect and that too in a Bcl-2-negative context (Henderson *et al* , 1991, Martin *et al* , 1993) Thus, whilst LMP-1 has been shown to upregulate Bcl-2 expression in EBV-negative BL lines which express low to negligible levels of Bcl-2 protein, such an effect is not evident in epithelial cells Also, the observation that EBV infection or LMP-1 expression in peripheral B lymphocytes (which constitutively express Bcl-2) does not lead to significant upregulation of Bcl-2 has led to the suggestion that the function of LMP-1 in this cellular context may be to maintain rather than induce Bcl-2 levels (Martin *et al* , 1993) LMP-1 has also been shown to upregulate IL-6 and IL-8 expression in epithelial cells in an NF- $\kappa$ B- and p38MAPK- dependent manner (Ehopoulos *et al* , 1997, 1999) Expression of LMP-1 has been shown to block morphological differentiation of epithelial cells suggesting that the expression of this EBV protein may participate in the development of EBV-associated epithelial malignancy such as NPC via a mechanism different from that observed in B-cell or fibroblast transformation, that of deregulation of morphological differentiation (Dawson *et al* , 1990) LMP1 can also induce expression of the epidermal growth factor receptor (EGFR) in epithelial cells and although this function maps to CTAR1 it is independent of NF- $\kappa$ B activation (Miller *et al* , 1995) Also, the ability of LMP-1 to upregulate the expression of proteins CD44 and MMP-9 suggests that this EBV protein may not only be involved in transformation but also promoting tumour spread (Gregory, 1995, Yoshizaki *et al* , 1998)

Transcription of LMP1 can be regulated by EBV gene products including EBNA2, EBNA1 and BARFO (Harada and Kieff, 1997; Kusano and Raab-Traub, 2001) through elements present within its promoter region and each of these may serve as an alternative route to inducing LMP1 expression. Thus, many of the effects of these gene products may be indirect and mediated by regulation of the expression of LMP1.

#### 1.6.6 LMP2A AND 2B

The genes encoding LMP2A and 2B have also been named terminal protein 1 and terminal protein 2 (TP1, TP2), because they are transcribed across the terminal repeat sequences of the linear viral genome of which are fused together upon infection to generate the intracellular episomal form of the viral genome (Laux *et al.*, 1988; Sample *et al.*, 1989). The two messages consist of different 5' exons and eight common exons and are predicted to encode nearly identical proteins differing only in the length of their hydrophilic amino termini (Ring, 1994). Transcription of the LMP2A starts 3 kb. downstream of the LMP1 transcription start site (Laux *et al.*, 1988; Sample *et al.*, 1989). The LMP2B and LMP1 promoters form bi-directional transcription units containing a common EBNA2 responsive element, while a separate EBNA2 response element regulates LMP2A transcription (Zimber-Strobl, *et al.*, 1993). LMP2A and 2B are 54 and 40 kDa in size respectively. Both proteins are predicted to encode twelve highly hydrophobic membrane spanning domains and are localized to patches in the plasma membrane of infected cells, in close association with LMP1 (Longnecker and Kieff, 1990). LMP2A and 2B are not required for EBV mediated immortalization, but LMP 2A appears to be important for maintaining viral latency (Longnecker *et al.*, 1992; Brielmeir *et al.*, 1996; Freuhling *et al.*, 1998, Longnecker and Miller, 1996). The LMP2 proteins are phosphorylated on serine, threonine and tyrosine residues (Longnecker *et al.*, 1991) and have been shown to interact with *src*-family tyrosine kinases in EBV-infected B cells (Burkhardt *et al.*, 1992). However, in epithelial cells, LMP2A does not appear to be phosphorylated by *src* kinases but by Csk (Scholle *et al.*, 1999). The LMP2s are also associated with another stably phosphorylated tyrosine kinase *sky*. These interactions suggest that the LMP2s play a role in transmembrane signal transduction (Kieff, 1996).

LMP2A has been shown to inhibit anti-immunoglobulin-mediated  $\text{Ca}^{2+}$  mobilization, PLC $\gamma$ 2 activation and anti-immunoglobulin-induced reactivation of the lytic cycle, which can be bypassed by TPA and calcium ionophores (Miller *et al* , 1994, 1995a) These data are consistent with a model in which LMP2A sequesters the B-cell receptor-associated tyrosine kinase, blocking its autophosphorylation and downstream signalling events (Miller *et al* , 1995a) LMP2A and LMP2B singly or LMP1/LMP2A coexpressed are capable of altering keratinocyte cell adhesion molecule expression (Farwell *et al* , 1999) *In vivo* studies indicate that LMP2A allows B-cell survival in the absence of normal B-cell receptor signals (Merchant *et al* , 2000) Although LMP2A can activate the protein kinase Akt in a phosphatidyl-inositol-3-kinase (PI3-K)-dependent pathway in an EBV-negative BL line, this well known regulator of cell survival in B lymphocytes does not appear to mediate the cell survival effect of LMP 2A (Swart *et al* , 2000) The activation of the PI3-kinase-Akt pathway by LMP2A has been demonstrated to contribute to the ability of this latent protein to transform epithelial cells and inhibit differentiation (Scholle *et al* , 2000)

#### **1.6.7 EBV ENCODED RNAS (EBERS)**

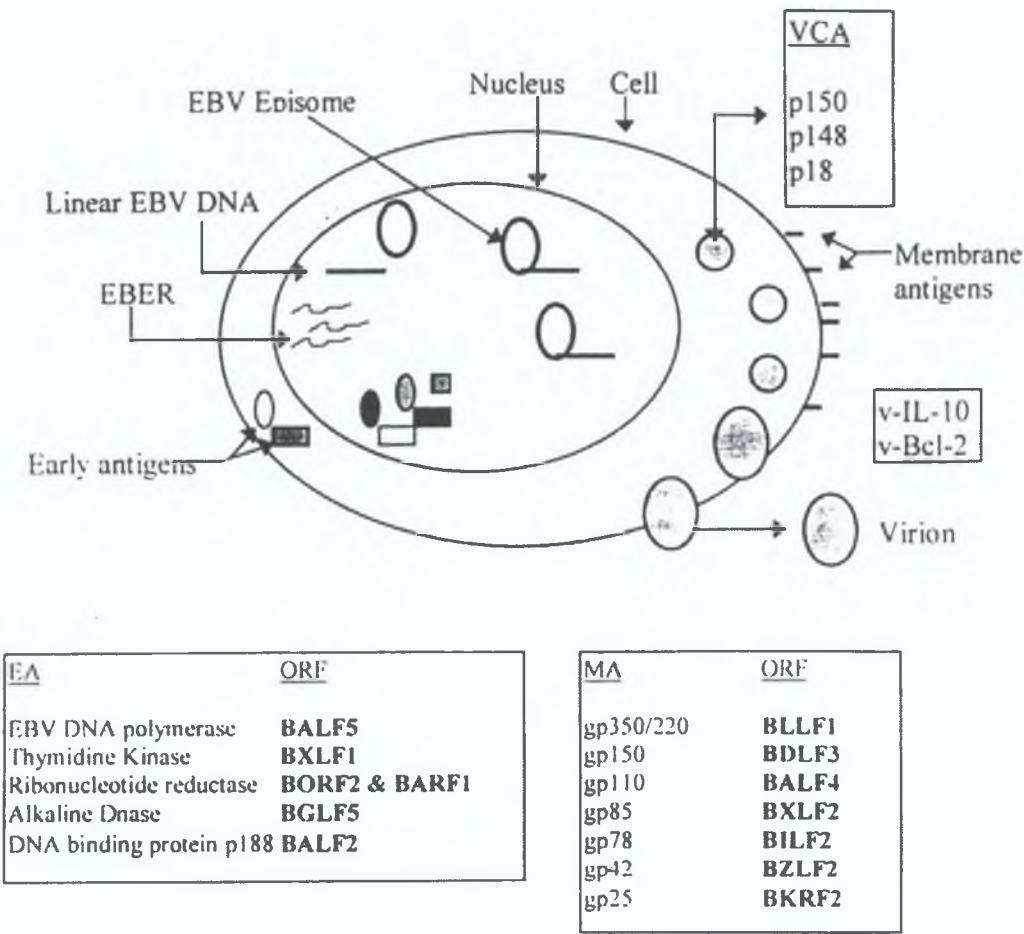
The two EBV-encoded, small nonpolyadenylated RNAs, EBER1 and EBER2, are by far the most abundant EBV RNAs in latently infected cells (Kieff, 1996) They are usually transcribed by RNA polymerase III although polymerase II may also be involved Most EBERs are located in the nucleus and are associated at the 3' terminus with the cellular La antigen (Howe and Steitz, 1986, Howe and Shu, 1989) EBER 1 and 2 have extensive sequence similarity to adenovirus VA1 and VA2 and cell U6 small RNAs, both of which form similar secondary structures and complex with La protein (Rosa *et al* , 1981, Ghckman *et al* , 1988) Stable expression of EBERs in the EBV-negative BL cell line, Akata, at levels comparable to those in EBV-positive cells, was found to confer enhanced tumorigenic potential (Komano *et al* , 1999, Ruf *et al* , 2000) The function of the EBERs is unclear but by analogy to the VA and U6 RNAs two alternative roles have been proposed for the EBERs In adenovirus infection VA1 RNA acts in the cytoplasm to directly inhibit activation of an interferon-induced protein kinase, which blocks

transcription by phosphorylating the protein-synthesis initiator factor eIF-2 $\alpha$ . EBER1 and 2 can partially complement the replication of an adenovirus with null mutation in VAI and VAII, but their effect on eIF-2 $\alpha$  kinase activity is significantly less and they are not found in the cytoplasm (Kieff, 1996). Both of the proposed functions of EBERs are somewhat incompatible with the observation that their expression is delayed until after EBNA and LMP gene expression and initiation of DNA synthesis (Alfieri *et al*, 1991). Nevertheless, the earlier events in primary B-cell infection are sensitive to interferon (IFN) and EBERs may play a role in blocking eIF-2 kinase (Thorley-Lawson, 1980, 1981).

## 1.7 GENES OF THE LYTIC VIRAL CYCLE

Only a small fraction of latently infected B-lymphocytes spontaneously enter the productive cycle and in these cells the viral DNA is amplified several hundred fold by a lytic origin of DNA replication, *ori Lyt* (Hammerschmidt and Sugden, 1988). EBV lytic infection is usually induced *in vitro* by chemicals (Luka *et al*, 1979, Saemundsen *et al*, 1980). Phorbol esters are among the most reproducible and most broadly applicable inducers, their effect is probably mediated by protein kinase C activation of Jun-Fos interactions with AP-1 sites upstream of the immediate early virus genes (Farell *et al*, 1983, 1989, Farell 1992, Laux *et al*, 1988). The Akata cell line which carries an LMP2A-deleted virus can be induced by cross-linking of surface immunoglobulins (sIg) to the extent that more than 50% of the cells enter the lytic cycle (Takada, 1984, Takada and Ono, 1989). A second approach to investigating viral replication is to induce the lytic cycle by superinfection of Raji cells with defective EBV from the P3HR-1 cell line (Mueller-Lantzsch *et al*, 1980). Raji is an EBV-positive BL cell with an unusually high EBV episome copy number. This cell line is defective for DNA replication and late gene expression and infection is thus tightly latent (Polack *et al*, 1984). Defective virions from P3HR-1 contain rearranged DNA molecules in which the intermediate early trans-activator of lytic cycle are expressed after superinfection (Cho *et al*, 1984, Miller *et al*, 1984). Studies with such cell lines has allowed the division of EBV replicative proteins into early antigens (EA), membrane antigens (MA) and virus capsid antigens (VCA)

(Figure 1.9). Early antigens are further subdivided into EA-D (diffuse) and EA-R (restricted) due to a different sensitivity to methanol fixation (Henle *et al.*, 1971a; 1971b). After induction, cells that have become permissive to viral replication undergo cytopathic changes characteristic of herpesviruses, including migration of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (IARC Monograph, 1997).



**Figure 1.9** 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.

Virus gene expression follows a temporal and sequential order (Farrell, 1992; Takada and Ono, 1989). Some virus genes are expressed independently of new protein synthesis; early after induction and are classified as immediate early genes. Early lytic virus genes

are expressed slightly later and their expression is not affected by inhibition of viral DNA synthesis (Kieff, 1996).

### 1.7.1 Immediate early genes

After P3HR-1 superinfection of Raji or sIg cross-linking of Akata cells in the presence of protein synthesis inhibitors, three leftward mRNAs are transcribed. The BZLF1, BRLF1 and BL'LF4 encoded proteins are potent transactivators of early EBV lytic gene expression (Takada and Ono, 1989; Marschall *et al.*, 1991; Kieff, 1996). BZLF1's functional and physical interaction with NF $\kappa$ B is an important regulator of LMP1-mediated effects in latent infection. BZLF1 can also downregulate the EBNA Cp promoter perhaps facilitating the transition from latent to lytic infection (Kenney *et al.*, 1989; Sinclair *et al.*, 1992).

### 1.7.2 Early genes

The early genes are expressed when lytic cycle is induced in the presence of inhibitors of DNA synthesis. By this criterion at least 30 EBV mRNAs are early gene products (Hummel and Kieff, 1982; Baer *et al.*, 1984). Two very abundant early proteins have been mapped to specific DNA sequences. The BALF2 protein is homologous to a HSV DNA binding protein and is important in DNA replication (Hummel and Kieff, 1982; Kieff, 1996). The BHRF1 protein which is expressed in moderate abundance, has extensive collinear homology with *bcl-2* (Pearson *et al.*, 1983; Austin *et al.*, 1988). BHRF1 can protect EBV negative BL cells from apoptosis (McCarthy *et al.*, 1996). However, EBV recombinants lacking the BHRF1 ORF are fully capable of initiating and maintain cell growth transformation and they can also enter the lytic cycle and produce virus (Lee and Yates, 1992; Marchini *et al.*, 1991). Several of the early genes are linked to DNA replication as indicated in Figure 1.9. 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 of cellular factors in regulating the productive cycle, at least in certain cell types (Marschall *et al.*, 1991).

### 1.7.3 LATE GENES

The late genes code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress (IARC Monograph, 1997). Among the non-glycoproteins the major nucleocapsid protein is encoded by BCLF1, BNRF1 encodes the major external nonglycoprotein of the virion and BXRFL is likely to encode a basic core protein. The BFRF3 ORF encodes the tegument protein, (Figure 1.1) and VCA p18, which is strongly immunogenic in humans (Kieff, 1996). The genes encoding the EBV glycoproteins are illustrated in bold figure 1.9. The late BCRF1 gene which is located in the middle of the EBNA regulatory domain between *ori-P* and Cp, is a close homolog of the human IL-10 gene, with nearly 90% collinear identity in amino-acid sequence (Moore *et al* , 1990, Vieira *et al* , 1991, Toutou *et al* , 1996). BCRF1 has most of the activity of human IL-10, including negative regulation of macrophages and NK cell functions and inhibition of IFN  $\gamma$  production. Thus, virally expressed IL-10 may have a local effect on these responses to reactivate infection (IARC Monograph, 1997).

## 1.8 ROLE OF APOPTOSIS IN EBV BIOLOGY

While the process of apoptosis is a genetically controlled pre-programmed event of cell death that eliminates cells during development when they have become redundant, it also functions as an emergency response to various agents including radiation damage, 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 severely reduce or eliminate spread of progeny virus in the host (Shen and Shen, 1995). Thus, many viruses have evolved strategies to evade or delay early apoptosis to allow persistence in the host and production of high yields of progeny virus. Within the last few years, both the biochemical basis of apoptosis and its regulation by some viral products have become clearer. For example, part of the apoptotic program includes the induction of cellular endonucleases which could target replicating viral DNA and prevent virus production at a very early stage. In order to combat such host cell

responses to infection many viruses have evolved genes that either themselves encode proteins or effect the expression of host cell apoptosis-related proteins that efficiently suppress the apoptotic program. In addition, many viruses are now known to induce apoptosis actively at late stages of infection. This process may represent a final and important step in the spread of progeny to neighbouring cells while also evading host immune inflammatory responses and protecting progeny virus from host enzymes and antibodies. Such virally-induced apoptosis may also contribute to some clinical manifestations and cytotoxicity associated with many human diseases of viral origin (Shen and Shenk, 1995).

EBV is unique in that it uses both pro- and anti-apoptotic properties to its advantage in its life cycle. This virus is able to effectively utilise these properties by either regulating the host cell apoptotic death machinery or encoding gene products that function similarly to anti-apoptotic proteins of the host cell. In the life cycle of EBV, protection against apoptosis serves two purposes. Firstly, as a mechanism that ensures its persistence in the host in the memory B-cell compartment by mimicking the normal process of B-lymphocyte selection in germinal centers. Secondly, the expression of particular virally encoded proteins in the lytic phase delays/suppresses cell death until sufficient progeny virions are made.

### **1.8.1 EBV induces the expression of anti-apoptotic proteins**

EBV-negative BL cells and type I BL cell lines phenotypically resemble germinal center B-lymphocytes (Gregory, 1995). This resemblance includes their ability to undergo apoptosis in the face of diverse stimuli including serum deprivation and calcium ionophore. On the other hand, type III EBV-positive BL lines that express the entire complement of latent proteins, display increased resistance to apoptosis (Gregory *et al.*, 1991). These observations prompted further studies aimed at understanding the molecular basis of the protection from apoptosis conferred by EBV to the BL cell lines. Thus, it was found that the apoptosis-sensitive EBV-positive group I BL lines (and primary BL cells) also have little or no detectable Bcl-2 expression, while the apoptosis resistant

group III BL lines were found to be Bcl-2-positive (Henderson *et al* , 1991) Identification of a candidate latent protein encoded by the EBV genome that may be responsible for the upregulation of Bcl-2 observed in the group III BL cells was achieved when clear correlations were found between LMP-1 expression and protection from apoptosis on the one hand and on the other hand between LMP-1 expression and upregulation of Bcl-2 expression in LMP-1- (but not EBNA-1-, EBNA-2-, EBNA-LP- or LMP-2-) transfectants derived from EBV-negative BL cell lines (Henderson *et al* , 1991) This study also reported that EBNA-2 can augment the effect of LMP-1 expression on induction of Bcl-2 in dual transfection experiments of BJAB-cells (Henderson *et al* , 1991) In another study, Martin *et al* (1993) reported the effect of ectopic expression of EBNA-2 on induction of Bcl-2 protein in transient transfection experiments of BJAB-cells, however, coexpression of the *LMP-1* and *EBNA-2* genes did not result in significantly higher levels of Bcl-2 expression than did either gene alone in these cells The induction of Bcl-2 expression by LMP-1 has been shown to be a delayed response relative to its effects on NF- $\kappa$ B activation and upregulation of several adhesion molecules Studies with cell lines in which LMP-1 is under inducible control or expressed transiently have shown that maximum upregulation of Bcl-2 occurs 48-72 h post-induction of LMP-1 (Rowe *et al* , 1994, Wang *et al* , 1996) There is also evidence that EBNA3B (EBNA4) can upregulate Bcl-2 expression in an EBV-negative cell line (Silins and Sculley, 1995)

While expression of LMP-1 can induce Bcl-2 expression in group I BL lines and protect them from apoptosis, EBV infection of primary B-cells does not significantly increase Bcl-2 levels despite the fact that LMP-1 is expressed in the infected cells (Martin *et al* , 1993) Compared to EBV-negative or group I BL lines, primary B lymphocytes express significant amount of Bcl-2 and it has therefore been suggested that induction of Bcl-2 expression by EBV (or LMP-1) may not be relevant in a Bcl-2-positive cellular context such as exists in peripheral B-lymphocytes Furthermore, it is possible that there is some cellular control on the levels of expression of Bcl-2 in peripheral B-lymphocytes, perhaps a negative feedback mechanism as occurs in the case of the A20 protein (Song *et al* , 1996) The role of LMP-1 in LCLs, therefore, may not be to induce, but rather to maintain, the high constitutive level of Bcl-2 that is found in the uninfected peripheral B-

cells. The observation that LMP-1 – mediated upregulation of Bcl-2 expression may be more relevant in a Bcl-2-negative cellular context also applies to epithelial and T cell lines since studies have shown that ectopic expression of LMP-1 in these cell lines does not further upregulate the constitutive Bcl-2 levels (Rowe *et al.*, 1994; Kawanishi, 1997).

Overexpression of Bcl-2 in group I BL cell lines following *bcl-2* gene transfer results in a change in the behaviour of the cells in apoptosis assays towards that of group III lines (Henderson *et al.*, 1991; Milner *et al.*, 1992). These *bcl-2*-transfectants display reduced apoptotic responses following serum starvation, calcium ionophore treatment or anti-smlg stimulation; the inhibition of cell death being proportional to the amount of Bcl-2 protein (Milner *et al.*, 1992). Interestingly, in a panel of group I, group I/*bcl-2* (*bcl-2* transfectants of group I cell lines), and group III sublines from the same BL parental line, it was found that, for the three different apoptosis triggers, the degree of protection imparted by the group III phenotype far outweighed that apparent from the levels of Bcl-2 protein measured in the group III cells. Since there were no gross differences observed between the distribution of Bcl-2 in the group I transfectants and the group III transfectants, these observations were interpreted to mean that either, in a group I cell, Bcl-2 function is antagonised or one or more additional Bcl-2-independent survival-pathways are operational in group III cells. In so far as the first possibility is concerned, *bcl-2* homologs, *bax* and *bcl-xS*, which can suppress Bcl-2 function have been identified (reviewed in Allday, 1996 and Korsmeyer, 1995). It is therefore conceivable that antagonism of Bcl-2 function by such homologous proteins occurs in the group I, but not in the group III environment. In so far as protein levels are concerned, there does not appear to be consistent differences in the levels of these Bcl-2 antagonists between type I and type III BL cells (Spender *et al.*, 1999). In support of the second possibility, it is plausible that additional EBV-induced apoptosis-suppressor proteins, such as the Bcl-2 homologs Bcl-xL, Mcl-1 or the A20 protein act in concert with Bcl-2 in group III cells to promote survival. In this regard, LMP-1 has been shown to upregulate the expression of several other anti-apoptotic proteins including Mcl-1 and A20 (Wang *et al.*, 1996; Laherty *et al.*, 1992), the former serving as a rapid and short-term effector of cell viability prior to the up-regulation of Bcl-2 (Wang *et al.*, 1996).

The upregulation of the anti-apoptotic zinc-finger protein, A20, by LMP-1 has been demonstrated in B- lymphocytes as well as epithelial cells and this effect is NF- $\kappa$ B dependent mapping to both CTAR1 and CTAR2 (Laherty *et al* , 1992, Fries *et al* , 1996, Miller *et al* , 1995, 1997) A20 is also constitutively expressed in EBV-immortalised B-cells (Spender *et al* , 1999) LMP-1-mediated protection of epithelial cells from p53-mediated apoptosis has been shown to involve A20 (Fries *et al* , 1996) A20 can also contribute to the antiapoptotic effect of LMP-1 expression in BL cell lines (Laherty *et al* , 1992) More recently, A20 was demonstrated to function in a negative feedback loop by inhibiting activation of NF- $\kappa$ B from both CTAR1 and CTAR2 domains of LMP-1 (Eliopoulos *et al* , 1999) This effect was mediated by the binding of A20 to TRAF2 and this effect by A20 has also been demonstrated in the case of signaling from some of the other members of the TNFR-family including CD40 which can also induce A20 expression in BL cells (Sarma *et al* , 1995)

### **1.8.2 EBV regulates components of the cell cycle machinery to protect against apoptosis**

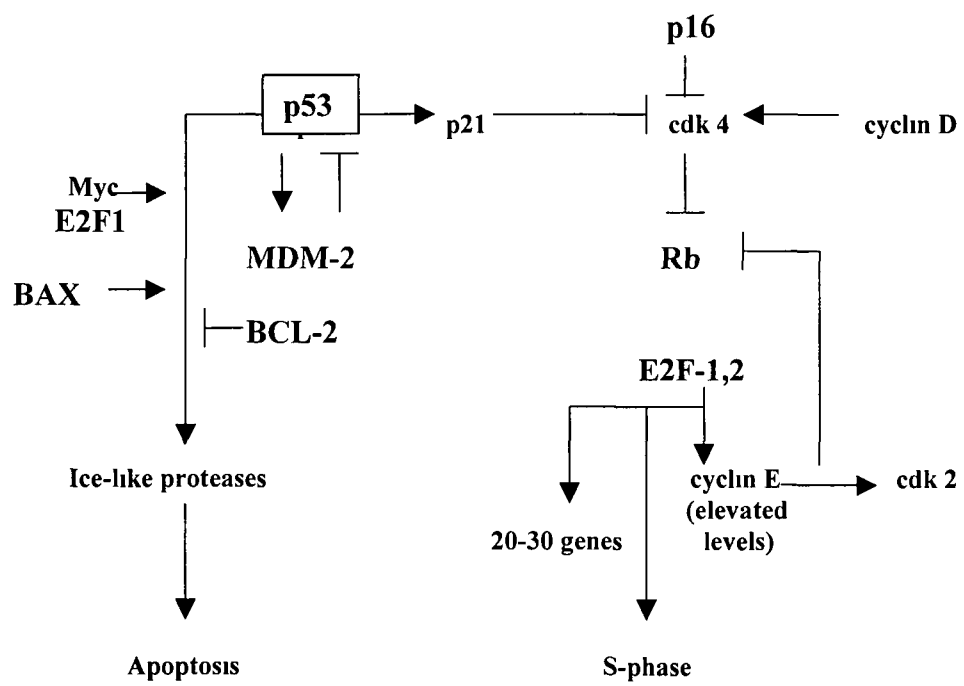
In addition to regulating apoptosis by inducing the expression of anti-apoptotic proteins, EBV and in particular LMP-1 can also control apoptosis by regulating the cell cycle, since the processes of apoptosis and cell cycle progression (proliferation) are coupled through the use and control of shared molecular machinery (Evan *et al* , 1995)

Many oncogenes that promote cell cycle progression also induce apoptosis This duality in function is exemplified by c-Myc which is a sequence-specific DNA-binding phosphoprotein that serves as a transcriptional activator and whose expression is required for passage through G1 and entry into the S-phase of the cell cycle (Gregory, 1995) In order to explain the ability of c-Myc to promote such opposed cellular functions as proliferation and death, a model has been proposed according to which c-Myc necessarily drives both processes, proliferation being favoured under appropriate conditions of availability of nutrients, space, growth and survival factors (Evan *et al* , 1995) Thus, this

model predicts that the activity of Myc in promoting proliferation is dependent on the route to its apoptosis-promoting activity being blocked. Accordingly, even normally proliferating cells may be regarded as being “primed” for apoptosis. This model can explain why c-Myc, being a potent inducer of cell death is strongly favoured during the carcinogenic process. Thus, it has been suggested that as soon as the affected cell acquires some form of apoptosis-suppressing mutation, the advantage afforded by c-Myc-induced proliferation outweighs any disadvantage conferred by c-Myc-induced apoptosis. Until that point, however, most cells with deregulated c-Myc are thought to delete themselves (by apoptosis). This theory gains support from observations made in transgenic animals expressing deregulated c-Myc. Lymphocytes from these animals were found to exhibit elevated levels of spontaneous apoptosis and an increased sensitivity to induction of apoptosis in lymphoid organs was also observed (Evan *et al*, 1995, Levine 1997). That cell death is an important restraint during carcinogenesis induced by c-Myc in the absence of an ‘anti-death factor’, is best exemplified by the occurrence in these animals of tumours displaying oncogenic synergy between *c-myc* and the anti-apoptotic gene *bcl-2* (reviewed in Allday, 1996). Further studies have shown that Bcl-2 suppresses only the apoptotic actions of c-Myc, leaving its ability to promote cell proliferation unaffected, thus leading to the suggestion that the proliferative and apoptotic arms of the c-Myc pathway must be distinct at the point at which Bcl-2 acts.

Further support for the theory that apoptosis and cell proliferation coincide comes from the observation that key tumour suppressor proteins such as pRb and p53 exert direct effects on cell viability and on cell cycle progression (reviewed in Allday, 1996, Levine, 1997, Hunter, 1993). Phosphorylation of pRb (the product of the retinoblastoma gene *rb*) inactivates the protein during late G1 at a point in the cell cycle that appears to be coincident with the late G1 restriction point (after which cells no longer require mitogenic signals to complete their cell cycle). pRb is the principal target for inactivation by several DNA tumour virus oncoproteins including Simian virus (SV) 40 large T antigen (Tag), human papilloma virus (HPV) E7 protein and adenoviral E1A. This suggests that active pRb acts as a restraint to the mitogenic actions of these virus proteins (Taya, 1997).

One of the functions of pRb is to suppress the action of the G1-specific E2F-DP transcription factors which coordinate expression of the late G1 phase and early S phase genes required for cell cycle progression. The activity of pRb is regulated by phosphorylation in the *p16-cyclinD1-cdk4-rb* pathway (Taya, 1996, Levine, 1997) (Figure 1 10). p16 is a negative regulator of cyclin D1-cdk4 and the gene is shut off in many cancer cells or mutated in others. Cyclin-D1-cdk4 complexes target pRb for cell cycle regulation by phosphorylating it. E2F-DP transcription factor complexes (E2F-1,-2 and -3 and DP-1,-2 and -3) whose activity is regulated by pRb, in turn regulate a number of genes [including those encoding cyclin E, cyclin A, dihydrofolate reductase and proliferating cell nuclear antigen (PCNA)] required to initiate or propagate the S phase of the cell cycle. Phosphorylation of pRb by cyclinD1-cdk4 releases E2F-DP proteins (E2F-DP proteins are bound by dephosphorylated pRb) from the pRb complex, relieving repression of these genes or activating their transcription.



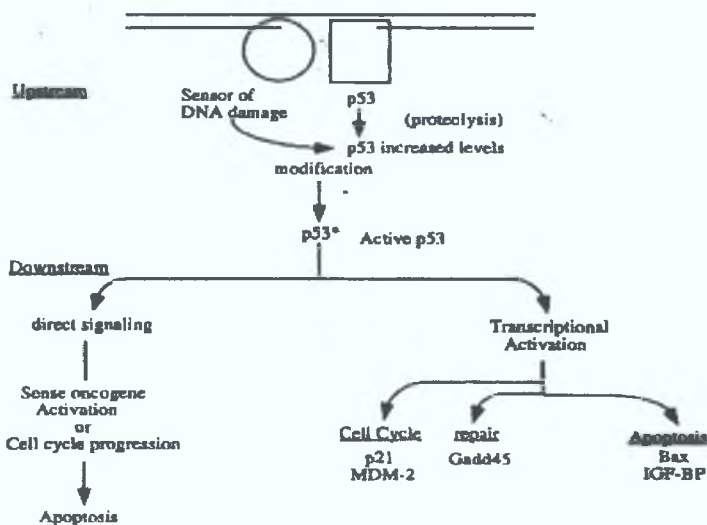
**Figure 1 10 The p53-Rb pathway** Inter-relationships among a number of oncogenes (Myc, MDM-2, BCL-2) and tumour suppressor genes (p16, Rb, p53) that regulate the G1-S phase restriction point, its relation to a DNA damage checkpoint mediated by p53, and the choice by p53 whether to initiate a G1 arrest (via p21) or apoptosis Available evidence suggests an important role for Rb and its two related gene products, p107 and p130 (along with E2F-4 and -5), in p53-mediated G1-S phase regulation Shown are the p53-MDM2 autoregulatory loop that reverses this checkpoint control and the gene products that positively or negatively act on the probability of entering apoptosis (Key - ⊥ inhibits activity , → induces activity) *Adapted from Levine, 1997*

By binding and inactivating pRb, thereby liberating active E2F, the viral oncoproteins E1A (adenovirus) and E7 (HPV) promote cell cycle progression (Levine, 1997) Consistent with this, is data showing that deregulated expression of E2F transcription factors induces both proliferation and apoptosis c-Myc can also override the growth-suppressive effects of pRb, however, there is some doubt as to whether c-Myc can interact with pRb *in vivo* Not only does a lack of functional pRb lead to apoptosis, but restoration of functional pRb to *rb*<sup>-</sup> cells directly inhibits it, under conditions where apoptosis is induced by radiation (i.e. p53-dependent) or even by dominant oncogenes This indicates that the effects of pRb must be exerted downstream of p53

p53 plays a key role in limiting the further expansion of cells containing damaged genomes and it achieves this by either enforcing cell cycle arrest or triggering apoptosis (reviewed in Levine, 1997 and Bates and Vousden, 1996) Which of these two alternate fates occurs in any particular instance appears to depend on a number of undefined factors, such as cell type, nature and intensity of insult and cytokine status The high frequency with which p53 is functionally inactivated in human tumours attests to the critical role that p53 plays in limiting neoplastic progression By transducing genome damage into growth arrest and/or apoptosis, p53 prevents the progression of potentially mutant cells together with the neoplastic risk they present Levels of p53 rapidly increase following DNA damage, mainly because the normally short-lived p53 protein is stabilised, and this appears to be an important component of the G1 arrest and apoptosis that follow DNA damage Tumour cells that have lost functional p53 exhibit resistance to induction of apoptosis by a range of genotoxic agents Moreover, because such cells

fail to arrest or die following DNA damage, they enter S phase with unrepaired DNA and sustain substantially elevated risks of further mutation.

p53 can act as a transcription factor, and most evidence suggests that it induces growth-arrest by the modulation of specific target genes (reviewed in Levine, 1997). Most prominent among these is the gene encoding the cyclin-dependent kinase (cdk) inhibitor p21/WAF-1/Cip-1, whose action arrests cells in late G1. p21 binds to a number of cyclin and cdk complexes: cyclinD1-cdk4, cyclin E-cdk2, cyclin A-cdk2 and cyclin A-cdc2 (Figures 1.10, 1.11). One molecule of p21 per complex appears to permit cdk activity, while any more inhibits kinase activity and cell cycle progression. p21 also binds to PCNA, and formation of p21-PCNA complexes blocks the role of PCNA in DNA replication (but not its role in DNA repair). Thus, p21 can act on cyclin-cdk complexes and PCNA to block DNA replication. p21-independent pathways may also contribute to p53-mediated growth arrest. In this respect, GADD45, which is encoded by a p53-responsive gene, can also bind PCNA.



**Figure 1.11 The Events in p53 activation.** DNA damage (indicated by the break in the double line at the top) is recognised by a “sensor” molecule that identifies a specific type of lesion and possibly by the p53 protein. The sensor modifies p53 (by phosphorylation) when both molecules correctly determine that there is damage. A modified p53 is more stable (increased half-life) and a steric or allosteric change in p53 permits DNA binding to a specific DNA sequence regulating several downstream genes [p21 (inhibits cdk

responsible for G1/S transition), MDM2 (negatively controls p53 expression), GADD45 (DNA replication), Bax (pro-apoptotic) and cyclin G] Two modes of signaling for cellular apoptosis are possible one requiring transcription and one involving direct signaling with no transcription of downstream genes required *Adapted from Levine, 1997*

It is less certain how p53 regulates apoptosis or what factors coordinate its actions as a cytostatic- versus a cytotoxic- agent Some evidence indicates that induction of apoptosis by p53 may not require transcriptional activation of genes, although transrepression has not been excluded (reviewed in Levine, 1997, Evan, 1995) Other data implicate p53-dependent transactivation of the *bax* gene, which encodes a member of the Bcl-2 family that can induce apoptosis It is well established that overexpression of Bcl-2 can block p53-mediated apoptosis Bax binds to Bcl-2 and antagonises its ability to block apoptosis, so a p53-dependent *bax* synthesis can tip the scales towards apoptosis A second p53-regulated gene product that could affect growth regulation is the insulin-like growth factor-binding protein-3 (IGF-BP3) IGF-BP3 blocks the IGF mitotic signaling pathway by binding to IGF and preventing its interaction with its receptor Thus, the blocking of IGF activity could enhance apoptosis or lower the mitogenic response of cells

p53 also acts to induce apoptosis in cells with oncogenic lesions such as loss of functional pRb or the expression of viral proteins such as E1A or deregulated c-Myc (reviewed by Evan *et al* , 1995) Mice in which pRb inactivation is targeted to the lens or photoreceptors of the developing eye exhibit the characteristic excessive proliferation and apoptosis which are associated with the inactivation of pRb Such apoptosis is inhibited in a *p53*-negative background and in this case, the animals develop tumours instead The expression of the adenovirus E1A protein in rat embryo fibroblasts stabilises and activates p53, the resulting cells die of apoptosis Adenoviruses normally express the E1B-55 kD protein, which binds to p53 and blocks its transcription factor activity, and the E1B-19kD protein, which acts like Bcl-2 to block apoptosis downstream of *p53* activation

EBV-negative BL cells which exhibit deregulated c-Myc expression as a result of the t (8;14) translocation proliferate rapidly in culture but readily undergo apoptosis under inappropriate conditions such as serum deprivation (Gregory, 1995). Expression of LMP-1 or EBNA2 in these cell lines has been shown to have growth inhibitory and anti-apoptotic effects which may at least in part be a consequence of downregulation of c-Myc expression as a result of downregulation of transcription of the adjacent translocated Ig- $\mu$  locus (Floettmann *et al.*, 1996; Jochner *et al.*, 1996). LMP-1 has also been demonstrated to protect Jurkat T cells from apoptosis induced by serum deprivation by downregulating the expression of c-Myc (Kawanishi, 1997).

TGF $\beta$  peptides belong to a family of structurally related molecules with a variety of biological functions including differentiation and apoptosis on a variety of cell and tissue types including human B and T lymphocytes (reviewed in Aravanitakis *et al.*, 1995). EBV-negative BL lines and EBV-positive BL lines with a group I phenotype are generally sensitive to the growth inhibitory effects of TGF- $\beta$  and this results from cells accumulating in G1 and undergoing apoptosis. However, EBV immortalised LCLs and EBV-positive BL lines that express all the latent viral proteins are resistant to the growth-inhibitory effects of TGF- $\beta$  (Altiok *et al.*, 1993; MacDonald *et al.*, 1996). Aravanitakis *et al.* (1995) were able to demonstrate that LMP-1 expression could independently reproduce the ability of EBV to confer TGF- $\beta$ -resistance on infected B-lymphocytes and group III BL lines by gene transfer into the EBV-negative BL cell line BL41. TGF- $\beta$  is known to arrest cells in the mid- to late- G1 phase of cell cycle in not only mitogen-activated human B-cells but also in non-lymphoid cell systems (reviewed in MacDonald *et al.*, 1995; Chaouchi *et al.*, 1995). This G1 arrest has been linked to an upregulation of at least three inhibitors of cyclin-dependent kinases (cdks) namely, p15<sup>INK4B</sup>, p27<sup>KIP1</sup> and p21<sup>WAF1</sup> and in some cells to a downregulation of cdk4 (reviewed in MacDonald *et al.*, 1996 and Hunter, 1993). Binding of these inhibitors to cdk inhibits the formation of cdk-cyclin complexes that are required for progression through the cell cycle. The inhibition of cyclin D function leads to a block in phosphorylation of the retinoblastoma gene product, pRb, and this in turn represses the activity of the E2F family of transcription factors, thus preventing cells from making the transition from G1- to S- phase of the cell

cycle The protection against TGF- $\beta$ -induced G1 arrest afforded by LMP-1 was shown to be related to the induction of cyclin D2 expression by LMP-1 leading to the maintenance of pRb in the hyperphosphorylated state In the early G1 phase of the cell cycle, the presence of hypophosphorylated pRb is associated with transcriptional activation of cyclin D expression in normal cells (Taya *et al* , 1997) D-type cyclins form complexes with cdk4 which then proceed to phosphorylate pRb and shut down cyclin D transcription, thus completing the feedback loop regulating progression from the G1 phase to the S phase of the cell cycle The maintenance of pRb in the hyperphosphorylated state by LMP-1-thus leads to cell cycle progression regardless of the negative growth regulatory signal imposed by TGF- $\beta$

Mutations and elevated expression of p53 are two factors that can play a role in malignant transformation or tumour cell growth of Burkitt's lymphoma Mutations in p53 have been found in approximately 33% of BL biopsies and in at least 63% of BL lines (Farrell *et al* , 1991) In 65% of BL lines studies the wild-type allele is lost and the cells express the mutant p53 allele at elevated levels In general, single nucleotide substitutions which result in aminoacid changes are found BL cells with p53 lesions have been shown to be relatively resistant to DNA-damaging drugs such as cisplatin compared to those cell lines harbouring wild-type p53 (Allday *et al* , 1995) Expression of LMP-1 can however protect against wild-type p53-mediated apoptosis and this has been demonstrated in both the BL and epithelial cell contexts based on the usage of cell lines expressing temperature-sensitive p53 In BL cells, protection by LMP-1 is most likely due to the induction of Bcl-2 expression (Okan *et al* , 1995) However, in the epithelial cells examined, protection against p53-mediated apoptosis was shown to be due to the induction of A20 expression (Fries *et al* , 1996) In both cases, protection by LMP-1 did not involve an effect on the transactivation function of p53

#### **1.8.2.1 EBV primes LCLs for apoptosis**

##### **c-Myc**

Activated peripheral blood B-cells ( IL-4 + anti-CD40 antibodies) cultured *in vitro* in the absence of serum exhibit little DNA synthesis, accumulate in the Go/G1 phase of the cell cycle and have a lower level of *c-myc* expression compared to when grown in the continuous presence of serum (reviewed in Cherney *et al.*, 1994). However, EBV-immortalised lymphoblastoid cell lines grown under autocrine growth factor-deprived conditions (cultured under low-density conditions) exhibit apoptosis by day 3 of growth (Allday, 1996). In fact, under such culture conditions, these cells continue to proliferate until apoptotic death and this change in growth properties is not accompanied by a change in the levels of c-Myc protein (or even p53 or Bcl-2). These observations led to the suggestion that deregulation of *c-myc* may mediate the apoptotic response of the growth factor-deprived LCLs. Indeed, it was demonstrated that antisense oligonucleotides to *c-myc* specifically inhibited apoptosis in the growth factor-deprived LCLs but suppressed growth of undeprived LCLs. These studies indicated that EBV latent gene expression must deregulate *c-myc* in B lymphocytes and this promotes both proliferation and apoptosis when there is a depletion of autocrine growth factors (Cherney *et al.*, 1994).

Studies based on the usage of an LCL established with a recombinant EBV conditional for the expression of EBNA-2, have shown that EBNA-2 is the likely EBV latent gene product responsible for deregulating c-Myc in LCLs. In this study by Kempkes *et al.* (1995), primary B-lymphocytes were infected with the recombinant virus such that in the presence of estrogen, growth transformation of the infected B-cells was achieved leading to the formation of estrogen-dependent LCLs expressing EBNA2 (as a fusion protein with the hormone binding domain of the estrogen receptor) in addition to the other EBV latent proteins. Removal of estrogen resulted in about half of the cells entering a quiescent non-proliferative state whereas the others underwent apoptotic death. Growth arrest occurred at both the G1 and G2 phases of the cell cycle which is indicative of a requirement of EBNA-2 function at both of these stages of the cell cycle. However, only those cells arrested in G1 could be rescued into cell cycle on induction of EBNA2, and this was shown to correlate with the induction of c-Myc and LMP-1 expression, followed by the induction of cyclin D2 (G1-specific cyclin) and cdk4 and then the phosphorylated

form of pRb. Concomitant with the appearance of the phosphorylated form of pRb, the E2F transcription factor, (which is maintained inactive by binding to the hypophosphorylated form of Rb and is required for DNA synthesis) became detectable. This study therefore demonstrated that in LCLs, functional EBNA-2 is responsible not only for the induction and maintenance of c-Myc in a deregulated state but also for proliferation. In order to investigate if this effect of EBNA-2 was due to an indirect effect on expression of LMP-1, Zimmer-Strobl *et al* (1996) have analysed the growth characteristics of primary B-cell transfectants that constitutively expressed *LMP-1* from a heterologous promoter that was carried on a plasmid vector containing a conditional (estrogen-dependent) EBNA-2 allele in addition to the other wild-type latent EBV genes (Zimmer-Strobl *et al*, 1996). In the absence of functional EBNA-2, it was found that LMP-1 was incapable of sustaining B-cell proliferation (at levels of expression similar to that observed in LCLs) but induced a phenotype consistent with prolonged cell viability. Proliferation of these transfectants resumed only in the presence of functional EBNA-2. Thus, it was demonstrated that LMP-1 is not sufficient to maintain proliferation of B-cells in the absence of EBNA-2 and that EBNA-2 acts beyond the upregulation of LMP-1 to maintain proliferation of EBV-immortalised B-cells perhaps by cooperating with other cellular and viral genes. Studies in EBV-immortalised B lymphocytes expressing a conditional LMP-1 have shown that the continuous expression of LMP-1 is essential for proliferation (Kilger *et al*, 1998).

A likely viral latent gene that may serve in the role of cooperating with EBNA-2 in initiating and maintaining B-cells in cycle is EBNA-LP. Resting B-cells, primed by CD21 cross-linking with the gp350 virus envelope protein to mimic virus infection and transfected with EBNA-2 and EBNA-LP expression vectors, enter and progress through the cell cycle and this ability of EBNA-2 and EBNA-LP is correlated with their ability to induce the synthesis of cyclin D2 (Sinclair *et al*, 1994). Expression of both EBNA-2 and EBNA-LP were required for this effect. Thus, by inducing cyclin D2 synthesis, EBNA-2 and EBNA-LP cooperate to induce a G0 to G1 transition. In this relation it is interesting to note that following EBV infection of resting primary B-cells, EBNA-2 and EBNA-LP are the first viral latent proteins to be expressed and that too in a coordinate fashion.

Studies by Kitay and Rowe have shown that EBNA-LP is phosphorylated throughout the cell cycle, but the extent of phosphorylation is increased during G2 phase and is maximal at the G2/M transition (Kitay and Rowe, 1996). Moreover, *in vitro* studies have shown that EBNA-LP can interact with p53 and pRb, two proteins known to play important roles in cell-cycle regulation. The significance of these latter observations are not known since EBNA-LP does not alter the function of these molecules when bound (Szekely *et al.*, 1993).

### p53

Investigations aimed at assessing the integrity of the p53/p21waf-1/ pRb/E2F pathway in B-cells immortalised by EBV have led to the same conclusion as that of the influence of EBV infection on *c-myc* expression in B-cells: EBV infection of B-cells primes the cells for apoptosis. Resting primary B-cells are essentially p53-negative (Allday *et al.*, 1995). Some of these cells spontaneously undergo apoptosis when placed in culture, through a p53-independent pathway. Normal peripheral B-cells treated with IL-4 and anti-CD40 are activated into proliferation and accumulate p53. EBV infection of resting B-cells similarly rescues them from spontaneous cell death and this process is also accompanied by an accumulation of p53 (Allday *et al.*, 1995). In the case of EBV-infected B-cells, accumulation of p53 requires viral gene expression (infection with UV-inactivated virus did not result in p53 accumulation) and it has been shown by Chen and Cooper (1996) that EBNA-2 and LMP-1 can independently transactivate p53. While IL-4/anti-CD40-activated B-cells respond to the DNA-damaging agent cisplatin by ceasing DNA synthesis (growth arrest), LCLs, respond with apoptosis (Allday *et al.*, 1995). Cisplatin-treatment of either IL-4/anti-CD40 activated B-cells or LCLs led to an elevation of p53 levels with similar kinetics. p53 accumulation in cisplatin-treated LCLs was shown to be due to post-translational modification leading to enhanced stability. Also, cisplatin-treatment of the LCLs did not alter Bcl-2 expression or the heterodimeric partner of Bcl-2, Bax (which is also known to be transcriptionally activated by p53 in some cell types). Ectopic expression of p53 in LCLs results in spontaneous apoptosis, indicating that the cisplatin-induced apoptosis observed in the LCLs is likely to be due to the increased accumulation of p53. Furthermore, in the latter experiment, in the cells

proceeding towards apoptosis, transcriptional activation of p21<sup>WAF-1</sup> by p53 correlated with the presence of dephosphorylated pRb. In normal fibroblasts, cisplatin-treatment correlates with the presence of hypophosphorylated pRb and G1-arrest since the cells are unable to proceed into S-phase (reviewed in Allday, 1995). However, the observation that the presence of hypophosphorylated pRb correlates with apoptosis in LCLs suggests that EBV must encode a function which enables the G1/S restriction point to be overcome and thus allow progression through the cell cycle, converting the p53 arrest response to apoptosis (Levine, 1997). Candidate mechanisms by which EBV may overcome the G1/S restriction point include deregulation of *c-myc* or utility of a virally-encoded E1A-like function which generates high levels of transcriptionally active E2F.

### 1.8.3 EBV lytic proteins encode anti-apoptotic functions

Unlike most viruses, EBV maintains a very stable persistent infection *in vivo* (Rowe *et al* , 1992). Another state in which EBV may be found is in the process of active replication and the production of progeny virions, this is the lytic cycle. Virus production is generally accepted to precede replication of viral DNA (Allday, 1996). This may be sensed by the host cell as damaged DNA and the p53 checkpoint response could then be activated. If the cells are driven to proliferate by EBV (as in type III latency) then apoptosis will be the most likely outcome. In this situation additional repressors of apoptosis can provide a selective advantage. Consistent with this model, there are at least two known virally encoded proteins, BHRF-1 and BZLF-1, that are synthesised during the lytic cycle that can provide a survival advantage to the infected cell (Allday, 1996). These lytic gene products can therefore delay apoptosis in order to maximise viral production. Expression of BHRF-1 in epithelial cells has been shown to confer enhanced resistance to cisplatin-induced apoptosis and can also inhibit differentiation (Dawson *et al* , 1990, 1995). In lymphoid cells, BZLF-1 has been shown to interact with p53 and inhibit its transactivating function, thereby providing a mechanism for preventing p53-mediated apoptosis (Zhang *et al* , 1994b). However, in epithelial cells, expression of this EBV protein was shown to result in growth arrest without inhibiting the transactivating function of p53 (Cayrol and Flemmington, 1996).

calcium, but not inositol triphosphate, have been reported in LMP-1 transfectants of a B lymphoma cell line and LMP-1 has also been shown to induce a calcium/calmodulin-dependent kinase in the same line (Wang *et al.*, 1988b; Mosialos *et al.*, 1994). LMP-1 has also been shown to upregulate the expression of matrix metalloproteinase 9 (MMP-9) and CD44, two proteins which are associated with increased invasiveness/ metastasis of tumours (Yoshizaki *et al.*, 1998; Gregory, 1995; Sneath and Mangham, 1998).

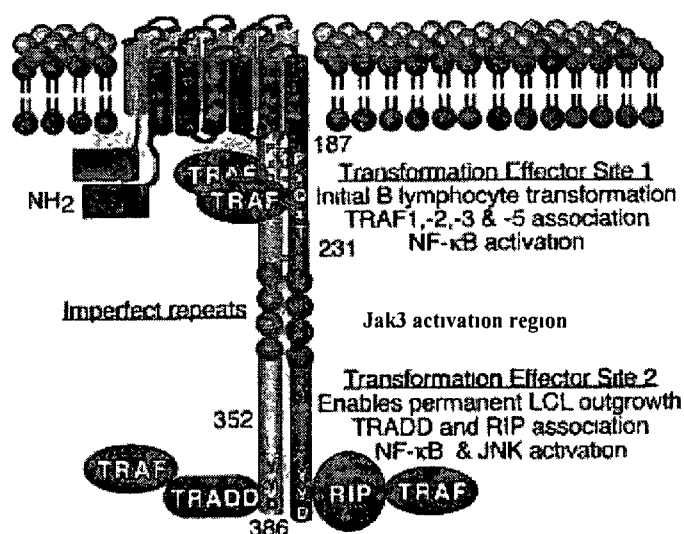
Constitutive LMP1 expression in EBV-immortalised B cells carrying a conditional (estrogen-regulatable) EBNA-2 gene has provided evidence that LMP1 in the absence of functional EBNA2 was incapable of sustaining B-cell proliferation (at levels of expression similar to that observed in LCLs) but induced a phenotype consistent with prolonged cell viability (Zimmer-Strobl *et al.*, 1996). Proliferation of these transfectants resumed only in the presence of functional EBNA-2. Studies involving conditional expression of LMP-1 in EBV-immortalised B cells (expressing all of the other latent genes) have revealed that LMP-1 expression was required for continuous proliferation. In the absence of LMP-1 expression, these cells remained in a resting state (Kilger *et al.*, 1998). Although expression of LMP-1 as the sole EBV gene can induce DNA synthesis in primary B-cells (Peng and Lundgren, 1992; Zimmer-Strobl *et al.*, 1996), the inability to sustain proliferation was shown to be due to the inability of the cells to progress through S phase (Zimmer-Strobl *et al.*, 1996). In studies with established EBV-negative BL cell lines in which expression of LMP-1 was regulated by tetracycline, induction of LMP-1 expression resulted in a transient cytostatic effect due to an accumulation of cells at the G2/M phase of the cell cycle and a decrease in c-Myc expression (Floettmann *et al.*, 1996).

Transfection studies with LMP-1 deletion mutants indicate that the cytoplasmic amino terminus is not responsible for the activating effects, the transmembrane domains are critical, due to their importance for LMP1 aggregation in the plasma membrane, and the carboxy-terminal domain is essential (Wang *et al.*, 1988b; Martin and Sugden, 1991; Moorthy and Thorley-Lawson, 1993a; 1993b). As expected, EBV recombinants lacking LMP1 were unable to induce growth transformation of primary B cells (Kaye *et al.*,

1993) Further experiments with EBV recombinants carrying deletion mutants of LMP-1 have shown that the transmembrane domain and the membrane proximal carboxy-terminal region are the principal LMP-1 components that mediate primary B-lymphocyte growth transformation while the distal region (last 155 amino acids) of the carboxy terminal region is critical for LCL outgrowth (Izumi *et al* , 1994, 1997, Izumi and Kieff, 1997, Kaye *et al* , 1993, 1995, 1999)

Genetic and biochemical data indicate that LMP-1 is equivalent to a constitutively activated receptor of the tumour necrosis Factor receptor (TNFR) family requiring oligomerisation for its activity in the absence of ligand (Floettmann and Rowe, 1997, Hatzivassiliou *et al* , 1998, Izumi and Kieff, 1997, Kilger *et al* , 1998, Mosialos *et al* , 1995, Gires *et al* , 1997) Members of the TNFR superfamily including TNFR, IL-1R, CD40, CD30, LMP-1 and lymphotoxin- $\beta$  receptor exert a number of effects including regulation of apoptosis and stress responses via signaling pathways that involve recruitment of adapter proteins called TNF receptor associated factors or TRAFs (Arch *et al* , 1998, Baker and Reddy, 1996) Mutational analysis has identified two functional domains in the carboxy terminus of LMP-1 CTAR (carboxy-terminal activation region)-1 or TES (Transformation effector site)-1 (residues 187-231) and CTAR-2/TES-2 (residues 351-386) (Huen *et al* , 1995, Mitchell and Sugden, 1995) (Figure 1 7) CTAR-1 engages the tumour necrosis factor receptor family-associated factors (TRAFs) through a TRAF interaction domain located within this region (Kaye *et al* , 1996, Devergne *et al* , 1996, 1998, Ehoupoulos *et al* , 1997, Miller *et al* , 1997, 1998, Brodeur *et al* , 1997, Sandberg *et al* , 1997) In EBV-transformed B cells or transiently transfected BL cells, TRAF-1, -2, -3, and -5 are associated with LMP-1 with engagement of TRAF-2 occurring largely via TRAF1 as TRAF1-TRAF2 heterodimers However, in epithelial cells, very little TRAF-1 is expressed and only TRAF-2, -3 and -5 are significantly complexed with LMP-1 Critical to the association of TRAFs with LMP1 is a P<sup>204</sup>XQ<sup>206</sup>XT<sup>208</sup> motif located within CTAR-1 Unlike CTAR-1, CTAR-2 does not directly interact with TRAFs However, LMP-1 associates with the tumour necrosis factor receptor adaptor protein TRADD (Izumi and Kieff, 1997) as well as receptor interacting protein RIP (Izumi *et al* , 1999) and it is via these adaptor proteins that

CTAR-2 is thought to recruit TRAF proteins. In contrast to TNFR1, LMP1 does not associate with the death domain (carboxy-terminus) of TRADD but with the N-terminus (Kieser *et al* , 1999)



**Figure 17 Schematic diagram of LMP-1** LMP1 consists of a 24-aminoacid N-terminal cytoplasmic domain, six transmembrane domains and a 200 amino acid C-terminal cytoplasmic tail. LMP-1 constitutively aggregates in the plasma membrane and associates with TRAFs via the P<sup>204</sup>XQ<sup>206</sup>XT<sup>208</sup> motif in TES (Transformation Effector Site)-1 or CTAR (Carboxy terminal activation region)-1 (residues 187 – 231) and with TRADD and RIP via the Y<sup>384</sup>Y<sup>385</sup>D<sup>386</sup> motif in TES-2 or CTAR-2 (residues 352 – 386). The region between aminoacids 275 and 307 of LMP-1 is responsible for activation of JAK3. Adapted from Izumi *et al* , 1999

One of the first signaling functions assigned to LMP-1 is activation of the transcription factor, NF-κB and this function could be demonstrated in both B and T-lymphocytes and epithelial cells (Hammaraskjold and Simurda, 1992, Huen *et al* , 1995, Mitchell and Sugden, 1995). With the discovery that LMP-1 associates with the TRAF signaling molecules and exhibits similar functions to members of the TNF-superfamily, deeper insight into the mechanism of activation of NF-κB by this EBV protein have been realised. Thus, CTAR1 was demonstrated to mediate 20-40% of the total activation of NF-κB by LMP-1 while CTAR-2 makes a greater contribution (60-80%) in this effect in different cell types. Furthermore, TRAF-2 has been shown to be an important mediator of NF-κB activation by both domains. TRAF-3 on the other hand has been shown to act



**Figure 1 8 Schematic diagram of aggregated LMP1 in the cell plasma membrane showing the pathway involved in activation of NF- $\kappa$ B downstream of TRAF2** TRAF1, TRAF2 and TRAF3 constitutively associate with the LMP-1-proximal NF- $\kappa$ B-activating region, CTAR-1 TRADD constitutively associates with the LMP-1 distal and major NF- $\kappa$ B-activating region, CTAR2 Aggregation of TRAF2 most likely activates NIK which in turn phosphorylates and activates IKK $\alpha$  and IKK $\beta$  IKK $\alpha$  and IKK $\beta$  then phosphorylate I $\kappa$ B $\alpha$ , leading to its ubiquitination and degradation and NF- $\kappa$ B activation  
*Adapted from Sylla et al , 1998*

CTAR-2 but not CTAR-1 is also responsible for the activation of the JNK (c-jun N-terminal kinase)-1 pathway by LMP-1 which leads to activation of the c-Jun-N-terminus component of the transcription factor AP-1 (Kieser *et al* , 1997, 1999, Eliopoulos and Young, 1998) While SEK (stress enhanced kinase)-1 has been shown to mediate activation of JNK1 by LMP-1 (Kieser *et al* , 1997, Eliopoulos and Young, 1998), the components of the pathway upstream of SEK1 are unknown and does not include MEK kinase (a mitogen activated protein kinase kinase kinase) (Kieser *et al* , 1997) However, there is controversy over the involvement of TRADD/TRAF2 in the signaling pathway from the CTAR2 domain of LMP1 to JNK1 Thus, while Eliopoulos *et al* (1999), have demonstrated the involvement of TRADD/TRAF2 in LMP1-mediated signaling to the JNK pathway, the study of Kieser *et al* (1999) demonstrates otherwise It is likely that this discrepancy is due to the differences in the methodology used (different TRAF2 dominant negative mutants and usage of a TRADD mutant in one case and not in the other) The activation of AP-1 has been shown to also be required for the effect of LMP-1 expression on upregulating MMP-9 (Takeshita *et al* , 1999) LMP-1 can also activate the p38 MAPK (mitogen-activated protein kinase) pathway and both CTAR-1 and CTAR-2 are responsible for mediating this effect in a TRAF2-dependent manner (Eliopoulos *et al* , 1999) This pathway is responsible for the upregulation of IL-6 and IL-8 production by LMP-1 in epithelial cells A third functional domain of LMP-1, CTAR3, has recently been identified which localises to a region of the carboxy terminus of LMP-1 (aa 275-307) between CTAR-1 and CTAR-2 (Figure 1 7) This domain is responsible for the interaction of LMP-1 with Janus kinase (JAK)-3 which in turn activates STAT (signal transducers and activators of transcription) proteins (Gires *et al* , 1999) However,

activation of JAK 3 may also require interaction between CTAR1 or CTAR2 and CTAR3 (Brennan *et al* , 2001) Activation of the p21-Rho-hke GTP-ase cdc-42 in fibroblasts has been identified as yet another signaling function of LMP-1, however, this function maps to the transmembrane domains of LMP-1 and unlike the TNFR1 signaling pathway is not responsible for mediating LMP-1 signaling to JNK (Gires *et al* , 1999) LMP-1 has also been shown to mediate activation of a Ras-MAPK-dependent pathway and this activity is required for cellular transformation (Roberts and Cooper, 1998)

There appear to be cell-type-specific differences in the effects of induction of LMP-1 For instance, upregulation of Bcl-2 expression by LMP-1 appears to be a B-cell dependent effect and that too in a Bcl-2-negative context (Henderson *et al* , 1991, Martin *et al* , 1993) Thus, whilst LMP-1 has been shown to upregulate Bcl-2 expression in EBV-negative BL lines which express low to negligible levels of Bcl-2 protein, such an effect is not evident in epithelial cells Also, the observation that EBV infection or LMP-1 expression in peripheral B lymphocytes (which constitutively express Bcl-2) does not lead to significant upregulation of Bcl-2 has led to the suggestion that the function of LMP-1 in this cellular context may be to maintain rather than induce Bcl-2 levels (Martin *et al* , 1993) LMP-1 has also been shown to upregulate IL-6 and IL-8 expression in epithelial cells in an NF- $\kappa$ B- and p38MAPK- dependent manner (Ehopoulos *et al* , 1997, 1999) Expression of LMP-1 has been shown to block morphological differentiation of epithelial cells suggesting that the expression of this EBV protein may participate in the development of EBV-associated epithelial malignancy such as NPC via a mechanism different from that observed in B-cell or fibroblast transformation, that of deregulation of morphological differentiation (Dawson *et al* , 1990) LMP1 can also induce expression of the epidermal growth factor receptor (EGFR) in epithelial cells and although this function maps to CTAR1 it is independent of NF- $\kappa$ B activation (Miller *et al* , 1995) Also, the ability of LMP-1 to upregulate the expression of proteins CD44 and MMP-9 suggests that this EBV protein may not only be involved in transformation but also promoting tumour spread (Gregory, 1995, Yoshizaki *et al* , 1998)

Transcription of LMP1 can be regulated by EBV gene products including EBNA2, EBNA1 and BARFO (Harada and Kieff, 1997; Kusano and Raab-Traub, 2001) through elements present within its promoter region and each of these may serve as an alternative route to inducing LMP1 expression. Thus, many of the effects of these gene products may be indirect and mediated by regulation of the expression of LMP1.

#### 1.6.6 LMP2A AND 2B

The genes encoding LMP2A and 2B have also been named terminal protein 1 and terminal protein 2 (TP1, TP2), because they are transcribed across the terminal repeat sequences of the linear viral genome of which are fused together upon infection to generate the intracellular episomal form of the viral genome (Laux *et al.*, 1988; Sample *et al.*, 1989). The two messages consist of different 5' exons and eight common exons and are predicted to encode nearly identical proteins differing only in the length of their hydrophilic amino termini (Ring, 1994). Transcription of the LMP2A starts 3 kb. downstream of the LMP1 transcription start site (Laux *et al.*, 1988; Sample *et al.*, 1989). The LMP2B and LMP1 promoters form bi-directional transcription units containing a common EBNA2 responsive element, while a separate EBNA2 response element regulates LMP2A transcription (Zimber-Strobl, *et al.*, 1993). LMP2A and 2B are 54 and 40 kDa in size respectively. Both proteins are predicted to encode twelve highly hydrophobic membrane spanning domains and are localized to patches in the plasma membrane of infected cells, in close association with LMP1 (Longnecker and Kieff, 1990). LMP2A and 2B are not required for EBV mediated immortalization, but LMP 2A appears to be important for maintaining viral latency (Longnecker *et al.*, 1992; Brielmeir *et al.*, 1996; Freuhling *et al.*, 1998, Longnecker and Miller, 1996). The LMP2 proteins are phosphorylated on serine, threonine and tyrosine residues (Longnecker *et al.*, 1991) and have been shown to interact with *src*-family tyrosine kinases in EBV-infected B cells (Burkhardt *et al.*, 1992). However, in epithelial cells, LMP2A does not appear to be phosphorylated by *src* kinases but by Csk (Scholle *et al.*, 1999). The LMP2s are also associated with another stably phosphorylated tyrosine kinase *sky*. These interactions suggest that the LMP2s play a role in transmembrane signal transduction (Kieff, 1996).

LMP2A has been shown to inhibit anti-immunoglobulin-mediated  $\text{Ca}^{2+}$  mobilization, PLC $\gamma$ 2 activation and anti-immunoglobulin-induced reactivation of the lytic cycle, which can be bypassed by TPA and calcium ionophores (Miller *et al* , 1994, 1995a) These data are consistent with a model in which LMP2A sequesters the B-cell receptor-associated tyrosine kinase, blocking its autophosphorylation and downstream signalling events (Miller *et al* , 1995a) LMP2A and LMP2B singly or LMP1/LMP2A coexpressed are capable of altering keratinocyte cell adhesion molecule expression (Farwell *et al* , 1999) *In vivo* studies indicate that LMP2A allows B-cell survival in the absence of normal B-cell receptor signals (Merchant *et al* , 2000) Although LMP2A can activate the protein kinase Akt in a phosphatidyl-inositol-3-kinase (PI3-K)-dependent pathway in an EBV-negative BL line, this well known regulator of cell survival in B lymphocytes does not appear to mediate the cell survival effect of LMP 2A (Swart *et al* , 2000) The activation of the PI3-kinase-Akt pathway by LMP2A has been demonstrated to contribute to the ability of this latent protein to transform epithelial cells and inhibit differentiation (Scholle *et al* , 2000)

### 1.6.7 EBV ENCODED RNAS (EBERS)

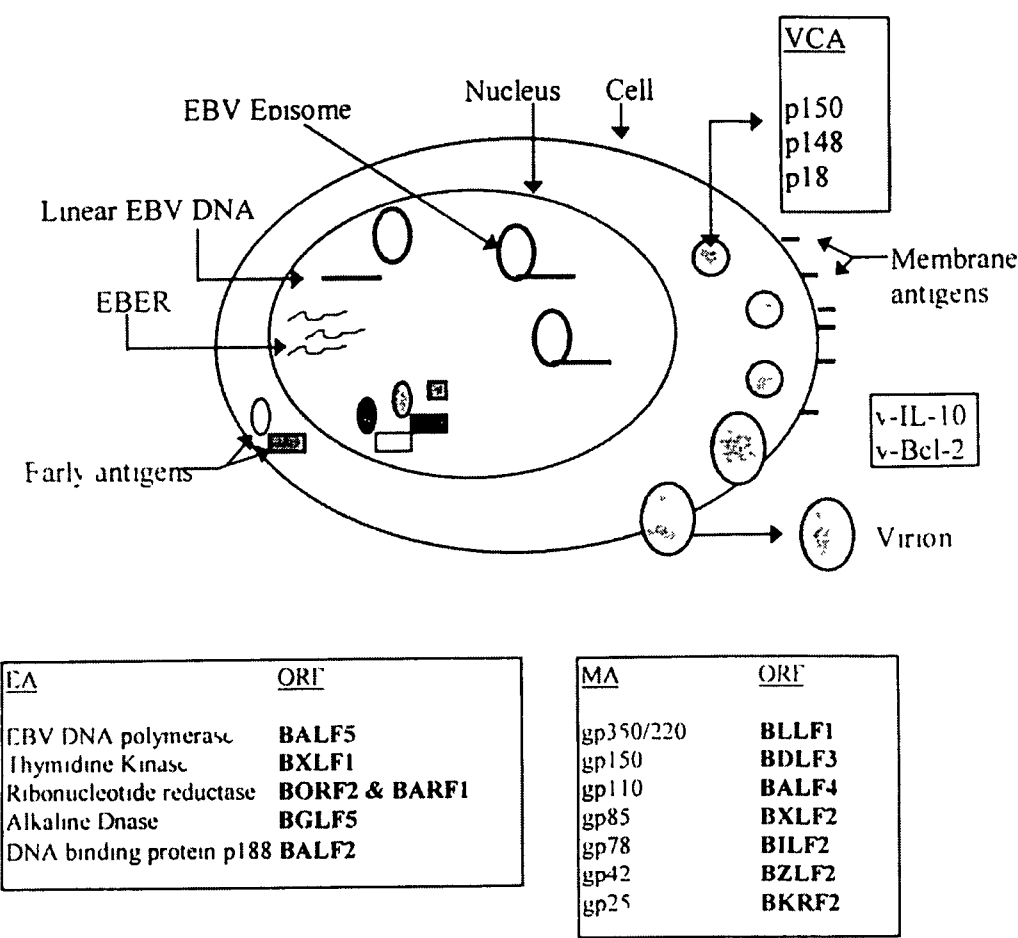
The two EBV-encoded, small nonpolyadenylated RNAs, EBER1 and EBER2, are by far the most abundant EBV RNAs in latently infected cells (Kieff, 1996) They are usually transcribed by RNA polymerase III although polymerase II may also be involved Most EBERs are located in the nucleus and are associated at the 3' terminus with the cellular La antigen (Howe and Steiz, 1986, Howe and Shu, 1989) EBER 1 and 2 have extensive sequence similarity to adenovirus VA1 and VA2 and cell U6 small RNAs, both of which form similar secondary structures and complex with La protein (Rosa *et al* , 1981, Glickman *et al* , 1988) Stable expression of EBERs in the EBV-negative BL cell line, Akata, at levels comparable to those in EBV-positive cells, was found to confer enhanced tumorigenic potential (Komano *et al* , 1999, Ruf *et al* , 2000) The function of the EBERs is unclear but by analogy to the VA and U6 RNAs two alternative roles have been proposed for the EBERs In adenovirus infection VA1 RNA acts in the cytoplasm to directly inhibit activation of an interferon-induced protein kinase, which blocks

transcription by phosphorylating the protein-synthesis initiator factor eIF-2 $\alpha$ . EBER1 and 2 can partially complement the replication of an adenovirus with null mutation in VAI and VAIL, but their effect on eIF-2 $\alpha$ , kinase activity is significantly less and they are not found in the cytoplasm (Kieff, 1996). Both of the proposed functions of EBERs are somewhat incompatible with the observation that their expression is delayed until after EBNA and LMP gene expression and initiation of DNA synthesis (Alfieri *et al.*, 1991). Nevertheless, the earlier events in primary B-cell infection are sensitive to interferon (IFN) and EBERs may play a role in blocking eIF-2 kinase (Thorley-Lawson, 1980; 1981).

### 1.7 GENES OF THE LYTIC VIRAL CYCLE

Only a small fraction of latently infected B-lymphocytes spontaneously enter the productive cycle and in these cells the viral DNA is amplified several hundred fold by a lytic origin of DNA replication, *ori Lyt* (Hammerschmidt and Sugden, 1988). EBV lytic infection is usually induced *in vitro* by chemicals (Luka *et al.*, 1979; Saemundsen *et al.*, 1980). Phorbol esters are among the most reproducible and most broadly applicable inducers, their effect is probably mediated by protein kinase C activation of Jun-Fos interactions with AP-1 sites upstream of the immediate early virus genes (Farell *et al.*, 1983; 1989; Farell 1992; Laux *et al.*, 1988). The Akata cell line which carries an LMP2A-deleted virus can be induced by cross-linking of surface immunoglobulins (sIg) to the extent that more than 50% of the cells enter the lytic cycle (Takada, 1984; Takada and Ono; 1989). A second approach to investigating viral replication is to induce the lytic cycle by superinfection of Raji cells with defective EBV from the P3HR-1 cell line (Mueller-Lantzsch *et al.*, 1980). Raji is an EBV-positive BL cell with an unusually high EBV episome copy number. This cell line is defective for DNA replication and late gene expression and infection is thus tightly latent (Polack *et al.*, 1984). Defective virions from P3HR-1 contain rearranged DNA molecules in which the intermediate early trans-activator of lytic cycle are expressed after superinfection (Cho *et al.*, 1984; Miller *et al.*, 1984). Studies with such cell lines has allowed the division of EBV replicative proteins into early antigens (EA), membrane antigens (MA) and virus capsid antigens (VCA)

(Figure 1 9) Early antigens are further subdivided into EA-D (diffuse) and EA-R (restricted) due to a different sensitivity to methanol fixation (Henle *et al* , 1971a, 1971b) After induction, cells that have become permissive to viral replication undergo cytopathic changes characteristic of herpesviruses, including migration of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (IARC Monograph, 1997)



**Figure 1 9 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

Virus gene expression follows a temporal and sequential order (Farrell, 1992, Takada and Ono, 1989) Some virus genes are expressed independently of new protein synthesis, early after induction and are classified as immediate early genes Early lytic virus genes

are expressed slightly later and their expression is not affected by inhibition of viral DNA synthesis (Kieff, 1996)

### 1.7.1 Immediate early genes

After P3HR-1 superinfection of Raji or sIg cross-linking of Akata cells in the presence of protein synthesis inhibitors, three leftward mRNAs are transcribed. The BZLF1, BRLF1 and BL'LF4 encoded proteins are potent transactivators of early EBV lytic gene expression (Takada and Ono, 1989, Marschall *et al* , 1991, Kieff, 1996). BZLF1's functional and physical interaction with NF $\kappa$ B is an important regulator of LMP1-mediated effects in latent infection. BZLF1 can also downregulate the EBNA Cp promoter perhaps facilitating the transition from latent to lytic infection (Kenney *et al* , 1989, Sinclair *et al* , 1992).

### 1.7.2 Early genes

The early genes are expressed when lytic cycle is induced in the presence of inhibitors of DNA synthesis. By this criterion at least 30 EBV mRNAs are early gene products (Hummel and Kieff, 1982, Baer *et al* , 1984). Two very abundant early proteins have been mapped to specific DNA sequences. The BALF2 protein is homologous to a HSV DNA binding protein and is important in DNA replication (Hummel and Kieff, 1982, Kieff, 1996). The BHRF1 protein which is expressed in moderate abundance, has extensive colinear homology with *bcl-2* (Pearson *et al* , 1983, Austin *et al* , 1988). BHRF1 can protect EBV negative BL cells from apoptosis (McCarthy *et al* , 1996). However, EBV recombinants lacking the BHRF1 ORF are fully capable of initiating and maintain cell growth transformation and they can also enter the lytic cycle and produce virus (Lee and Yates, 1992, Marchim *et al* , 1991). Several of the early genes are linked to DNA replication as indicated in Figure 1.9. 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 of cellular factors in regulating the productive cycle, at least in certain cell types (Marschall *et al* , 1991).

### 1.7.3 LATE GENES

The late genes code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress (IARC Monograph, 1997). Among the non-glycoproteins the major nucleocapsid protein is encoded by BCLF1, BNRF1 encodes the major external nonglycoprotein of the virion and BXRf1 is likely to encode a basic core protein. The BFRF3 ORF encodes the tegument protein, (Figure 1.1) and VCA p18, which is strongly immunogenic in humans (Kieff, 1996). The genes encoding the EBV glycoproteins are illustrated in bold figure 1.9. The late BCRF1 gene which is located in the middle of the EBNA regulatory domain between *ori-P* and Cp, is a close homolog of the human IL-10 gene, with nearly 90% collinear identity in amino-acid sequence (Moore *et al* , 1990, Vieira *et al* , 1991, Touitou *et al* , 1996). BCRF1 has most of the activity of human IL-10, including negative regulation of macrophages and NK cell functions and inhibition of IFN  $\gamma$  production. Thus, virally expressed IL-10 may have a local effect on these responses to reactivate infection (IARC Monograph, 1997).

### 1.8 ROLE OF APOPTOSIS IN EBV BIOLOGY

While the process of apoptosis is a genetically controlled pre-programmed event of cell death that eliminates cells during development when they have become redundant, it also functions as an emergency response to various agents including radiation damage, 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 severely reduce or eliminate spread of progeny virus in the host (Shen and Shenk, 1995). Thus, many viruses have evolved strategies to evade or delay early apoptosis to allow persistence in the host and production of high yields of progeny virus. Within the last few years, both the biochemical basis of apoptosis and its regulation by some viral products have become clearer. For example, part of the apoptotic program includes the induction of cellular endonucleases which could target replicating viral DNA and prevent virus production at a very early stage. In order to combat such host cell

responses to infection many viruses have evolved genes that either themselves encode proteins or effect the expression of host cell apoptosis-related proteins that efficiently suppress the apoptotic program. In addition, many viruses are now known to induce apoptosis actively at late stages of infection. This process may represent a final and important step in the spread of progeny to neighbouring cells while also evading host immune inflammatory responses and protecting progeny virus from host enzymes and antibodies. Such virally-induced apoptosis may also contribute to some clinical manifestations and cytotoxicity associated with many human diseases of viral origin (Shen and Shenk, 1995).

EBV is unique in that it uses both pro- and anti-apoptotic properties to its advantage in its life cycle. This virus is able to effectively utilise these properties by either regulating the host cell apoptotic death machinery or encoding gene products that function similarly to anti-apoptotic proteins of the host cell. In the life cycle of EBV, protection against apoptosis serves two purposes. Firstly, as a mechanism that ensures its persistence in the host in the memory B-cell compartment by mimicking the normal process of B-lymphocyte selection in germinal centers. Secondly, the expression of particular virally encoded proteins in the lytic phase delays/suppresses cell death until sufficient progeny virions are made.

### **1.8.1 EBV induces the expression of anti-apoptotic proteins**

EBV-negative BL cells and type I BL cell lines phenotypically resemble germinal center B-lymphocytes (Gregory, 1995). This resemblance includes their ability to undergo apoptosis in the face of diverse stimuli including serum deprivation and calcium ionophore. On the other hand, type III EBV-positive BL lines that express the entire complement of latent proteins, display increased resistance to apoptosis (Gregory *et al* , 1991). These observations prompted further studies aimed at understanding the molecular basis of the protection from apoptosis conferred by EBV to the BL cell lines. Thus, it was found that the apoptosis-sensitive EBV-positive group I BL lines (and primary BL cells) also have little or no detectable Bcl-2 expression, while the apoptosis resistant

group III BL lines were found to be Bcl-2-positive (Henderson *et al* , 1991) Identification of a candidate latent protein encoded by the EBV genome that may be responsible for the upregulation of Bcl-2 observed in the group III BL cells was achieved when clear correlations were found between LMP-1 expression and protection from apoptosis on the one hand and on the other hand between LMP-1 expression and upregulation of Bcl-2 expression in LMP-1- (but not EBNA-1-, EBNA-2-, EBNA-LP- or LMP-2-) transfectants derived from EBV-negative BL cell lines (Henderson *et al* , 1991) This study also reported that EBNA-2 can augment the effect of LMP-1 expression on induction of Bcl-2 in dual transfection experiments of BJAB-cells (Henderson *et al* , 1991) In another study, Martin *et al* (1993) reported the effect of ectopic expression of EBNA-2 on induction of Bcl-2 protein in transient transfection experiments of BJAB-cells, however, coexpression of the *LMP-1* and *EBNA-2* genes did not result in significantly higher levels of Bcl-2 expression than did either gene alone in these cells The induction of Bcl-2 expression by LMP-1 has been shown to be a delayed response relative to its effects on NF- $\kappa$ B activation and upregulation of several adhesion molecules Studies with cell lines in which LMP-1 is under inducible control or expressed transiently have shown that maximum upregulation of Bcl-2 occurs 48-72 h post-induction of LMP-1 (Rowe *et al* , 1994, Wang *et al* , 1996) There is also evidence that EBNA3B (EBNA4) can upregulate Bcl-2 expression in an EBV-negative cell line (Silins and Sculley, 1995)

While expression of LMP-1 can induce Bcl-2 expression in group I BL lines and protect them from apoptosis, EBV infection of primary B-cells does not significantly increase Bcl-2 levels despite the fact that LMP-1 is expressed in the infected cells (Martin *et al* , 1993) Compared to EBV-negative or group I BL lines, primary B lymphocytes express significant amount of Bcl-2 and it has therefore been suggested that induction of Bcl-2 expression by EBV (or LMP-1) may not be relevant in a Bcl-2-positive cellular context such as exists in peripheral B-lymphocytes Furthermore, it is possible that there is some cellular control on the levels of expression of Bcl-2 in peripheral B-lymphocytes, perhaps a negative feedback mechanism as occurs in the case of the A20 protein (Song *et al* , 1996) The role of LMP-1 in LCLs, therefore, may not be to induce, but rather to maintain, the high constitutive level of Bcl-2 that is found in the uninfected peripheral B-

cells The observation that LMP-1 – mediated upregulation of Bcl-2 expression may be more relevant in a Bcl-2-negative cellular context also applies to epithelial and T cell lines since studies have shown that ectopic expression of LMP-1 in these cell lines does not further upregulate the constitutive Bcl-2 levels (Rowe *et al* , 1994, Kawanishi, 1997)

Overexpression of Bcl-2 in group I BL cell lines following *bcl-2* gene transfer results in a change in the behaviour of the cells in apoptosis assays towards that of group III lines (Henderson *et al* , 1991, Milner *et al* , 1992) These *bcl-2*-transfectants display reduced apoptotic responses following serum starvation, calcium ionophore treatment or anti-smIg stimulation, the inhibition of cell death being proportional to the amount of Bcl-2 protein (Milner *et al* , 1992) Interestingly, in a panel of group I, group I/*bcl-2* (*bcl-2* transfectants of group I cell lines), and group III sublines from the same BL parental line, it was found that, for the three different apoptosis triggers, the degree of protection imparted by the group III phenotype far outweighed that apparent from the levels of Bcl-2 protein measured in the group III cells Since there were no gross differences observed between the distribution of Bcl-2 in the group I transfectants and the group III transfectants, these observations were interpreted to mean that either, in a group I cell, Bcl-2 function is antagonised or one or more additional Bcl-2-independent survival-pathways are operational in group III cells In so far as the first possibility is concerned, *bcl-2* homologs, *bax* and *bcl-xS*, which can suppress Bcl-2 function have been identified (reviewed in Allday, 1996 and Korsmeyer, 1995) It is therefore conceivable that antagonism of Bcl-2 function by such homologous proteins occurs in the group I, but not in the group III environment In so far as protein levels are concerned, there does not appear to be consistent differences in the levels of these Bcl-2 antagonists between type I and type III BL cells (Spender *et al* , 1999) In support of the second possibility, it is plausible that additional EBV-induced apoptosis-suppressor proteins, such as the Bcl-2 homologs Bcl-xL, Mcl-1 or the A20 protein act in concert with Bcl-2 in group III cells to promote survival In this regard, LMP-1 has been shown to upregulate the expression of several other anti-apoptotic proteins including Mcl-1 and A20 (Wang *et al* , 1996, Laherty *et al* , 1992), the former serving as a rapid and short-term effector of cell viability prior to the up-regulation of Bcl-2 (Wang *et al* , 1996)

The upregulation of the anti-apoptotic zinc-finger protein, A20, by LMP-1 has been demonstrated in B- lymphocytes as well as epithelial cells and this effect is NF- $\kappa$ B dependent mapping to both CTAR1 and CTAR2 (Laherty *et al.*, 1992; Fries *et al.*, 1996, Miller *et al.*, 1995; 1997). A20 is also constitutively expressed in EBV-immortalised B-cells (Spender *et al.*, 1999). LMP-1-mediated protection of epithelial cells from p53-mediated apoptosis has been shown to involve A20 (Fries *et al.*, 1996). A20 can also contribute to the antiapoptotic effect of LMP-1 expression in BL cell lines (Laherty *et al.*, 1992). More recently, A20 was demonstrated to function in a negative feedback loop by inhibiting activation of NF- $\kappa$ B from both CTAR1 and CTAR2 domains of LMP-1 (Eliopoulos *et al.*, 1999). This effect was mediated by the binding of A20 to TRAF2 and this effect by A20 has also been demonstrated in the case of signaling from some of the other members of the TNFR-family including CD40 which can also induce A20 expression in BL cells (Sarma *et al.*, 1995).

### **1.8.2 EBV regulates components of the cell cycle machinery to protect against apoptosis**

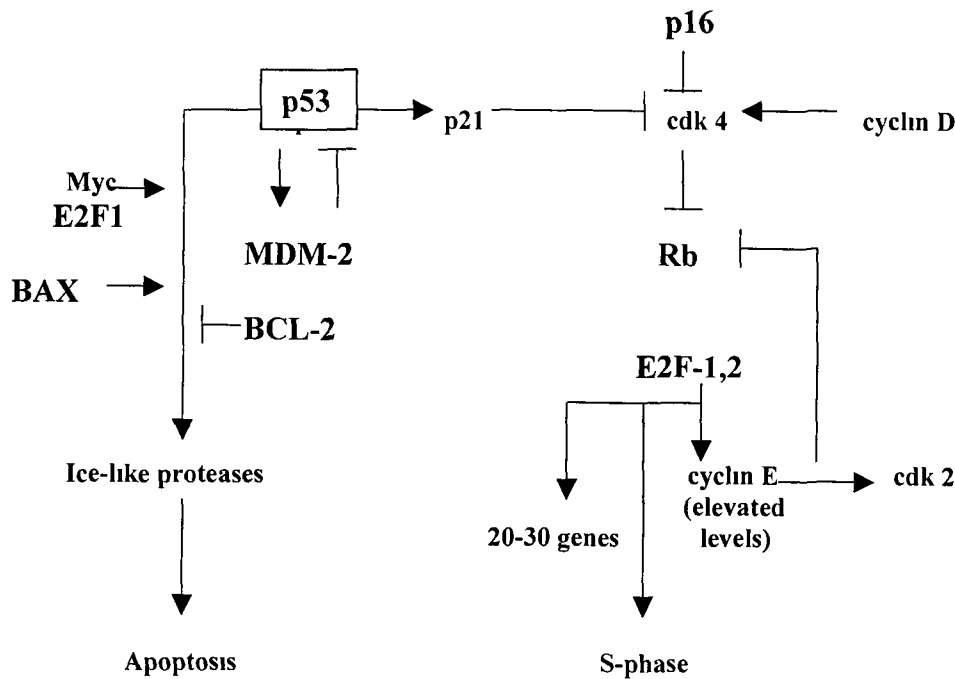
In addition to regulating apoptosis by inducing the expression of anti-apoptotic proteins, EBV and in particular LMP-1 can also control apoptosis by regulating the cell cycle, since the processes of apoptosis and cell cycle progression (proliferation) are coupled through the use and control of shared molecular machinery (Evan *et al.*, 1995).

Many oncogenes that promote cell cycle progression also induce apoptosis. This duality in function is exemplified by c-Myc which is a sequence-specific DNA-binding phosphoprotein that serves as a transcriptional activator and whose expression is required for passage through G1 and entry into the S-phase of the cell cycle (Gregory, 1995). In order to explain the ability of c-Myc to promote such opposed cellular functions as proliferation and death, a model has been proposed according to which c-Myc necessarily drives both processes, proliferation being favoured under appropriate conditions of availability of nutrients, space, growth and survival factors (Evan *et al.*, 1995). Thus, this

model predicts that the activity of Myc in promoting proliferation is dependent on the route to its apoptosis-promoting activity being blocked. Accordingly, even normally proliferating cells may be regarded as being “primed” for apoptosis. This model can explain why c-Myc, being a potent inducer of cell death is strongly favoured during the carcinogenic process. Thus, it has been suggested that as soon as the affected cell acquires some form of apoptosis-suppressing mutation, the advantage afforded by c-Myc-induced proliferation outweighs any disadvantage conferred by c-Myc-induced apoptosis. Until that point, however, most cells with deregulated c-Myc are thought to delete themselves (by apoptosis). This theory gains support from observations made in transgenic animals expressing deregulated c-Myc. Lymphocytes from these animals were found to exhibit elevated levels of spontaneous apoptosis and an increased sensitivity to induction of apoptosis in lymphoid organs was also observed (Evan *et al*, 1995, Levine 1997). That cell death is an important restraint during carcinogenesis induced by c-Myc in the absence of an ‘anti-death factor’, is best exemplified by the occurrence in these animals of tumours displaying oncogenic synergy between *c-myc* and the anti-apoptotic gene *bcl-2* (reviewed in Allday, 1996). Further studies have shown that Bcl-2 suppresses only the apoptotic actions of c-Myc, leaving its ability to promote cell proliferation unaffected, thus leading to the suggestion that the proliferative and apoptotic arms of the c-Myc pathway must be distinct at the point at which Bcl-2 acts.

Further support for the theory that apoptosis and cell proliferation coincide comes from the observation that key tumour suppressor proteins such as pRb and p53 exert direct effects on cell viability and on cell cycle progression (reviewed in Allday, 1996, Levine, 1997, Hunter, 1993). Phosphorylation of pRb (the product of the retinoblastoma gene *rb*) inactivates the protein during late G1 at a point in the cell cycle that appears to be coincident with the late G1 restriction point (after which cells no longer require mitogenic signals to complete their cell cycle). pRb is the principal target for inactivation by several DNA tumour virus oncoproteins including Simian virus (SV) 40 large T antigen (Tag), human papilloma virus (HPV) E7 protein and adenoviral E1A. This suggests that active pRb acts as a restraint to the mitogenic actions of these virus proteins (Taya, 1997).

One of the functions of pRb is to suppress the action of the G1-specific E2F-DP transcription factors which coordinate expression of the late G1 phase and early S phase genes required for cell cycle progression. The activity of pRb is regulated by phosphorylation in the *p16-cyclinD1-cdk4-rb* pathway (Taya, 1996, Levine, 1997) (Figure 1 10). p16 is a negative regulator of cyclin D1-cdk4 and the gene is shut off in many cancer cells or mutated in others. Cyclin-D1-cdk4 complexes target pRb for cell cycle regulation by phosphorylating it. E2F-DP transcription factor complexes (E2F-1,-2 and -3 and DP-1,-2 and -3) whose activity is regulated by pRb, in turn regulate a number of genes [including those encoding cyclin E, cyclin A, dihydrofolate reductase and proliferating cell nuclear antigen (PCNA)] required to initiate or propagate the S phase of the cell cycle. Phosphorylation of pRb by cyclinD1-cdk4 releases E2F-DP proteins (E2F-DP proteins are bound by dephosphorylated pRb) from the pRb complex, relieving repression of these genes or activating their transcription.



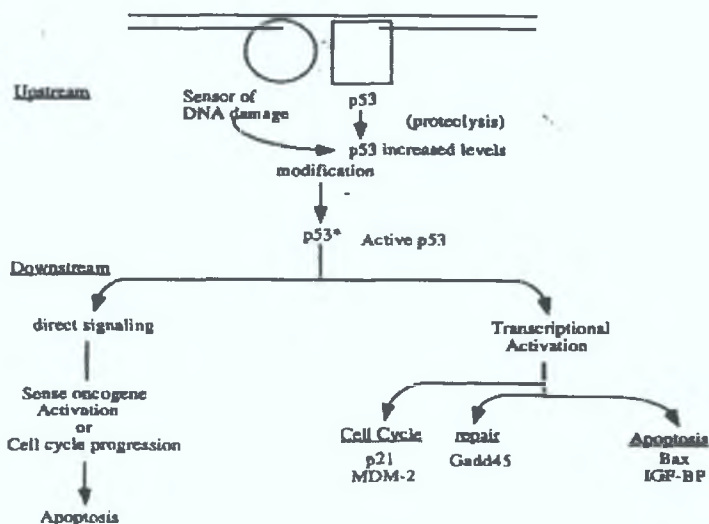
**Figure 1.10 The p53-Rb pathway.** Inter-relationships among a number of oncogenes (Myc, MDM-2, BCL-2) and tumour suppressor genes (p16, Rb, p53) that regulate the G1-S phase restriction point, its relation to a DNA damage checkpoint mediated by p53, and the choice by p53 whether to initiate a G1 arrest (via p21) or apoptosis. Available evidence suggests an important role for Rb and its two related gene products, p107 and p130 (along with E2F-4 and -5), in p53-mediated G1-S phase regulation. Shown are the p53-MDM2 autoregulatory loop that reverses this checkpoint control and the gene products that positively or negatively act on the probability of entering apoptosis. (Key:- ⊥ inhibits activity ; → induces activity) *Adapted from Levine, 1997.*

By binding and inactivating pRb, thereby liberating active E2F, the viral oncoproteins E1A (adenovirus) and E7 (HPV) promote cell cycle progression (Levine, 1997). Consistent with this, is data showing that deregulated expression of E2F transcription factors induces both proliferation and apoptosis. c-Myc can also override the growth-suppressive effects of pRb; however, there is some doubt as to whether c-Myc can interact with pRb *in vivo*. Not only does a lack of functional pRb lead to apoptosis, but restoration of functional pRb to *rb*<sup>-</sup> cells directly inhibits it, under conditions where apoptosis is induced by radiation (i.e. p53-dependent) or even by dominant oncogenes. This indicates that the effects of pRb must be exerted downstream of p53.

p53 plays a key role in limiting the further expansion of cells containing damaged genomes and it achieves this by either enforcing cell cycle arrest or triggering apoptosis (reviewed in Levine, 1997 and Bates and Vousden, 1996). Which of these two alternate fates occurs in any particular instance appears to depend on a number of undefined factors, such as cell type, nature and intensity of insult and cytokine status. The high frequency with which p53 is functionally inactivated in human tumours attests to the critical role that p53 plays in limiting neoplastic progression. By transducing genome damage into growth arrest and/or apoptosis, p53 prevents the progression of potentially mutant cells together with the neoplastic risk they present. Levels of p53 rapidly increase following DNA damage, mainly because the normally short-lived p53 protein is stabilised, and this appears to be an important component of the G1 arrest and apoptosis that follow DNA damage. Tumour cells that have lost functional p53 exhibit resistance to induction of apoptosis by a range of genotoxic agents. Moreover, because such cells

fail to arrest or die following DNA damage, they enter S phase with unrepaired DNA and sustain substantially elevated risks of further mutation.

p53 can act as a transcription factor, and most evidence suggests that it induces growth-arrest by the modulation of specific target genes (reviewed in Levine, 1997). Most prominent among these is the gene encoding the cyclin-dependent kinase (cdk) inhibitor p21/WAF-1/Cip-1, whose action arrests cells in late G1. p21 binds to a number of cyclin and cdk complexes: cyclinD1-cdk4, cyclin E-cdk2, cyclin A-cdk2 and cyclin A-cdc2 (Figures 1.10, 1.11). One molecule of p21 per complex appears to permit cdk activity, while any more inhibits kinase activity and cell cycle progression. p21 also binds to PCNA, and formation of p21-PCNA complexes blocks the role of PCNA in DNA replication (but not its role in DNA repair). Thus, p21 can act on cyclin-cdk complexes and PCNA to block DNA replication. p21-independent pathways may also contribute to p53-mediated growth arrest. In this respect, GADD45, which is encoded by a p53-responsive gene, can also bind PCNA.



**Figure 1.11 The Events in p53 activation.** DNA damage (indicated by the break in the double line at the top) is recognised by a “sensor” molecule that identifies a specific type of lesion and possibly by the p53 protein. The sensor modifies p53 (by phosphorylation) when both molecules correctly determine that there is damage. A modified p53 is more stable (increased half-life) and a steric or allosteric change in p53 permits DNA binding to a specific DNA sequence regulating several downstream genes [p21 (inhibits cdk

responsible for G1/S transition), MDM2 (negatively controls p53 expression), GADD45 (DNA replication), Bax (pro-apoptotic) and cyclin G]. Two modes of signaling for cellular apoptosis are possible: one requiring transcription and one involving direct signaling with no transcription of downstream genes required. Adapted from Levine, 1997

It is less certain how p53 regulates apoptosis or what factors coordinate its actions as a cytostatic- versus a cytotoxic- agent. Some evidence indicates that induction of apoptosis by p53 may not require transcriptional activation of genes, although transrepression has not been excluded (reviewed in Levine, 1997, Evan, 1995). Other data implicate p53-dependent transactivation of the *bax* gene, which encodes a member of the Bcl-2 family that can induce apoptosis. It is well established that overexpression of Bcl-2 can block p53-mediated apoptosis. Bax binds to Bcl-2 and antagonises its ability to block apoptosis, so a p53-dependent *bax* synthesis can tip the scales towards apoptosis. A second p53-regulated gene product that could affect growth regulation is the insulin-like growth factor-binding protein-3 (IGF-BP3). IGF-BP3 blocks the IGF mitotic signaling pathway by binding to IGF and preventing its interaction with its receptor. Thus, the blocking of IGF activity could enhance apoptosis or lower the mitogenic response of cells.

p53 also acts to induce apoptosis in cells with oncogenic lesions such as loss of functional pRb or the expression of viral proteins such as E1A or deregulated c-Myc (reviewed by Evan *et al* , 1995). Mice in which pRb inactivation is targeted to the lens or photoreceptors of the developing eye exhibit the characteristic excessive proliferation and apoptosis which are associated with the inactivation of pRb. Such apoptosis is inhibited in a *p53*-negative background and in this case, the animals develop tumours instead. The expression of the adenovirus E1A protein in rat embryo fibroblasts stabilises and activates p53, the resulting cells die of apoptosis. Adenoviruses normally express the E1B-55 kD protein, which binds to p53 and blocks its transcription factor activity, and the E1B-19kD protein, which acts like Bcl-2 to block apoptosis downstream of *p53* activation.

EBV-negative BL cells which exhibit deregulated c-Myc expression as a result of the t (8,14) translocation proliferate rapidly in culture but readily undergo apoptosis under inappropriate conditions such as serum deprivation (Gregory, 1995). Expression of LMP-1 or EBNA2 in these cell lines has been shown to have growth inhibitory and anti-apoptotic effects which may at least in part be a consequence of downregulation of c-Myc expression as a result of downregulation of transcription of the adjacent translocated Ig- $\mu$  locus (Floettmann *et al* , 1996, Jochner *et al* , 1996). LMP-1 has also been demonstrated to protect Jurkat T cells from apoptosis induced by serum deprivation by downregulating the expression of c-Myc (Kawanishi, 1997).

TGF $\beta$  peptides belong to a family of structurally related molecules with a variety of biological functions including differentiation and apoptosis on a variety of cell and tissue types including human B and T lymphocytes (reviewed in Aravanitakis *et al* , 1995). EBV-negative BL lines and EBV-positive BL lines with a group I phenotype are generally sensitive to the growth inhibitory effects of TGF- $\beta$  and this results from cells accumulating in G1 and undergoing apoptosis. However, EBV immortalised LCLs and EBV-positive BL lines that express all the latent viral proteins are resistant to the growth-inhibitory effects of TGF- $\beta$  (Altiook *et al* , 1993, MacDonald *et al* , 1996). Aravanitakis *et al* (1995) were able to demonstrate that LMP-1 expression could independently reproduce the ability of EBV to confer TGF- $\beta$ -resistance on infected B-lymphocytes and group III BL lines by gene transfer into the EBV-negative BL cell line BL41. TGF- $\beta$  is known to arrest cells in the mid- to late- G1 phase of cell cycle in not only mitogen-activated human B-cells but also in non-lymphoid cell systems (reviewed in MacDonald *et al* , 1995, Chaouchi *et al* , 1995). This G1 arrest has been linked to an upregulation of at least three inhibitors of cyclin-dependent kinases (cdks) namely, p15<sup>INK4B</sup>, p27<sup>KIP1</sup> and p21<sup>WAF1</sup> and in some cells to a downregulation of cdk4 (reviewed in MacDonald *et al* , 1996 and Hunter, 1993). Binding of these inhibitors to cdk inhibits the formation of cdk-cyclin complexes that are required for progression through the cell cycle. The inhibition of cyclin D function leads to a block in phosphorylation of the retinoblastoma gene product, pRb, and this in turn represses the activity of the E2F family of transcription factors, thus preventing cells from making the transition from G1- to S- phase of the cell

cycle The protection against TGF- $\beta$ -induced G1 arrest afforded by LMP-1 was shown to be related to the induction of cyclin D2 expression by LMP-1 leading to the maintenance of pRb in the hyperphosphorylated state In the early G1 phase of the cell cycle, the presence of hypophosphorylated pRb is associated with transcriptional activation of cyclin D expression in normal cells (Taya *et al* , 1997) D-type cyclins form complexes with cdk4 which then proceed to phosphorylate pRb and shut down cyclin D transcription, thus completing the feedback loop regulating progression from the G1 phase to the S phase of the cell cycle The maintenance of pRb in the hyperphosphorylated state by LMP-1-thus leads to cell cycle progression regardless of the negative growth regulatory signal imposed by TGF- $\beta$

Mutations and elevated expression of p53 are two factors that can play a role in malignant transformation or tumour cell growth of Burkitt's lymphoma Mutations in p53 have been found in approximately 33% of BL biopsies and in at least 63% of BL lines (Farrell *et al* , 1991) In 65% of BL lines studies the wild-type allele is lost and the cells express the mutant p53 allele at elevated levels In general, single nucleotide substitutions which result in aminoacid changes are found BL cells with p53 lesions have been shown to be relatively resistant to DNA-damaging drugs such as cisplatin compared to those cell lines harbouring wild-type p53 (Allday *et al* , 1995) Expression of LMP-1 can however protect against wild-type p53-mediated apoptosis and this has been demonstrated in both the BL and epithelial cell contexts based on the usage of cell lines expressing temperature-sensitive p53 In BL cells, protection by LMP-1 is most likely due to the induction of Bcl-2 expression (Okan *et al* , 1995) However, in the epithelial cells examined, protection against p53-mediated apoptosis was shown to be due to the induction of A20 expression (Fries *et al* , 1996) In both cases, protection by LMP-1 did not involve an effect on the transactivation function of p53

#### **1.8.2.1 EBV primes LCLs for apoptosis**

##### **c-Myc**

Activated peripheral blood B-cells ( IL-4 + anti-CD40 antibodies) cultured *in vitro* in the absence of serum exhibit little DNA synthesis, accumulate in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle and have a lower level of *c-myc* expression compared to when grown in the continuous presence of serum (reviewed in Cherney *et al* , 1994) However, EBV-immortalised lymphoblastoid cell lines grown under autocrine growth factor-deprived conditions (cultured under low-density conditions) exhibit apoptosis by day 3 of growth (Allday, 1996) In fact, under such culture conditions, these cells continue to proliferate until apoptotic death and this change in growth properties is not accompanied by a change in the levels of c-Myc protein (or even p53 or Bcl-2) These observations led to the suggestion that deregulation of *c-myc* may mediate the apoptotic response of the growth factor-deprived LCLs Indeed, it was demonstrated that antisense oligonucleotides to *c-myc* specifically inhibited apoptosis in the growth factor-deprived LCLs but suppressed growth of undeprived LCLs These studies indicated that EBV latent gene expression must deregulate *c-myc* in B lymphocytes and this promotes both proliferation and apoptosis when there is a depletion of autocrine growth factors (Cherney *et al* , 1994)

Studies based on the usage of an LCL established with a recombinant EBV conditional for the expression of EBNA-2, have shown that EBNA-2 is the likely EBV latent gene product responsible for deregulating c-Myc in LCLs In this study by Kempkes *et al* (1995), primary B-lymphocytes were infected with the recombinant virus such that in the presence of estrogen, growth transformation of the infected B-cells was achieved leading to the formation of estrogen-dependent LCLs expressing EBNA2 (as a fusion protein with the hormone binding domain of the estrogen receptor) in addition to the other EBV latent proteins Removal of estrogen resulted in about half of the cells entering a quiescent non-proliferative state whereas the others underwent apoptotic death Growth arrest occurred at both the G<sub>1</sub> and G<sub>2</sub> phases of the cell cycle which is indicative of a requirement of EBNA-2 function at both of these stages of the cell cycle However, only those cells arrested in G<sub>1</sub> could be rescued into cell cycle on induction of EBNA2, and this was shown to correlate with the induction of c-Myc and LMP-1 expression, followed by the induction of cyclin D2 (G<sub>1</sub>-specific cyclin) and cdk4 and then the phosphorylated

form of pRb. Concomitant with the appearance of the phosphorylated form of pRb, the E2F transcription factor, (which is maintained inactive by binding to the hypophosphorylated form of Rb and is required for DNA synthesis) became detectable. This study therefore demonstrated that in LCLs, functional EBNA-2 is responsible not only for the induction and maintenance of c-Myc in a deregulated state but also for proliferation. In order to investigate if this effect of EBNA2 was due to an indirect effect on expression of LMP-1, Zimmer-Strobl *et al.* (1996) have analysed the growth characteristics of primary B-cell transfectants that constitutively expressed *LMP-1* from a heterologous promoter that was carried on a plasmid vector containing a conditional (estrogen-dependent) EBNA-2 allele in addition to the other wild-type latent EBV genes (Zimmer-Strobl *et al.*, 1996). In the absence of functional EBNA-2, it was found that LMP-1 was incapable of sustaining B-cell proliferation (at levels of expression similar to that observed in LCLs) but induced a phenotype consistent with prolonged cell viability. Proliferation of these transfectants resumed only in the presence of functional EBNA-2. Thus, it was demonstrated that LMP-1 is not sufficient to maintain proliferation of B-cells in the absence of EBNA-2 and that EBNA-2 acts beyond the upregulation of LMP-1 to maintain proliferation of EBV-immortalised B-cells perhaps by cooperating with other cellular and viral genes. Studies in EBV-immortalised B lymphocytes expressing a conditional LMP-1 have shown that the continuous expression of LMP-1 is essential for proliferation (Kilger *et al.*, 1998).

A likely viral latent gene that may serve in the role of cooperating with EBNA-2 in initiating and maintaining B-cells in cycle is EBNA-LP. Resting B-cells, primed by CD21 cross-linking with the gp350 virus envelope protein to mimic virus infection and transfected with EBNA-2 and EBNA-LP expression vectors, enter and progress through the cell cycle and this ability of EBNA-2 and EBNA-LP is correlated with their ability to induce the synthesis of cyclin D2 (Sinclair *et al.*, 1994). Expression of both EBNA-2 and EBNA-LP were required for this effect. Thus, by inducing cyclinD2 synthesis, EBNA-2 and EBNA-LP cooperate to induce a G0 to G1 transition. In this relation it is interesting to note that following EBV infection of resting primary B-cells, EBNA-2 and EBNA-LP are the first viral latent proteins to be expressed and that too in a coordinate fashion.

Studies by Kitay and Rowe have shown that EBNA-LP is phosphorylated throughout the cell cycle, but the extent of phosphorylation is increased during G2 phase and is maximal at the G2/M transition (Kitay and Rowe, 1996). Moreover, *in vitro* studies have shown that EBNA-LP can interact with p53 and pRb, two proteins known to play important roles in cell-cycle regulation. The significance of these latter observations are not known since EBNA-LP does not alter the function of these molecules when bound (Szekely *et al* , 1993).

### **p53**

Investigations aimed at assessing the integrity of the p53/p21waf-1/ pRb/E2F pathway in B-cells immortalised by EBV have led to the same conclusion as that of the influence of EBV infection on *c-myc* expression in B-cells. EBV infection of B-cells primes the cells for apoptosis. Resting primary B-cells are essentially p53-negative (Allday *et al* , 1995). Some of these cells spontaneously undergo apoptosis when placed in culture, through a p53-independent pathway. Normal peripheral B-cells treated with IL-4 and anti-CD40 are activated into proliferation and accumulate p53. EBV infection of resting B-cells similarly rescues them from spontaneous cell death and this process is also accompanied by an accumulation of p53 (Allday *et al* , 1995). In the case of EBV-infected B-cells, accumulation of p53 requires viral gene expression (infection with UV-inactivated virus did not result in p53 accumulation) and it has been shown by Chen and Cooper (1996) that EBNA-2 and LMP-1 can independently transactivate p53. While IL-4/anti-CD40-activated B-cells respond to the DNA-damaging agent cisplatin by ceasing DNA synthesis (growth arrest), LCLs, respond with apoptosis (Allday *et al* , 1995). Cisplatin-treatment of either IL-4/anti-CD40 activated B-cells or LCLs led to an elevation of p53 levels with similar kinetics. p53 accumulation in cisplatin-treated LCLs was shown to be due to post-translational modification leading to enhanced stability. Also, cisplatin-treatment of the LCLs did not alter Bcl-2 expression or the heterodimeric partner of Bcl-2, Bax (which is also known to be transcriptionally activated by p53 in some cell types). Ectopic expression of p53 in LCLs results in spontaneous apoptosis, indicating that the cisplatin-induced apoptosis observed in the LCLs is likely to be due to the increased accumulation of p53. Furthermore, in the latter experiment, in the cells

proceeding towards apoptosis, transcriptional activation of p21<sup>WAF-1</sup> by p53 correlated with the presence of dephosphorylated pRb. In normal fibroblasts, cisplatin-treatment correlates with the presence of hypophosphorylated pRb and G1-arrest since the cells are unable to proceed into S-phase (reviewed in Allday, 1995). However, the observation that the presence of hypophosphorylated pRb correlates with apoptosis in LCLs suggests that EBV must encode a function which enables the G1/S restriction point to be overcome and thus allow progression through the cell cycle, converting the p53 arrest response to apoptosis (Levine, 1997). Candidate mechanisms by which EBV may overcome the G1/S restriction point include deregulation of *c-myc* or utility of a virally-encoded E1A-like function which generates high levels of transcriptionally active E2F.

### **1.8.3 EBV lytic proteins encode anti-apoptotic functions**

Unlike most viruses, EBV maintains a very stable persistent infection *in vivo* (Rowe *et al.*, 1992). Another state in which EBV may be found is in the process of active replication and the production of progeny virions; this is the lytic cycle. Virus production is generally accepted to precede replication of viral DNA (Allday, 1996). This may be sensed by the host cell as damaged DNA and the p53 checkpoint response could then be activated. If the cells are driven to proliferate by EBV (as in type III latency) then apoptosis will be the most likely outcome. In this situation additional repressors of apoptosis can provide a selective advantage. Consistent with this model, there are at least two known virally encoded proteins, BHRF-1 and BZLF-1, that are synthesised during the lytic cycle that can provide a survival advantage to the infected cell (Allday, 1996). These lytic gene products can therefore delay apoptosis in order to maximise viral production. Expression of BHRF-1 in epithelial cells has been shown to confer enhanced resistance to cisplatin-induced apoptosis and can also inhibit differentiation (Dawson *et al.*, 1990; 1995). In lymphoid cells, BZLF-1 has been shown to interact with p53 and inhibit its transactivating function, thereby providing a mechanism for preventing p53-mediated apoptosis (Zhang *et al.*, 1994b). However, in epithelial cells, expression of this EBV protein was shown to result in growth arrest without inhibiting the transactivating function of p53 (Cayrol and Flemmington, 1996).

## **CHAPTER 2**

### **MATERIALS AND METHODS**

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## 2.1 BIOLOGICAL MATERIALS

### 2.1.1 Cell lines

Table 2.1 Cell lines used in this study

Cell Line	EBV Status	Cell Classification	Description
DG75	-	EBV negative BL	Lymphoid B cell line derived from an Israeli Burkitt-like lymphoma case (Ben-Bassat <i>et al</i> , 1977)
DG75 tTA	-	BL (stable transfectant)	Clonal derivative of DG75 stably transfected with the tetracycline-regulated transactivator only (Floettmann <i>et al</i> , 1997)
DG75 tTA LMP1	-	BL (Stable transfectant)	Tetracycline-regulated system established in DG75 whereby the expression of LMP1 can be induced by the removal of tetracycline from the growth media (Floettmann <i>et al</i> , 1997)
Mutu 1	+	Type I BL	Early passage BL cell line expressing EBNA1 as the sole viral latent gene (Gregory <i>et al</i> , 1990)
Mutu 3 c95	+	Type III BL	A clone of Mutu I cells that have upon serial passage in culture, "drifted" to express the entire complement of EBV latent genes (Gregory <i>et al</i> , 1990)
Mutu 3 (LMP1-)	+	Type III BL	This is a partially characterised Mutu 3 clone which no longer expresses LMP-1
BL41, BL41-B95 8 IARC 171	- + +	EBV-negative BL Type III phenotype LCL	These cell lines are a matched set BL41-B95 8 was established by infection of BL41 cells with the EBV strain B95 8 and expresses all of the EBV latent genes (Calendar <i>et al</i> , 1987, Andersson <i>et al</i> , 1991)) IARC 171 is a LCL established by infection of B lymphocytes from the same patient from whom the BL41 cell line was

			derived with the B95 8 strain (Lenoir <i>et al</i> , 1985)
OKU BL OKU LCL	+ +	Type I LCL	These cells are a matched pair OKU BL is a group I BL cell line expressing only EBNA1 OKU LCL was established by infection of peripheral B lymphocytes from the same patient from whom OKU BL was derived with the EBV strain B95 8
IARC 290B	+	LCL	A spontaneously transformed LCL (Lenoir <i>et al</i> , 1985)
IB4	+	LCL	An LCL established by infection of fetal cord blood lymphocytes with the EBV strain B95 8 (King <i>et al</i> , 1980)
X50-7	+	LCL	A spontaneously transformed LCL (Wilson and Miller, 1979)
Ag876	+	Type III BL	Type III BL cell line expressing all of the EBV latent genes
Jurkat	-	T cell	Acute T-lymphocytic leukemic cell line (Brattsand <i>et al</i> , 1990)
Jurkat tTA LMP-1	-	T cell (stable transfectant)	Tetracycline-regulated system established in Jurkat cells whereby the expression of LMP1 can be induced by the removal of tetracycline from the growth medium
C33A, C33A Neo, C33A LMP1	- - -	Epithelial cell	These are cervical epithelial cell lines C33A is the parental cell line C33A Neo is the control transfectant expressing the neomycin-resistance gene only C33A LMP1 is stably transfected with a vector constitutively expressing LMP1 and the neomycin resistance gene (Miller <i>et al</i> , 1995)

All BL cell lines, LCLs, the Jurkat and the Jurkat tTA LMP-1 cell lines were obtained from Professor Martin Rowe, University of Wales, Cardiff The epithelial cell line C33A and its derivatives were a gift from Dr Nancy Raab-Traub, University of North Carolina, USA

2.1.2 Antibodies

The monoclonal antibodies against LMP-1, CS1-4, was a generous gift from Professor Martin Rowe, University of Wales, Cardiff The monoclonal antibodies against Bcl-2, Bcl2 100/124 was a gift from Professor David Mason, John Radcliffe Hospital, University of Oxford, Oxford The rabbit polyclonal antibodies to HA was a gift from Professor Luke O'Neill, Trinity College Dublin The monoclonal antibodies against the CD40 receptor, G28 5, was obtained from Professor Edward Clark, (affinity-purified antibodies) and Professor Martin Rowe (ascites) The monoclonal antibodies against phosphotyrosine, PT66, was a gift from Dr Deirdre Cooke, Dublin City University The alkaline-phosphatase (AP) conjugated anti-mouse antibodies was purchased from Promega and the alkaline-phosphatase (AP) conjugated anti-rabbit antibodies was purchased from Boehringer Mannheim The polyclonal antibodies against p65 and c-rel were purchased from Chemicon Int and the polyclonal antibodies against p50 was a gift from Dr Katherine Fitzgerald, Trinity College Dublin

2.1.3 Bacterial Strains

*E coli* DH5 $\alpha$ , genotype F-, *end* A1, *hsd*R17 (*r*<sub>k</sub>-,*m*<sub>k</sub>=), *sup*E44, *thi* -1, $\lambda$ -, *rec* A1, *gyr* A96, *rel* A1,  $\phi$  80*lac* Z $\delta$ M15

2.1.4 Plasmids

Table 2 2 Plasmids used in this study

PLASMID	GIFT FROM	DESCRIPTION
pEFCX pEFCXLMP-1 pEFCXI $\kappa$ B $\alpha$ DN	Dr Peter Brodin, Umea University, Sweden	pEFCXLMP-1 is an LMP-1 expression plasmid in which the LMP-1 gene (from the B95 8 virus) was cloned in front of the strong polypeptide chain elongation factor 1 $\alpha$ promoter contained in pEFCX pEFCX I $\kappa$ B $\alpha$ DN expresses a dominant negative I $\kappa$ B $\alpha$ in which serines 32 and 36 were mutated to alanines (Liljeholm <i>et al</i> , 1998)

pSG5 pSG5 LMP-1 pSG5 LMP AAA pSG5 LMP G pSG5 LMP AAAG	Professor Martin Rowe, University of Wales College of Medicine, Cardiff, U K	pSG5 LMP-1 is an LMP-1 expression plasmid in which the LMP-1 gene from the B95 8 virus was cloned in front of the SV40 promoter contained in pSG5 (Stratagene) PSG5 LMP AAA expresses LMP-1 with amino acids proline 204, glutamine 206 and threonine 208 mutated to alanine PSG5 LMP G expresses LMP-1 with aminoacid 384 changed from tyrosine to glycine PSG5 LMP AAAG expresses LMP-1 with the combination of mutations present in LMP-1 AAA and LMP-1 G (Floettmann <i>et al</i> , 1998)
pSG5 TRAF2Δ(6-86)	Professor Martin Rowe, University of Wales College of Medicine, Cardiff, U K	Expresses a dominant-negative TRAF2 molecule that carries an N-terminal deletion of aminoacids 6 to 86 (Eliopoulos <i>et al</i> , 1999)
pSG5 IκBαDN	Professor Martin Rowe, University of Wales College of Medicine, Cardiff, U K	Expresses a dominant negative IκBα in which serines 32 and 36 were mutated to alanines
pSG5 A20	Professor Martin Rowe, University of Wales College of Medicine, Cardiff, U K	Expression plasmid for the full- length A20 protein (Eliopoulos <i>et al</i> , 1999)
p-1374/+81Bfl-1 CAT p-1374/+81Bfl-1 mκB CAT p-1240/+81Bfl-1 CAT p-367/+81Bfl-1 CAT p-129/+81Bfl-1 CAT	Dr Celine Gelin, School of Medicine and Dentistry, University of New Jersey, New Jersey U S A	p-1374/+81Bfl-1 CAT contains the region -1374 to +81 of the human <i>bfl-1</i> gene cloned in the promoterless vector, pCAT-basic, expressing a CAT reporter gene p-1374/+81Bfl-1 mκB CAT contains the -1374 to +81 of the <i>bfl-1</i> gene but with a mutated NF-κB site at position -833 (GTTTATTACC) p -1240/+81 Bfl-1 CAT, p-367/+81 Bfl-1 CAT and p-129/+81 Bfl-1 CAT contain the regions -1240 to +81, -367 to +81 and -129 to +81 of <i>bfl-1</i> cloned into pCAT-basic (Zong <i>et al</i> , 1999)
3 x enh κB-luc	Professor Martin Rowe, University of Wales College of Medicine, Cardiff U K	Contains three κB elements upstream of a minimal conalbumin promoter driving the expression of the firefly luciferase gene (Floettmann and Rowe, 1997)
pcDNA3 HABfl-1	Dr G Chinnadurai, Saint Louis University School of Medicine, Missouri U S A	Expresses Bfl-1 protein tagged to the influenza virus HA epitope (D'Sa-Eipper <i>et al</i> , 1996)

2.1.5 Oligonucleotides

Sigma Genosys Ltd

-54/-41Bfl-1 NF-κB

Forward oligonucleotide

5' ACA GAA ATT CCA CC 3'

Reverse oligonucleotide

5' GGT GGA ATT TCT GT 3'

Promega

'consensus' NF-κB oligonucleotide

5' AGT TGA GGG GAC TTT CCC AGG C 3'

3' TCA ACT CCC CTG AAA GGG TCC G 5'

2.1.6 Commercial kits and restriction enzymes

Restriction enzymes	Roche Biochemicals and New England Biolabs
In Vitro Transcription kit	Pharmingen
BCA Protein assay kit	Pierce
Qiagen Tip-100	Qiagen
RPA kit	Pharmingen
Wizard PCR Preps DNA purification system	Promega

2.2 CHEMICAL MATERIALS

Protein prestained markers	NEB
α <sup>32</sup> P labeled UTP	Amersham
γ <sup>32</sup> P labeled ATP	New England Biolabs

dNTPs	Pharmacia Biotech
Rnase A	Pharmacia Biotech
Poly(dI-dC) Poly(dI-dC)	Pharmacia Biotech
MicroSpin™ G-25 columns	Pharmacia Biotech
Marvel	Premier Beverages
Chloroform	ROMIL
Isopropanol	ROMIL

Sigma-Aldrich-Fluka Chemical Co

Urea, Dithiothreitol, Coomassie blue R, BCIP/NBT, Tetracycline, Liquid Phenol, Nitocellulose, Ampicillin, Potassium acetate, Tween-20, BSA, Sodium azide, Sigmacote, Ammonium phosphate, Mineral oil,  $\alpha$ -Thiol-glycerol, PMSF, MOPS, BCS *E coli* tRNA, Aprotinin, Formaldehyde, Micophenohc acid, BCS, DEAE-dextran, okadaic acid

Merck

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

BDH

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

Boehringer Mannheim

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

Calbiochem-Novobiochem

Okadaic acid

Oxoid

Agar technical, Bacto-Tryptone, Yeast extract

KODAK

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

National diagnostics

Acrylagel, Bis-acrylagel, Accugel

Gibco-BRL

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

Promega

100bp DNA ladder, T4 polynucleotide kinase, NF-kappaB oligonucleotide

## **2.3 DNA MANIPULATION**

Preparation of all solutions used in chapter two are outlined in the appendix

### **2.3.1 Storage of DNA samples**

DNA samples were stored in TE buffer pH 8.0 at 4°C. EDTA was used to chelate heavy metal ions that are needed for DNase activity while storage at pH 8.0 minimises deamidation. DNA was also stored in sterile distilled H<sub>2</sub>O (dH<sub>2</sub>O).

### **2.3.2 Equilibration of phenol**

Before use, phenol was equilibrated to pH 8.0 with TrisCl pH 8.0 as DNA partitions into the organic phase at <pH 7.8. Solid phenol was melted at 68°C, hydroxyquinoline was added to a final concentration of 0.1% (w/v) (acts as an antioxidant, a chelator of metal ions and an RNase inhibitor). An equal volume of buffer (0.5 M TrisHCl pH 8.0) was added to the liquified phenol and stirred for 15 mins. The two phases were then allowed

to equilibrate and as much as possible of the upper aqueous phase was removed. The extraction was repeated using equal volumes of 0.1 M TrisHCl pH 8.0 until the pH of the phenol was > 7.8. An equal volume of 0.1 M TrisHCl pH 8.0 and 0.2% (w/v)  $\beta$ -mercapthoethanol were added to the phenol, which was then stored at 4°C in the dark until required.

### **2.3.3 Phenol/chloroform extraction and ethanol precipitation**

Phenol/chloroform extraction and ethanol precipitation was carried out to concentrate nucleic acid samples or change the buffers in which a sample was dissolved. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution, mixed by vortexing and centrifuged for 10 min at 13,000 x g. The upper aqueous phase was removed, taking care not to take any material from the interphase and this was placed in a fresh autoclaved eppendorf tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous phase, vortexed as before and centrifuged for 5 min at 13,000 x g. Again the upper aqueous phase was removed to a fresh tube. One tenth volume of 3 M sodium acetate (pH 5.2) was added to the solution of DNA, mixed and then 2 and a half volumes of 100% (v/v) ethanol was added. This mixture was vortexed briefly and incubated at room temperature for 5 min. The DNA samples were then centrifuged for 30 min at 12,000 x g at 4°C, the supernatant was removed and pellets were washed with 1 ml 70% (v/v) ethanol to remove excess salts. The tube was centrifuged for 5 min at 10,000 x g, the supernatant was removed and pellets were air dried for approximately 10 min. Pellets were resuspended in an appropriate volume of sterile Tris-EDTA (TE) (pH 8.0) or dH<sub>2</sub>O.

### **2.3.4 Restriction enzyme digestion of DNA**

Restriction enzymes bind specifically to and cleave double-stranded DNA at specific sites within or adjacent to a particular sequence known as the recognition site. The restriction enzymes used were supplied with incubation buffers at a concentration of 10X (working concentration 1X). DNA was digested with restriction endonucleases for

identification purposes or to linearise or cut fragments from a plasmid DNA digests were performed by adding

200 ng – 1 µg of DNA (Final concentration of <300 ng/µl)  
1 µl of enzyme/µg of DNA solution (10 U)  
10 X buffer to a final concentration of 1X  
dH<sub>2</sub>O to the final volume required

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

### **2.3.5 Ligation reactions**

Two fragments of DNA may be ligated in one of two ways. The majority of restriction endonucleases digest DNA leaving either a 5' or a 3' overhang. If the ends of the linear DNA molecules to be ligated are compatible then they may be ligated by cohesive ligation. If they are not, blunt-end ligation may be required. During ligation, the ligase enzyme catalyses the formation of a phosphodiester bond between adjacent nucleotides only if one contains a 5' phosphate group and the other contains a 3' hydroxyl group. Since recircularisation of vector DNA can be a potential problem, it is necessary to carry out a dephosphorylation reaction on the vector DNA prior to including it in the ligation reaction. Removal of 5' phosphate groups was carried out by treatment of vector DNA with Calf Intestinal phosphatase (CIP). Digested vector DNAs (<100ng/µl) were dephosphorylated using CIP in a 100µl volume (CIP was added at 1 unit/100 pmoles for cohesive termini and 1 unit/2 pmoles for blunt termini). The solution was mixed gently and incubated for 30 min at 37°C. This was followed by an enzyme denaturation step achieved by heating to 75°C for 10 min. DNA was then purified by phenol/chloroform extraction and ethanol precipitation (section 2.3.3).

Cohesive-end ligations of equimolar amounts of vector and insert DNA (<1µg) were generally carried out overnight at 16°C in a commercial ligation buffer (5mM ATP) with 10 units of T4 DNA ligase /ml in a total reaction volume of 10µl. After ligation, the

samples were heated at 70°C for 10 min to inactivate the ligase and stored on ice until required in transformation

Blunt-end ligations are usually less desirable than cohesive-end ligations due to their much lower efficiency. Nonetheless, blunt-end ligations were required in cases where restriction enzymes generated blunt ended molecules. Also, if two compatible cohesive ends cannot be generated, it is necessary to 'fill-in' the unmatched bases at the 5' or 3' overhangs and then carry out a blunt-ended ligation reaction. DNA repair was achieved by using the Klenow fragment of *E coli* DNA polymerase I (New England Biolabs) as follows. The DNA was resuspended at a concentration of 50 µg/ml in 1X Eco Pol buffer (supplied with the Klenow), dNTPs were added to a final concentration of 33 µM each, 1 µl of Klenow was added and the reaction was placed at 25°C for 15 min. The enzyme was inactivated by heating to 70°C for 10 min. This DNA was then purified by phenol/chloroform extraction and ethanol precipitation (see Section 2.3.3). Ligation reactions were then performed as for cohesive-end reactions except that a 1:3 to 1:5 molar ratio of vector:insert DNA was used in the reactions to improve the efficiency of ligation.

### **2.3.6 Preparation of competent cells**

The Calcium chloride (CaCl<sub>2</sub>) method was employed to prepare competent cells. An *E coli* strain (DH5α) was streaked from a glycerol stock on to a 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 SOB (see Appendix) broth. This culture was incubated in a shaking incubator at 200 rpm overnight 37°C. An aliquot of this starter culture (2 ml) was then used to inoculate 100 ml of sterile SOB and incubated at 37°C with shaking until the O.D. of the culture at 640 nm was between 0.4 and 0.8 (approximately 2 hr 15 min). The cells were then transferred to two sterile 50 ml falcon tubes and incubated on ice for 10 min followed by centrifugation at 4,000 x g, 4°C for 10 min. The resulting pellets were resuspended in 25 ml of 100 mM CaCl<sub>2</sub>, mixed gently and incubated on ice for a further 20 min. Centrifugation was carried out as before.

(4,000 x g at 4°C for 10 min) followed by removal of the supernatant and the pellet was then resuspended in a 1% (w/v) volume of CaCl<sub>2</sub>. The competent cells were stored on ice and used within 24 hr.

### **2.3.7 Transformations**

Two hundred microliters of competent cells were placed in a pre-chilled microcentrifuge tube containing 10 µl of DNA at a concentration of approximately 100 ng/10 µl. The contents were mixed gently and incubated on ice for 30 min, during which time an aliquot of SOC (see Appendix) was pre-heated to 42°C. After 30 min on ice the cells were heat-pulsed at 42°C for 90 s followed by incubation on ice for a further 2 min. One milliliter of preheated SOC was then added to the cells and incubated at 37°C in a shaking incubator for 1 hr 10 min. The cells were concentrated by centrifugation following which 800 µl of the supernatant was removed and discarded. The cells were resuspended in the remaining supernatant and plated out with the appropriate controls on LB plates containing ampicillin and incubated overnight at 37°C. Only bacteria that took up plasmid DNA encoding ampicillin-resistance grew on the LB amp plates. Recombinant colonies were thus used to inoculate 5 ml of LB containing ampicillin, incubated overnight at 37°C and DNA minipreparations were carried out as described in section 2.3.8. Glycerol stocks of all bacterial cultures were prepared by the addition of 0.5 ml of a 50% (v/v) glycerol solution to 0.5 ml of an overnight bacterial culture of interest and storing at -80°C.

### **2.3.8 Small scale preparation of plasmid DNA (miniprep)**

This method is a modification of a protocol from Sambrook *et al* (1989). A single bacterial colony was used to inoculate 5 ml of LB medium (with appropriate antibiotic) and incubated overnight at 37°C. An aliquot (1.5 ml) of this culture was added to a sterile microfuge tube and centrifuged for 30 s at room temperature, the remainder was stored at 4°C. The medium was removed from the tube, leaving the pellet as dry as possible. The pellet was resuspended thoroughly in 100 µl of solution I by vigorous vortexing. To this,

200  $\mu$ l of freshly prepared solution II was added, the tube contents were mixed by inverting the tube rapidly a number of times. Ice-cold solution III (150  $\mu$ l) was added and the tubes were vortexed gently for 10 s.

The lysate was centrifuged for 5 min at 12,000  $\times$  g, the supernatant was transferred to a fresh tube, taking care not to carry over any of the white precipitate. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed by vortexing and centrifuged for 5 min at 12,000  $\times$  g. The upper aqueous phase was removed to a fresh microfuge tube, to which 2 and a half volumes of 100% (v/v) ethanol were added, the solution was vortexed and centrifuged for 5 min at 12,000  $\times$  g. The supernatant was discarded and the pellet was washed with 1 ml 70% (v/v) ethanol, centrifuged as before and the supernatant removed. The pellet was air-dried for 10-15 min. After resuspending in 50  $\mu$ l of TE (pH 8.0), 1  $\mu$ l of DNase-free RNase A (20  $\mu$ g/ml) was added, the tubes vortexed, incubated at 37°C for 1 hr and then stored at 4°C.

### **2.3.9 Qiagen<sup>TM</sup> plasmid DNA purification protocol**

Plasmid DNA was purified using the QIAGEN-tip 100 solution system from Promega. All buffers used are described in the Appendix. A glycerol stock of the bacteria of interest was streaked out on LB-ampicillin agar and incubated overnight at 37°C, an isolated colony from this plate was used to inoculate a 5 ml LB-ampicillin starter culture and incubated in a shaking incubator at (300rpm) 37°C for 8 hrs. One millilitre of the starter culture was used to inoculate 25 ml of LB-ampicillin 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  $\lambda = 600$  nm. The following centrifugation steps were carried out using a JA-20 rotor in a Beckman centrifuge. The bacterial culture was transferred to a centrifuge tube and subjected to centrifugation at 6,000  $\times$  g for 15 min at 4°C. The supernatant was removed and the pellet was dried by inverting the tube on tissue paper and allowing any remainder of the supernatant to drain off. The bacterial pellet was resuspended completely in 4 ml of ice-cold Buffer P1 containing RNaseA (100 $\mu$ g/ml) and then 4 ml of freshly prepared Buffer P2 was added and incubated at room temperature for 5 min.

Following incubation, 10 ml of prechilled Buffer P3 was added, mixed by gentle inversion of the tube (5-6 times) and then incubated on ice for 20 min. The mixture was then centrifuged for 1 hr at 20,000 x g at 4°C.

The Qiagen-tip 100 was equilibrated by applying 4 ml of QBT buffer and allowing the column to empty by gravity. The column does not dry out at this stage as the flow of buffer will stop when the buffer reaches the upper filter. After the centrifugation step, the supernatant was removed immediately from the tube without disturbing the pelleted material and applied to the column after filtering through 1MM filter paper. The QIAGEN-tip was washed with 2 x 10 ml of Buffer QC. DNA was then eluted with 5 ml of Buffer QF. DNA was precipitated by adding 0.7 volumes of room-temperature isopropanol and centrifuged immediately at 15,000 x g for 30 min at 4°C and the supernatant was carefully removed. The resulting pellet was washed with 70% (v/v) ethanol, allowed to air dry for 5 min and redissolved in a suitable volume of TE or dH<sub>2</sub>O. DNA was then quantified by spectrophotometric analysis as described in section 2.3.13.

### **2.3.10 Agarose gel electrophoresis of DNA**

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

An appropriate quantity of agarose or low melt agarose was added to 100 ml 1X TBE/TAE buffer based on the percentage agarose gel required. Increasing the percentage agarose (1.8-2.0%) in the gel was generally used to improve resolution of smaller DNA fragments while separation of larger DNA molecules was best achieved by using lower percentage gels (0.7%-0.8%). The agarose was completely dissolved by boiling and after sufficient cooling (~60°C) the gel was cast into the Hybaid horizontal gel electrophoresis system and a comb was inserted for formation of wells. The gel was allowed to set for at least 1 hour before filling the chamber with 1X TBE/TAE and removing the comb. Sample buffer containing bromophenol blue as a tracking marker was added to each sample before loading up to a maximum volume of 20µl per well. DNA sample buffer

was also added to 500 ng of a 1Kb or 100 bp DNA ladder which was loaded as a size marker. The gel was run at constant voltage (5 V/cm), usually at 100V, for 1-2 hr. When complete, the gel was stained in ethidium bromide (0.5 mg/ml) for 30 min, placed in distilled water to destain for 15 min and viewed under UV illumination.

### **2.3.11 Isolation of DNA from agarose gels**

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

### **2.3.12 Purification of DNA from low melt agarose**

All DNA fragments of interest were purified from agarose using the Wizard PCR Preps DNA Purification System (Promega) as follows: the agarose gel slice (300 mg) was placed in a sterile microcentrifuge tube and heated to 70°C until the agarose had melted completely. One millilitre of the resin provided was added to the liquified solution and mixed gently but thoroughly for 20 s (not vortexed).

A 2 ml syringe (plunger removed) was attached to the Wizard minicolumn and the DNA/agarose/resin mix was added to the syringe. The plunger was replaced and the mix was pushed gently through the column. The column was washed with 2 ml of 80% (v/v) isopropanol and then subjected to centrifugation at 10,000 x g for 2 min to dry the resin. The DNA was eluted from the resin by adding 50 µl of TE or dH<sub>2</sub>O to the minicolumn,

incubating for 1 min at room temperature followed by centrifugation at 10,000 x g for 20s. The purified DNA was stored at 4°C or at -20°C.

### **2.3.13 Spectrophotometric analysis of nucleic acids**

DNA and RNA concentration was determined by measuring the absorbance at 260 nm, which is the wavelength at which nucleic acids absorb maximally ( $\lambda_{\max}$ ). A 50 µg/ml preparation of pure DNA has an absorbance of 1 unit at 260 nm while 40 µg/ml of pure RNA also has an absorbance reading of 1 at this wavelength. The purity of an RNA or DNA preparation was determined by reading absorbance at 260 nm, the  $\lambda_{\max}$  for nucleic acids and at 280 nm, the  $\lambda_{\max}$  for proteins and obtaining the ratio for these absorbances. Pure DNA and RNA have  $A_{260}/A_{280}$  ratios of 1.8 and 2.0 respectively. Lower ratios indicate the presence of protein while higher ratios often indicate residues of organic reagents.

## **2.4 CELL CULTURE METHODS**

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

### **2.4.1 Culture of cells in suspension**

All media compositions and media supplements are given in the Appendix. The cell lines DG75, DG75 tTA, DG75 tTA EBNA 2, DG75 tTA LMP1, BJABtTA, Mutu 1, Mutu 3 c95, Mutu 3 LMP1-, X50-7, BL41, BL41-B958, IARC 171, Rael BL, IARC 290B, Ag876 III, OKUBL, OKU LCL, Jurkat and Jurkat tTA LMP-1 were maintained in supplemented RPMI 1640. Additional supplements were added to some culture media (see section 2.4.4). Cultures were seeded at a density of  $2 \times 10^5$  to  $5 \times 10^5$  cells per ml in 25 cm<sup>2</sup> flasks and expanded in 75 cm<sup>2</sup> flasks. Cells were sub-cultured two or three times per week by harvesting into a sterile centrifuge tube and centrifuging at 1000 x g for 5

min at room temperature. The cell pellet was resuspended gently in an appropriate volume of fresh media and replaced into the tissue culture flask. All cell lines were incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37°C in a Heraeus cell culture incubator.

#### **2.4.2 Culture of adherent cells**

C33A, C33A Neo and C33A LMP1 were maintained in high glucose DMEM supplemented with 10% (v/v) FCS, penicillin (100 units/ml) and streptomycin (1 µg/ml). In addition, the growth medium for latter two cell lines was supplemented with 600 µg/ml geneticin. Cultures were seeded into 25 cm<sup>2</sup> and 75 cm<sup>2</sup> tissue culture flasks. As the cells were strongly adherent trypsinisation was required for harvesting prior to sub-culturing. For trypsinisation the medium was decanted and the cells were washed with 2 ml of sterile 1X PBS to remove any residual FCS which contains a trypsin-inhibitor activity (α<sub>2</sub>-macroglobulin). Two millilitres of 1X trypsin EDTA solution was placed in each flask and the flasks incubated at 37°C for 5 min or until all the cells could be visualised as having detached from the flask surface. The cell suspension was then decanted into a sterile centrifuge tube containing 5 ml of sterile supplemented media (FCS inhibits trypsin) and centrifuged at 1000 x g for 5 min. Cells were resuspended in supplemented medium at 2 to 5 x 10<sup>5</sup> cells/ml, using 5 ml per 25 cm<sup>2</sup> flask and 15 ml per 75 cm<sup>2</sup> flask. Cells were then incubated as in section 2.4.1.

#### **2.4.3 Preparation of conditioned medium**

Conditioned medium was prepared by regrowing cells in exponential phase of growth in fresh medium for 24 h. After centrifugation of the cell suspension at 1,000 x g for 5 min at room temperature, the supernatant was filtered through a 0.45 µm filter (Gelman Sciences) and then used immediately for testing.

#### 2.4.4 Media supplements

Supplements were added to the growth media of certain cell lines to (a) improve cellular proliferation or (b) to select cells containing transfected plasmids (all media supplements are outlined in the Appendix). L-cysteine is required for the survival and proliferation of many group I BL cell lines. However, L-cysteine is rapidly oxidised under normal culture conditions. To improve proliferation of the group I Burkitt's lymphoma cell line Mutu 1,  $\alpha$ -thioglycerol was added to growth media as a stable substitute for L-cysteine. The  $\alpha$ -thioglycerol was dissolved in bathocuprine disulfonic acid (BCS) which effectively prevents auto-oxidation of thiols in liquid solutions. Sodium pyruvate was also added to protect against  $H_2O_2$  which may be generated. HEPES was added to maintain an alkaline pH of 7.4. The cell lines DG75 tTA LMP1 and Jurkat tTA LMP1 are tetracycline-responsive cell lines in which the LMP-1 gene is cloned downstream of a promoter containing a binding site for a hybrid tetracycline regulated transactivator (tTA) which is constitutively expressed from a second co-transfected plasmid (Floettmann *et al.*, 1996). Tetracycline binds the tTA and prevents it binding to the promoter which remains silent, but upon removal of tetracycline from the growth medium the tTA binds the promoter sequence and activates transcription. These cell lines were maintained in supplemented RPMI containing 1  $\mu$ g/ml of tetracycline. Every three weeks the transfectants were reselected by the addition of 800  $\mu$ g/ml of hygromycin to DG75 tTA cultures, 800  $\mu$ g/ml of hygromycin and 2,000  $\mu$ g/ml of geneticin (G418) to DG75 tTA LMP1 and 300  $\mu$ g/ml of hygromycin and 2,000  $\mu$ g/ml geneticin to Jurkat tTA LMP1 cells.

The stably transfected cell lines C33A Neo and C33A LMP1 were maintained in supplemented high-glucose DMEM containing 600  $\mu$ g/ml of geneticin. The parental cell line C33A was maintained in supplemented high glucose DMEM.

#### 2.4.5 Cell counts

Cell counts were performed using an improved Neubauer haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viability. Ten microlitres of trypan blue was added to 90  $\mu$ l of a cell suspension and mixed. A sample of this mixture was added to the counting chamber of the haemocytometer and cells were visualised by light microscopy. Viable cells exclude the dye and remained clear while dead cells stained blue. Cell numbers were ascertained by multiplying the average cell count (of three individual counts) by the dilution factor (usually 1:1) and again by the volume of the haemocytometer chamber ( $1 \times 10^4$ /ml). Thus, cell counts were expressed as number of cells per ml.

#### 2.4.6 Cell storage and recovery

Cell stocks were prepared for long term storage as follows. Suspension cells  $1 \times 10^7$  cells in exponential phase were pelleted and resuspended in 800  $\mu$ l of supplemented RPMI to which 100  $\mu$ l of FCS was added, then placed on ice for 10 min. DMSO was added to a final concentration of 10% (v/v), mixed gently and transferred to a sterile cryotube. Adherent cells one confluent 75 cm<sup>2</sup> flask of adherent cells was used per cell stock. Adherent cells were washed with 1X PBS followed by trypsinisation and resuspension in 900  $\mu$ l of FCS and 100  $\mu$ l of DMSO. The cells were 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 prewarmed supplemented media. The cells were centrifuged at 1000 x g for 5 min, the pellet was resuspended in 5-10 ml of fresh supplemented medium, transferred to a culture flask and incubated at 37°C in 5% CO<sub>2</sub>.

### **2.4.7 Induction of gene expression in the tetracycline-regulated cell system**

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

### **2.4.8 Transient transfections**

Transient transfection of cells was performed by the DEAE-dextran protocol or by electroporation. In both cases, cells were seeded at a density of  $5 \times 10^5$  per ml of medium 24 h prior to transfection. Total DNA for transfection was normally coprecipitated in 100% ethanol, then washed in 70% ethanol and resuspended in a final volume of 30  $\mu$ l TE buffer pH 7.4 (this pH is very important), using the same total quantity of DNA per transfection.

#### **2.4.8.1 Electroporation of B lymphocytes**

On the day of transfection, cells were centrifuged at 1,000 x g for 5 min, washed once in a small (one-fifth) volume of complete RPMI medium and resuspended in complete RPMI medium at a density of  $2 \times 10^7$  cells per ml. For each transfection, a 60 mm culture dish with 5 ml complete medium (RPMI 1640/glutamine/10 %FCS) was preincubated at 37°C for at least 1 h prior to transfection. The DNA to be transfected (dissolved in 30  $\mu$ l TE pH 7.4) was added to the bottom of labeled cuvettes (Biorad, 0.4 mm) placed on ice. 500  $\mu$ l of the cell suspension ( $= 1 \times 10^7$  cells) was then dispensed into the cuvettes and mixed with the DNA. Each cell/DNA mix was then pulsed at either 270 V in the case of Mutu I cells or 300 V in the case of IARC 290B and IB4 cells with a capacitance of 960  $\mu$ F in a Biorad electroporator with a capacitance extender, and the cuvettes returned to ice immediately after electroporation. Cells must not stay longer than 10 min on ice.

before being transferred to media. Contents of cuvettes were transferred to culture dishes using a micropipette and yellow tip, treating the cells gently during the process. Cuvettes were washed with media from the culture dish, and placed at 37°C in a 5% CO<sub>2</sub> incubator for the required amount of time.

#### **2.4.8.2 DEAE Dextran-mediated transfection**

Details of all solutions required for this protocol are given in the Appendix section. The day before transfection, cells were seeded at  $5 \times 10^5$  cells/ml. After 24 h in culture, cells were counted again – it was essential for cell numbers to have almost doubled before beginning the transfection, thus ensuring that cell growth is in logarithmic phase and that cells are at their optimum for the uptake of DNA during transfection.  $1.4 \times 10^7$  cells were used for each transfection. On the day of transfection, cells were centrifuged at 1,000 x g for 5 min, resuspended in sterile TBS and  $1.4 \times 10^7$  cells dispensed into 25 ml sterile tubes. After centrifugation, all of the supernatant was removed with any traces of TBS carefully (without disturbing the cell pellet) removed using a pipette tip. The DNA-DEAE dextran mixes were prepared in microfuge tubes as follows. A total of between 6 and 16.0 µg of DNA made up to a volume of 30 µl with TE buffer pH 7.4 was used per transfection and 570 µl TBS was then added to the DNA in a microfuge tube. For any transfection set, the total amount of DNA was kept constant with any deficiencies made up with the corresponding empty vector. Then, 600 µl of 1 mg/ml DEAE dextran solution made up in TBS was mixed with the DNA solution and the cell pellet gently resuspended in the DNA-DEAE dextran mix. The transfection cocktails were incubated at room temperature for 30 min (40 min in the case of Jurkat T cells) with gentle swirling every 5-10 min to allow homogenisation. Transfections were terminated by adding 10 ml of warm (37°C) complete RPMI medium and the cells pelleted by centrifugation at 1,000 x g for 5 min. The cells were then washed once more with 10 ml complete medium and then transferred in 10 ml of medium to 25 cm<sup>2</sup> cell culture flasks for incubation.

#### 2.4.8.3 Luciferase assays

Cells for luciferase assay were normally harvested 24 or 48 h post-transfection. Cells were pelleted by centrifugation at 1,000 x g for 5 min at room temperature, washed once in sterile PBS and then transferred to a microfuge tube in 1 ml of sterile PBS. After centrifugation at 5,000 x g for 5 min at room temperature, the supernatant was completely removed and the cell pellet resuspended in 100 µl of Reporter lysis buffer (1 X, Promega). The tubes were vortexed for 10-15 s and lysis allowed to proceed for 15 min on ice. The lysates were subjected to one freeze-thaw cycle and then clarified by centrifugation at 12,000 x g for 5 min and the supernatant saved in a fresh tube. Samples were stored at -80°C until required, when 20 µl was taken for assay. Samples are stable in lysis buffer over several freeze-thaw cycles. At the time of assay, it was important to allow sufficient time for the detection reagent (stored at -80°C) to equilibrate to room temperature. Subsequently, 100 µl of detection reagent was added to the sample, mixed by repetitive pipetting (3 times) and light emission integrated over a period of 60 s after lag period of 10 s in a luminometer. Luciferase activity levels were adjusted for transfection efficiencies, estimated using β-galactosidase activities expressed from pCMVlacZ construct (1µg) included in the transfections (section 2.4.8.4). Normalised luciferase values were expressed as fold activation relative to control and the average from 2 independent experiments were usually plotted unless indicated otherwise.

#### 2.4.8.4 β-galactosidase assay

The β-galactosidase assay was performed using the same lysates as that used for measuring the luciferase activity. Mock-transfected (lacking DNA) cells were included as a control to account for endogenous enzyme activity and these values used to calculate the activity due to transfection of the *lac Z* containing expression vector. Cell extract (30 µl) was added to 3 µl 100X Mg solution, 66 µl ONPG (0-nitrophenyl β-D galactopyranoside) and 201 µl 0.1 M sodium phosphate pH7.5 (Appendix, Sambrook *et al*, 1989) and incubated at 37°C for 30 min or until a faint yellow colour developed. A reaction tube was included in which ONPG substrate was omitted in order to obtain a

background reading. Reactions were terminated by adding 500  $\mu$ l 1 M  $\text{Na}_2\text{CO}_3$ . Optical densities were read at 420nm over a linear range of 0.2-0.8.

## **2.4.9 Stable transfections**

### **2.4.9.1 Establishment of sensitivity of cells to geneticin (G418)**

Mutu I cells, in logarithmic phase of growth, were seeded at a density of  $2 \times 10^5$  cells per ml in media containing 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 or 2000  $\mu$ g of active G418 per ml and grown for 12 days under standard conditions. Media was changed every 4 days during this time and cell viability measured every 3 days by trypan blue exclusion. The minimum concentration of G418 which killed > 95% of the cells after the 12 day period was determined and this concentration then used in subsequent selection procedures for the generation of stable transfectants (section 2.4.9.2).

### **2.4.9.2 Generation of a pool of transfectants of Mutu I cells Expressing HA-Bfl-1**

Stable transfectants of Mutu I expressing HA-tagged Bfl-1 or HA were established by electroporation of the cells with 10  $\mu$ g of pcDNA3HA-Bfl-1 or pcDNA3HA as described in section 2.4.9.1. A mock transfection (without DNA) was also set up and the cells maintained under similar experimental conditions as the *HA-Bfl-1* – or *HA*- transfectected cells. At 48 h post-electroporation, the media was replaced with selection medium i.e. normal growth medium but containing 1,400  $\mu$ g active G418 per ml. Media containing antibiotic was changed every 3 – 4 days for the next 21 days by which time all of the mock-transfected cells were killed. The transfected population of cells was then examined for the expression of HABfl-1 and its functional consequence within the next 14 days.

## **2.4.10 Apoptosis assays**

### **2.4.10.1 DNA fragmentation analysis**

For the DNA ladder assay, cells were washed once in PBS and then lysed in a buffer comprising 0.8 % sodium lauryl sarcosinate, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0 and 0.5 mg/ml proteinase K for 12 h at 50°C followed by treatment with 0.5 mg/ml RNase A for 4 h at 37°C. The DNA was mixed with DNA loading dye (Appendix) and electrophoresed through a 2% agarose – 1 x TBE gel at 60 V for 3 h in 1 x TBE buffer. The gel was stained in an ethidium bromide bath before visualisation under UV light.

### **2.4.10.2 Acridine orange staining of cells**

Ninety-five µl of the cell suspension mixed with 5 µl of a 100 µg/ml solution of acridine orange was placed on a glass slide and then immediately visualised with a fluorescence microscope. Non-apoptotic cells display a diffuse chromatin staining pattern while apoptotic cells display a characteristic condensed chromatin staining pattern (Gregory *et al* , 1991). The percentage of apoptotic cells was determined from counts on at least 150 cells per individual culture.

## **2.5 RNA ANALYSIS**

### **2.5.1 RNase-free environment**

RNA is easily degraded by ubiquitous RNase enzymes and thus stringent measures were employed to avoid this potential hazard. All glassware and metal spatulas were baked prior to use at 180°C for 16 - 24 h in order to inactivate any RNases. Sterile disposable plasticware is generally considered RNase-free and thus did not require treatment. RNases are resistant to autoclaving but they can be deactivated by the chemical diethylpyrocarbonate (DEPC). DEPC was added to solutions at a final concentration of 0.1 % (v/v), incubated at room temperature for 18 hr and then autoclaved. Solutions which contain amines such as Tris cannot be DEPC-treated as the amines are inactivated by DEPC. Solutions containing these chemicals were prepared using DEPC-treated upH<sub>2</sub>O followed by autoclaving. Hands are a major source of RNase contamination, thus, 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 2.4.5. RNA was extracted from cultured cells using the commercial reagent RNA ISOLATOR™ (Genosys). Cells grown in suspension were pelleted and then lysed in RNA ISOLATOR™ by repetitive pipetting. One millilitre of RNA ISOLATOR™ was used per  $1 \times 10^7$  cultured cells. Cells grown in monolayers were lysed directly in the cell culture plates as trypsin can lead to the introduction of RNases. The lysate was collected by a sterile cell scraper and pipette and then homogenized as above. The homogenised sample was incubated at room temperature for 5-10 min to allow complete dissociation of nuclear protein complexes (the procedure may be stopped at this point by storing samples at -70°C). Phase separation was achieved by adding 0.2 ml of chloroform per 1 ml of RNA ISOLATOR. The samples were covered and shaken gently but thoroughly for 15 s or until completely emulsified. Samples were incubated at room temperature for 15 min.

The resulting mixture was centrifuged at 12,000 x g for 15 min at 4°C. Following centrifugation the mixture separates into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. The aqueous phase, which contains the RNA, was removed to a fresh tube and RNA was precipitated by adding 0.5 ml of isopropanol per ml of RNA ISOLATOR used initially. The samples were stored for 10 min at room temperature, then centrifuged at 12,000 x g for 10 min at 4°C. The resulting RNA pellet was washed using 1 ml of 75% (v/v) ethanol by inverting the tube 5 times. The pellets were then recentrifuged at 10,000 x g for 5 min at 4°C and the 75% (v/v) ethanol was removed. Pellets were air dried and dissolved in DEPC-treated upH<sub>2</sub>O. The resulting RNA preparation was heated at 60°C and mixed gently to ensure a homogeneous solution prior to aliquoting. An aliquot was removed for spectrophotometric and gel electrophoretic analysis (sections 2.3.13, 2.5.3).

### **2.5.3 RNA analysis by gel electrophoresis**

In order to check the integrity of RNA, isolated samples were run on a 1.6 % (w/v) agarose – 1 x TAE gels. These gels were prepared as outlined in section 2.3.10. The RNA samples (5 µl) were prepared for electrophoresis by adding 15 µl of RNA sample buffer and 3 µl of RNA loading buffer. The samples were heated to 65°C for 10 min prior to loading on the gel. The gel was run in 1X TAE as described in section 2.3.10. As ethidium bromide is included in the RNA loading buffer the gels did not require further staining and could be visualized directly on a UV transilluminator. The presence of two strongly staining bands represent the 28 S (approx. 5.0 kb) and the 18 S (approx. 1.8 kb) ribosomal RNAs, which indicated intact RNA. Degradation is observed by a smear running down the length of the gel.

### **2.5.4 Northern blotting**

Northern blotting was carried out according to Sambrook *et al.*, (1989). RNA samples were first size-fractionated on a formaldehyde gel.

#### 2.5.4.1 Treatment of electrophoresis apparatus

Prior to electrophoresis of RNA through formaldehyde gels, the electrophoresis apparatus was treated to remove any RNase. The tank, gel tray, comb and lid were washed in detergent, rinsed well in DEPC-treated H<sub>2</sub>O and then air dried in 100% (v/v) ethanol. The tank, gel tray and comb were then immersed in a 3% (v/v) solution of Hydrogen peroxide for 15 minutes. The apparatus was then rinsed thoroughly in DEPC-treated upH<sub>2</sub>O and allowed to air-dry.

#### 2.5.4.2 Electrophoresis of RNA through gels containing formaldehyde

As formaldehyde vapours are toxic these gels were prepared in a fume hood. The gel was prepared by melting the appropriate amount of agarose in DEPC-treated water, cooling to 60°C and adding 5X formaldehyde gel running buffer and formaldehyde to give a final concentration of 1 X and 2.2 M respectively (Appendix). The gel was allowed to set for at least 1 h. The samples were prepared by mixing the following in a microcentrifuge tube:

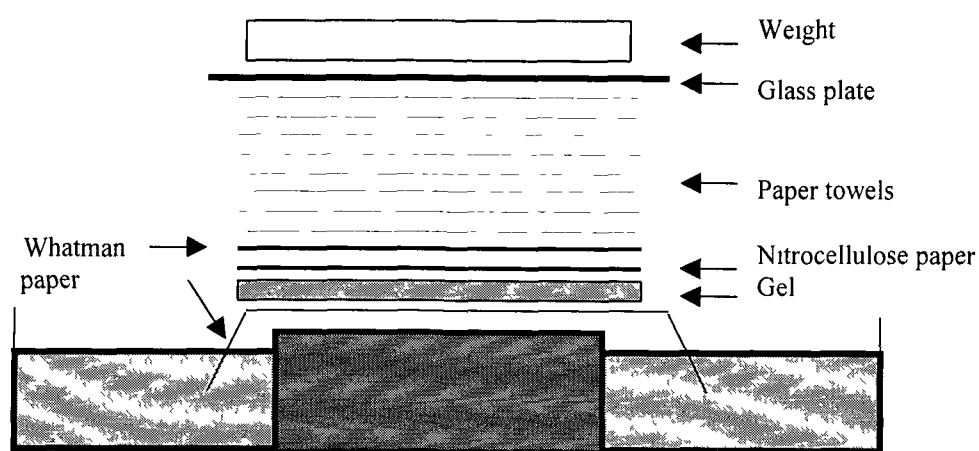
RNA (up to 30 µg)	4.5 µl
5X formaldehyde gel running buffer	2.0 µl
Formaldehyde	3.5 µl
Formamide	10 µl

The samples were incubated at 65°C for 15 min, chilled on ice then centrifuged briefly to collect the sample. Ethidium bromide was added to the samples (0.01 µg/sample) to enable examination of the quality of the RNA briefly under UV (70% strength) after electrophoresis. Sterile DEPC-treated gel loading buffer (2 µl) was added to each sample and applied to the gel immediately after pre-running the gel for five minutes at 5V/cm. Gels were run while submerged in 1X formaldehyde gel running buffer for approximately 3 hr or until the dye front had migrated two-thirds of the way down the gel. The gel was

removed from the buffer and viewed under UV light. The gel was then processed for blotting as described in section 2.5.4.3.

#### 2.5.4.3 Transfer of denatured RNA to nitrocellulose filters

Gels containing formaldehyde were first washed in several changes of DEPC-treated H<sub>2</sub>O. As the percentage agarose used to prepare the gel was greater than 1% (w/v), the gel was soaked in 0.05 M NaOH for 20 min. This treatment hydrolyses the RNA and improves the efficiency of transfer. The gel was then rinsed in DEPC-treated H<sub>2</sub>O and soaked in 20X SSC for 45 min (Sambrook *et al*, 1989). Unused areas of the gel were trimmed away and the top left-hand corner was cut for orientation purposes in this and succeeding operations. Capillary transfer was used to transfer the RNA onto the filter; transfer was set up as illustrated in Figure 2.1.



**Figure 2.1** Capillary transfer of nucleic acids from agarose gels to solid supports. Adapted from Sambrook *et al*, 1989.

A solid support was placed in a bath of 20X SSC. A sheet of 3MM Whatman paper was cut to cover the support and dip down either side into the buffer. The washed gel was then placed (wells facing down) on to the Whatman and covered by a piece of

nitrocellulose membrane cut to the size of the gel. This membrane was pre-wetted in deionised water and then soaked in 20X SSC for at least 5 min prior to placing it on the gel. The buffer chamber was then covered with cling film. Two pieces of 3MM Whatman paper which had been soaked in 2X SSC were then placed on top of the filter paper, care was taken at all times to ensure that no bubbles were trapped when preparing the transfer. A stack of paper towels were then placed on top of the gel and held in place by a weight. Transfer was allowed to proceed overnight. After transfer was completed, the saturated paper towels were removed as were the Whatman paper sheets. The gel and the filter paper were removed together and turned upside down on a clean piece of towel. The position of the wells were marked using a ball point pen and the gel was then discarded. The filter was washed briefly in 6X SSC to remove any remnants of the gel that may have adhered to the membrane, then placed on a fresh sheet of Whatman paper and allowed to dry for at least 30 min. The dried filter was then placed between two pieces of 3MM Whatman paper and baked in an oven at 80°C for 2 hr. The filter was then used directly for prehybridisation or stored at room temperature wrapped in aluminium foil.

#### **2.5.4.4 Purification of $\alpha^{32}\text{P}$ -labelled riboprobes from denaturing polyacrylamide gels**

Riboprobes generated by *in vitro* transcription of the APO-2 template set (section 2.5.6.1) were size-fractionated on a 5% polyacrylamide-urea gel (section 2.5.6.4) and those corresponding to the genes of interest (*bcl-2*, *bfl-1*, *bcl-x* and *GAPDH*) were excised from the gel by the 'crush and soak' method (Sambrook *et al.*, 1989) for use in Northern analysis. After polyacrylamide gel electrophoresis of the set of *in vitro* transcribed fragments, the plates were disassembled. 'Orientation' markers were prepared by carefully streaking  $\alpha^{32}\text{P}$ -UTP-containing loading dye (containing bromophenol blue) across a piece of filter paper in a recognisable shape and wrapping the filter paper tightly with selotape. Two of such 'orientation' markers were placed directly on the gel at positions away from the lanes containing the samples of interest. The gel was then wrapped in cling film and exposed to X-ray film for a length of time (usually 1-2 min) that permitted visualisation of the bands of interest and the orientation markers. Once the

appropriate exposure of the gel was achieved, the X-ray film was placed on the gel and aligned using the 'orientation' markers. The DNA fragments of interest could thus be located in the gel and were excised using a clean sharp razor blade. After excision of the gel slices, the fragments were eluted by crushing the gel slices in 2 volumes (approximately 300µl) of elution buffer (see Appendix) in a microfuge tube. After a short vortex, the tubes were centrifuged briefly and incubated at 37°C for 3-4 hours, with intermittent crushing and vortexing every 30-40 minutes. The samples were then subjected to centrifugation at 13,000 x g for 20 min and the supernatant saved. A half-volume (75 µl) of elution buffer was added to the crushed gel slice, the sample vortexed briefly, and subjected to centrifugation as before and incubated at 37°C for an hour. After centrifugation, the supernatant was saved and combined with the first eluant fraction. The riboprobes were precipitated by the addition of 2µg of yeast tRNA and 2 volumes of ice-cold ethanol and incubated at -20°C for at least 2 hours. After centrifugation at 13,000 x g for 30 min at room temperature, the pellet was washed with 75% ethanol, air dried and dissolved in 50µl of DEPC-treated upH<sub>2</sub>O. A one µl sample of the purified riboprobe was used to measure the level of incorporation of radiolabel using a scintillation counter.

#### **2.5.4.5 Prehybridization and hybridization protocol**

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

#### **2.5.4.6 Stringency washing**

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

**Table 2 3 Northern blot stringency washes**

Wash solution	Temperature	Time
1X SSC, 0 1% (w/v) SDS	Room temperature	30 s
1X SSC, 0 1% (w/v) SDS	22°C	30 min
1X SSC, 0 1% (w/v) SDS	22°C	30 min
0 1X SSC, 0 1% (w/v) SDS	65°C	30 min
0 1X SSC, 0 1% (w/v) SDS	65°C	30 min

After washing the membrane was removed gently from the hybridization bottle and placed in a stomacher bag in a cassette. The blot was kept moist at all times with 0 1X SSC, 0 1% (w/v) SDS. The membrane was exposed to X-ray film for 24-96 hr at -80°C.

### **2.5.5 Determination of *bfl-1* mRNA half-lives**

The half-life of *bfl-1* mRNA in various cell lines was determined by monitoring the rate of decay of *bfl-1* mRNA by Northern blotting (section 2 5 4) of RNA extracted from cells (seeded at  $10^6$  cells per ml) at various times after treatment with an inhibitor of RNA synthesis, actinomycin D (5µg/ml). *Bfl-1* mRNA levels in treated cells were determined by densitometric analysis of autoradiograms after Northern blotting using the LISCAP capture and image analysis program (Pharmacia), and values were corrected for variations due to loading after estimation of the 18S rRNA levels in each lane (as determined by densitometric analysis of ethidium bromide – stained gels). Values thus obtained were expressed as a percentage of the *bfl-1* mRNA level in untreated cells (taken as 100%). The half-life of *bfl-1* mRNA (i.e. the time taken for the mRNA to decay to 50% of the level observed in untreated cells) was then determined from the best-fit semilogarithmic line of the graph of these values plotted against time (hours) of exposure to actinomycin D.

### 2.5.6 RNase protection assay

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

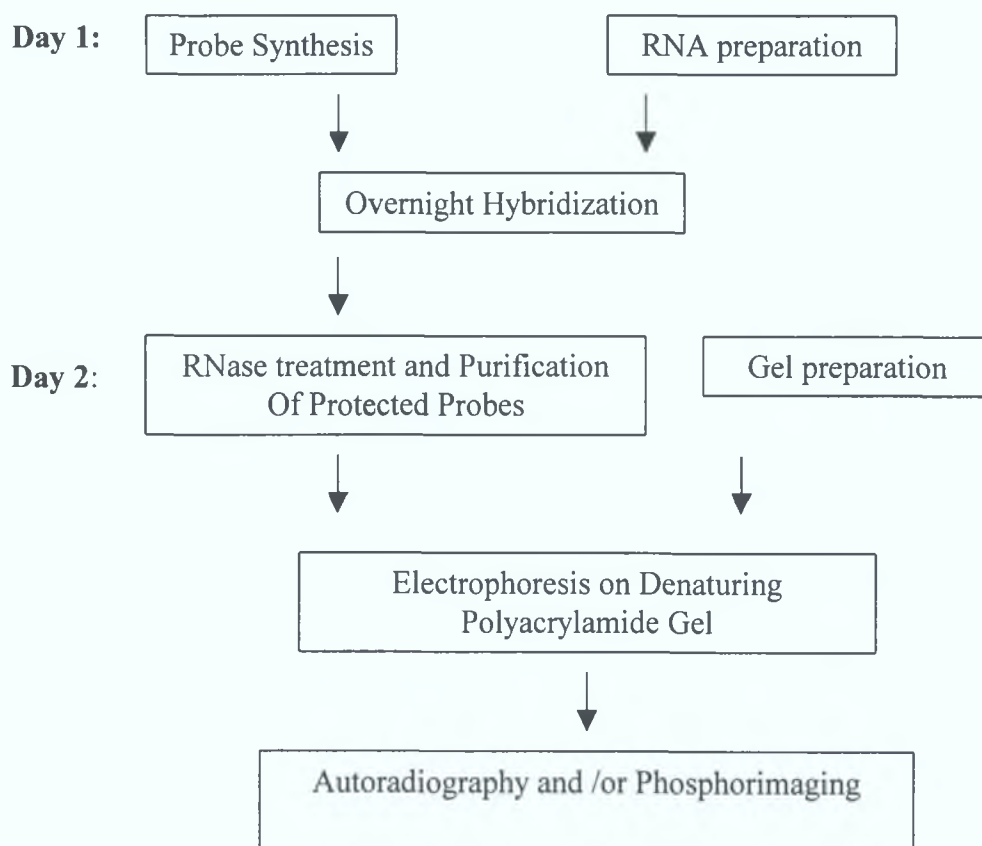


Figure 2.2 Overview of the ribonuclease protection assay protocol.

#### 2.5.6.1 Probe synthesis

The [ $\alpha$ - $^{32}$ P]UTP, GACU nucleotide pool, DTT, 5X transcription buffer and the template DNA set was brought to room temperature prior to setting up the reactions. The following were added to a 1.5 ml microcentrifuge tube for each probe synthesis:

Rnasin	1μl
GACU pool	1μl
DTT	2μl
5X transcription buffer	4μl
Template DNA	1μl
[ $\alpha$ - <sup>32</sup> P]UTP(3000Ci/mmol, 10mCi/ml)	10μl
T7 RNA polymerase	1μl

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

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

The contents were vortexed into an emulsion and centrifuged for 5 min at room temperature. The upper phase was transferred to a fresh tube containing 50μl of chloroform isoamyl alcohol (50:1), the contents of the tube mixed by vortexing and the tube subjected to centrifugation (top speed) for 2 min at room temperature. The upper aqueous phase was transferred to a sterile 1.5 ml tube to which 50μl of 4M ammonium acetate and 250μl of ice cold 100% (v/v) ethanol was added. The contents of the tube were mixed by inversion and the tube incubated at -70°C for 30 min followed by centrifugation at 4°C for 15 min. The supernatant was removed and the pellet was washed with 100μl of ice cold 90% (v/v) ethanol after which the pellet was air-dried for 5-10 min. The pellet was solubilized by the addition of 50 μl of hybridization buffer and gentle vortexing. Duplicate one μl samples of the labelled probe were quantified in a scintillation counter. A maximum yield of  $\sim 3 \times 10^6$  Cherenkov counts/μl with an

acceptable lower limit of  $-3 \times 10^5$  Cherenkov counts/ $\mu\text{l}$  was expected. The probe was stored at  $-20^\circ\text{C}$  until required. Generally probe can only be used for two overnight hybridizations when labeled with [ $\alpha$ - $^{32}\text{P}$ ]UTP.

#### **2.5.6.2 RNA preparation and hybridization**

RNA was prepared using the RNA isolation method outlined in section 2.5.2. Fifteen  $\mu\text{g}$  of total RNA was used for each probe hybridization. Each RNA sample was diluted to 50  $\mu\text{l}$  with DEPC-treated  $\text{upH}_2\text{O}$  to which 50  $\mu\text{l}$  of 4 M ammonium acetate and 250  $\mu\text{l}$  of ice cold 100% (v/v) ethanol were added. The samples were mixed by inverting and stored at  $-70^\circ\text{C}$  for one hr or  $-20^\circ\text{C}$  overnight. The precipitated RNA was collected by centrifugation at  $12,000 \times g$  for 30 min at  $4^\circ\text{C}$  and the pellet was washed with 90% (v/v) ice-cold ethanol. After removal of the supernatant and subsequent air-drying, the pellet was resuspended in 8  $\mu\text{l}$  of hybridization buffer by gently vortexing for 3-4 min followed by a brief centrifugation step. Two microlitres of the probe was then added to each RNA sample and mixed by pipetting. A drop of mineral oil was added to each sample and the tubes were centrifuged briefly in the microfuge. Samples were then placed in a heating block for 3 min which had been preheated to  $90^\circ\text{C}$ , and then immediately turned down to  $56^\circ\text{C}$ , allowing the temperature to ramp down slowly, and hybridisation allowed to proceed for 12-16 hr. The temperature of the heating block was then turned down to  $37^\circ\text{C}$  prior to RNase treatment allowing it to ramp down slowly and the tubes maintained at  $37^\circ\text{C}$  for 15 min.

#### **2.5.6.3 RNase treatments**

An RNase mixture was prepared by adding 2.5 ml of RNase buffer to 6  $\mu\text{l}$  of RNase A + T1 mix, per 20 RNA samples (RNase A 80ng/ $\mu\text{l}$ , RNase T1 250 U/ $\mu\text{l}$ ). The RNA samples were removed from the heating block and 100  $\mu\text{l}$  of the RNase cocktail was added underneath the oil into the aqueous layer (bubble). The tubes were

microcentrifuged for 10 s and incubated for 45 min at 30°C. Before the RNase treatment was completed a Proteinase K mixture was prepared (per 20 samples),

Proteinase K buffer 1 X      390 µl

Proteinase K (10 mg/ml)      30 µl

Yeast tRNA (2 mg/ml)      30 µl

The mixture was mixed together and 18 µl aliquots were added to a sterile 1.5 ml microcentrifuge tubes. The RNase-treated samples were extracted from underneath the oil and transferred to the tube containing the proteinase K mixture (avoiding transfer of oil). The RNase/Proteinase K mixture was vortexed briefly, subjected to brief centrifugation and incubated for 15 min at 37°C. Tris-saturated phenol pH 8.0 (65 µl) and 65 µl of chloroform isoamyl alcohol (50:1) were added to the samples, vortexed into an emulsion then centrifuged for 5 min at room temperature. The upper aqueous phase was extracted, avoiding the interphase, and transferred to a fresh tube to which 120 µl of 4 M ammonium acetate and 650 µl of ice cold 100% (v/v) ethanol was then added. The contents of the tubes were mixed by inversion and the tubes were subsequently incubated at -70°C for 30 min. Samples were subjected to centrifugation for 5 min at 4°C and the pellet was then washed with ice cold 90% (v/v) ethanol and allowed to air-dry for 5-10 min. The pellets were resuspended in 5 µl of 1 X loading buffer (provided in the kit). Prior to loading onto the gel the samples were heated to 90°C for 3 min and placed immediately in an ice bath.

#### **2.5.6.4 Denaturing polyacrylamide gel electrophoresis**

Five percent (w/v) polyacrylamide-urea gels were prepared according to the formula given in the Appendix. A Biorad gel electrophoresis system was used in this study. The plates were washed with detergent, rinsed first with tap water and then with dH<sub>2</sub>O and finally wiped in one direction with tissue soaked with 100% (v/v) ethanol. One plate was siliconsided using Sigmacote (Sigma). The apparatus was set up, the gel cast and the comb

inserted as per manufacturer's instructions. Prior to loading of the samples, the gel was pre-run in 1 x TBE at a constant power of 50 W for 45 min – 1 h. The wells were washed several times with electrophoresis buffer (1 x TBE) to remove unpolymerised acrylamide and urea that would otherwise interfere with loading and/or electrophoresis of the sample. After loading the samples, the gel was electrophoresed at 50 W for approximately 3 h. After electrophoresis, the gel was carefully removed, placed on two sheets of 3MM Whatmann paper cut to the size of the gel, covered with Saran Wrap and vacuum dried at 80°C for 1-2h. The dried gel was then exposed to X-ray film in a cassette with two intensifying screens was exposed at -70°C for an appropriate length of time.

## **2.6 WESTERN BLOT ANALYSIS**

### **2.6.1 Preparation of cellular protein extracts**

Prior to protein isolation the viability of the cells was examined and the viable cell count determined as described in Section 2.4.5. Four different methods were employed to isolate cellular protein with the method used depending on the ability to extract the protein of interest.

#### **2.6.1.1 Preparation of cellular protein - Method A**

This method was employed to isolate total cellular protein (i.e. cytoplasmic and nuclear proteins) and was used for the extraction of LMP-1 and Bcl-2 proteins. Cells were pelleted at  $1,000 \times g$  for 5 min and washed with 5 ml of ice-cold PBS. The cells were then transferred to a microfuge tube in 1 ml of ice-cold PBS, pelleted at  $3,000 \times g$  for 5 min and all of the supernatant removed. Adherent cells were washed twice with 5 ml of ice-cold PBS, scraped into 1 ml of PBS and then pelleted by centrifugation at  $3,000 \times g$  for 5 min. The cell pellet was resuspended in ice-cold suspension buffer (0.1 M NaCl, 0.01 M Tris-HCl pH 7.6, 0.001 M EDTA pH 8.0,  $1 \mu\text{g/ml}$  aprotinin,  $1 \mu\text{g/ml}$  leupeptin and  $100 \mu\text{g/ml}$  PMSF) using 200  $\mu\text{l}$  of suspension buffer for every  $5 \times 10^6$  cells. An equal volume of 2 $\times$  SDS gel loading buffer [100 mM Tris-HCl pH 7.6, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.2% (w/v) bromophenol blue] was immediately added to the cell suspension, after which the sample becomes extremely viscous. The sample was then placed in a boiling water bath for 10 min and then subjected to sonication for 1 min on full power to shear the DNA. The lysate was clarified by centrifugation at  $12,000 \times g$  for 10 min at room temperature. The supernatant was aliquoted and stored at  $-20^\circ\text{C}$ . Samples were analysed by SDS-PAGE, loading equivalent amounts of protein from approximately  $5 \times 10^5$  cells per lane.

#### **2.6.1.2 Preparation of cellular proteins– Method B**

This method extracts cytoplasmic and some membrane proteins but not nuclear proteins and was used for the extraction of HA-tagged Bfl-1 protein (D'sa Eipper *et al* , 1996) Cells were harvested as described in Section 2.6.1.1 Cell lysis was performed by resuspending the cell pellet in ice-cold lysis buffer [ 1%(v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1mM sodium orthovanadate, 100 µg/ml PMSF and 2 µg/ml leupeptin] using 100 µl of lysis buffer for every  $5 \times 10^6$  cells Cell lysis was carried out for 30 min on ice with occasional mixing Lysates were clarified by centrifugation at 13,000 x g for 15 min at 4°C The supernatant was saved, dispensed into aliquots and stored at –20°C Protein concentration of the lysates was determined by using the BCA protein assay as described in section 2.6.2 and equal amounts of protein (30-50 µg) used in SDS-PAGE

#### **2.6.1.3 Preparation of cellular proteins – Method C**

This method was used to extract the membrane protein CD40 (Baker *et al* , 1998) Cells were harvested as described in Section 2.6.1.1 Cell lysis was performed by resuspending the cell pellet in ice-cold lysis buffer [1% (v/v) Triton X-100, 150 mM NaCl, 25 mM Tris-HCl (pH 7.6), 1 mM disodium EDTA, 1mM sodium orthovanadate, 50 mM NaF, 100 µg/ml PMSF and 2 µg/ml leupeptin] using 100 µl of lysis buffer for every  $5 \times 10^6$  cells Cell lysis was carried out for 30 min on ice with occasional mixing Lysates were clarified by centrifugation at 13,000 x g for 15 min at 4°C The supernatant was saved, dispensed into aliquots and stored at –20°C Protein concentration of the lysates was determined by using the BCA protein assay as described in section 2.6.2 and equal amounts of protein (30-50 µg) was used in SDS-PAGE

#### **2.6.1.4 Preparation of cellular proteins – Method D**

This method was used to extract the phosphorylated and non-phosphorylated forms of the Bcl-2 protein from cells and has been described previously (Haldar *et al* , 1995) Cells

were harvested as described in Section 2.6.1.1. Cell lysis was performed by resuspending the cell pellet in ice-cold lysis buffer [3% (v/v) Triton X-100, 150 mM NaCl, 10 mM sodium phosphate (pH 7.4), 5 mM disodium EDTA, 1 mM sodium orthovanadate, 50 mM NaF, 10 mM sodium pyrophosphate, 100 µg/ml PMSF and 2 µg/ml leupeptin] using 100 µl of lysis buffer for every  $5 \times 10^6$  cells. The extracts were subjected to three freeze-thaw cycles and lysis then continued on ice for 30 min with occasional mixing. Lysates were clarified by centrifugation at 13,000 x g for 15 min at 4°C. The supernatant was saved, dispensed into aliquots and stored at -20°C. Protein concentration of the lysates was determined by using the BCA protein assay as described in section 2.6.2 and equal amounts of protein (30-50 µg) used in SDS-PAGE.

### **2.6.2 Estimation of protein concentration**

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

### 2.6.3 Protein electrophoresis, preparation of SDS-PAGE gels

A two phase SDS-PAGE system was used to analyse proteins with a 5% stacking gel and either a 10% (LMP-1, phosphotyrosine containing proteins, CD40), or a 15 0% (Bcl-2, HA-Bfl-1) resolving gel prepared as outlined below

#### Resolving Gel

<u>10 ml</u>	<u>10% (ml)</u>	<u>15% (ml)</u>
acrylagel	3 33	5 0
bis-acrylagel	1 35	2 025
1 5 M Tris (pH8 8)	2 5	2 5
dH <sub>2</sub> O	2 61	0 265
10% (v/v) SDS	0 10	0 10
10% (v/v) APS	0 10	0 10
TEMED	0 01	0 01

#### Stacking Gel

<u>2 5 ml</u>	<u>5% (ml)</u>
acrylagel	0 42
bis-acrylagel	0 168
1 M Tris (pH6 8)	0 312
dH <sub>2</sub> O	1 5475
10% (v/v) SDS	0 025
10% (v/v) APS	0 025
TEMED	0 0025

#### **2.6.4 Polyacrylamide gel electrophoresis (PAGE)**

An ATTO protein gel electrophoresis system was used in this study. Glass plates were washed with detergent, rinsed first with tap water and then with dH<sub>2</sub>O and finally wiped in one direction with tissue soaked with 100 (v/v) ethanol. The gasket was placed about the ridged plate, the plates were put together and secured with clamps. The depth of the resolving gel was marked on the outer plate. The resolving gel was then poured to within 2 cm of the top of the larger plate and overlaid with 100% (v/v) ethanol. When set, the ethanol was removed and the stacking gel was poured. A clean comb was inserted and the gel was allowed to polymerise for 45 min-1 hr. The electrophoresis tank was filled with 1X Tris-glycine running buffer to the level of the horizontal rubber gasket. After polymerisation the gaskets clamp stands and comb were removed. Unpolymerised gel was removed by gently rinsing the wells with running buffer and the wells were then straightened using a needle. The prepoured gels were lowered into the buffer at an angle to exclude air bubbles from the gel-buffer interface. The chamber formed by the inner plates was filled with 1X running Buffer, the samples were loaded (after addition of SDS loading buffer containing 2-mercaptoethanol to a final concentration of 1 x and heating to 95°C for 5 min) and the electrodes were attached. The gels were electrophoresed at a constant current of 30 mA per gel for approximately 1 hr in the case of the 10% resolving gel or 1h 30 min in the case of the 15% resolving gel. After electrophoresis, the plates were disassembled and the gel was either placed in transfer buffer prior to western blotting or stained in Coomassie blue (Appendix). Staining took place for 30 min, agitating constantly. The gel was then washed several times in destain solution (see Appendix) with constant agitation, until all background staining was removed.

#### **2.6.5 Western blot analysis**

An SDS-PAGE gel was used for electrophoresis of protein samples together with pre-stained molecular weight markers (New England Biolabs) as described in section 2.6.4. Two pieces of 3MM filter paper were cut to size of the gel as was the nitrocellulose membrane. The sponges from the transfer apparatus along with 2 pieces of 3 MM filter

paper cut to size and the SDS gel were soaked in transfer buffer. One pre-soaked sponge was placed on one side of the transfer apparatus, followed by one piece of filter paper. The gel was then placed on top of the filter paper. The nitrocellulose membrane which had been pre-wet in distilled water and then soaked in transfer buffer for 5 min was placed on top of the gel, ensuring that no bubbles were trapped between any of the layers. The second stack of filter paper and sponges were placed on top of the membrane, the transfer apparatus was assembled and placed in the blotting apparatus with the gel on the side of the negative (black) electrode and the nitocellulose on the side of the positive (red) electrode. Protein transfer was allowed to take place at a constant voltage of 75 V for 2 h with the buffer kept chilled by an ice pack (placed in the transfer apparatus) and recirculated. After transfer, the apparatus was disassembled and the membrane was washed briefly in TBS to remove any traces of gel. The membrane was then incubated in Blocking buffer for 1 - 2 h at room temperature followed by incubation with the appropriate antibody (diluted in Blocking buffer-Table 2.4) 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 2.4: Incubation conditions for antibodies used in western blotting**

Antibody	Name	Dilution/concentration	Blocking buffer
Anti -LMP1	CS1-4*	1/100	Blotto
Anti - HA	HA	1/1000	Blotto
Anti-Bcl2	Bcl2100/124*	1/10	Blotto
Anti-phosphotyrosine	PT-66	0.5µg/ml	5%(w/v)BSA, 0.05% (v/v) Tween20 in TBS
Anti-CD40	G28.5	1µg/ml	Blotto

\* Tissue culture supernatant

After overnight incubation, the membrane was washed three times in TBST (0.1%(v/v) Tween-20 in TBS) for 10 min and then incubated for 10 min in blocking buffer. The

filter was then incubated with the appropriate secondary antibody (an alkaline phosphatase-conjugate anti-mouse antibody (Promega) diluted 1/5000 in Blocking buffer or an alkaline phosphatase-conjugate anti-rabbit antibody (Boehringer Mannheim) diluted 1/1250 in Blocking buffer, for 1 hr at room temperature, followed by washing three times with TBST for 10 min each. All of the above incubations were carried out with agitation. Membranes were then placed in a clean container and covered with BCIP/NBT substrate. The container was placed in the dark at room temperature without shaking for 30 min or longer if required. The filter was then rinsed in distilled water to stop the reaction, photographed and then wrapped in cling film and stored in the dark.

## **2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)**

### **2.7.1 Preparation of nuclear extracts**

Nuclear extracts were prepared essentially according to the method of Dignam *et al* with modifications (1983). The composition of all buffers used is described in the Appendix. Briefly,  $5 \times 10^6$  cells were pelleted at  $1,000 \times g$  for 5 min and washed with 5 ml of ice-cold PBS. The pellet of cells was then resuspended in 1 ml of ice-cold PBS and transferred to a microfuge tube. After centrifugation at  $5000 \times g$  for 4 min at  $4^\circ \text{C}$ , the cells were resuspended in 1 ml of Buffer A. The cells were pelleted at  $5000 \times g$  for 10 min at  $4^\circ \text{C}$ , the supernatant removed and the cells were then lysed in  $20 \mu\text{l}$  of Buffer A containing 0.1% NP 40 for 15 min on ice. The nuclei were pelleted by centrifugation at  $13000 \times g$  for 10 min at  $4^\circ \text{C}$ , the supernatant removed and the nuclei lysed in  $15 \mu\text{l}$  of a hypertonic buffer (Buffer C) for 15 min on ice. The extracts were clarified by centrifugation at  $13000 \times g$  for 10 min at  $4^\circ \text{C}$  after which the supernatant was mixed with  $50 \mu\text{l}$  of Buffer D in a fresh microfuge tube. The concentration of protein in the extracts was determined by using the BCA assay (section 2.6.2) and the extracts were aliquoted and stored at  $-80^\circ \text{C}$  until use.

## 2.7.2 Nature of the oligonucleotide probes and their labeling for use in EMSA

Unlabeled double stranded oligonucleotide probes for use in EMSA were either purchased (a 22 bp oligonucleotide containing the 'consensus' NF-kappa B binding site 5'-GGGGACTTCC-3' from the Immunoglobulin kappa light chain enhancer region and called NF-κB-con), synthesised as single stranded oligonucleotides and then annealed in solution (a 14bp oligonucleotide corresponding to -54 to -41 of the *bfl-1* promoter region containing the NF-kappaB-like site 5'AGAAATTCCA-3' and called -54/-41Bfl-1 NF-κB) or prepared by restriction digestion of the *bfl-1* promoter region -129 to +81 contained in the reporter plasmid -129/+81 Bfl-1CAT. In all three cases labeling was based on the usage of bacteriophage T4 polynucleotide kinase which catalyses the transfer of the radiolabeled  $\gamma$ -phosphate of  $\gamma$ -<sup>32</sup>P ATP to the 5' terminus of the dephosphorylated double-stranded substrate DNA. NF-κB-con is double stranded and contains 5'-hydroxyl blunt ends. For preparation of the double stranded -54/-41Bfl-1 NF-κB oligonucleotide, equimolar amounts of the complimentary -54/-41 NF-κB oligonucleotides (containing 5'-hydroxyl termini) in 1X T4 polynucleotide kinase buffer were mixed in a microfuge tube and the tube placed in a heating block at 95°C for 2 minutes to denature the oligonucleotides. The heating block was then removed from the apparatus and allowed to cool to room temperature (60 – 75 min) for annealing of the oligonucleotides to ensue. The annealed oligonucleotides were either used immediately in the labeling reaction or stored at -20°C. The preparation of restriction fragments from the -129 to +81 region of *bfl-1* for use as probes in EMSA is described in a following section (Section 2.7.2.1).

The labeling reaction was performed by adding

- 10 pmol of substrate DNA containing 5' -hydroxyl termini
- 10X kinase buffer to give a final concentration of 1X
- 50 pmol of  $\gamma$ -<sup>32</sup>P ATP (3000Ci/mmol, 10mCi/ml)
- 20 U T4 polynucleotide kinase
- dH<sub>2</sub>O to a final reaction volume of 50μl

The reaction was incubated at 37°C for 20 min after which end-labeled oligonucleotides were purified away from unincorporated labeled nucleotides by spin-column chromatography through MicroSpin™ G-25 columns essentially according to manufacturer's specifications (Amersham Pharmacia Biotech)

### **2.7.2.1 Preparation of restriction DNA fragments and their labeling for use as probes in EMSA**

The -129/+81 region of the *bfl-1* gene was cloned as a *SalI* – *XbaI* fragment in the pG CAT basic vector (Zong *et al* , 1999) Probes spanning the entire -129/+81 region (*SalI*-*XbaI*) as well as overlapping parts within this region (*SalI*-*ApoI*, *SalI*-*AluI* and *ApoI*-*XbaI*) were generated for use in EMSA (Figure 3 49)

Fifteen µg (equivalent to approximately 10pmol) of the -129/+81Bfl-1 CAT plasmid was linearised with either *SalI* or *XbaI* enzyme (both of which generate 5' protruding ends suitable for the subsequent labeling reaction with T4 polynucleotide kinase) The linearised fragments were then dephosphorylated as described in Section 2 3 5 Following purification by phenol-chloroform extraction and ethanol precipitation, the fragments were end-labeled using T4 polynucleotide kinase and  $\gamma$ -<sup>32</sup>P ATP as described in Sections 2 3 3 and 2 7 2 End-labeled fragments were separated from unincorporated labeled nucleotides by phenol-chloroform extraction and ethanol precipitation in the presence of 0 5M ammonium acetate Following salt removal with 70% ethanol, the pellets were air-dried and dissolved in dH<sub>2</sub>O The 5'-end-labeled *SalI* fragments were cleaved with either *ApoI*, *AluI* or *XbaI* while the 5'-end-labeled *XbaI* fragments were cleaved with *ApoI* The now dual restriction enzyme digested fragments were size-fractionated by non-denaturing PAGE (section 2 7 4) followed by autoradiography to locate and excise the fragments of interest from the gel

### **2.7.3 Binding reaction**

The composition of all buffers used in the binding reaction are given in the Appendix

The binding of nuclear proteins to labeled DNA probe was performed in a reaction comprising

10 X Binding reaction buffer	2μl
non-specific competitor DNA poly dI-dC (2μg/μl)	1μl
<sup>32</sup> P-labeled DNA probe (15,000-20,000 cpm/μl)	1μl
upto 15μg of nuclear extract (in a volume made up to 16μl with Buffer D)	

A 'control reaction' lacking nuclear extract but containing all other components of the binding reaction was also set up. The reaction components were mixed gently and incubated at room temperature for 30 minutes. The reactions were terminated by the addition of sample buffer (DNA loading buffer lacking bromophenol blue).

#### **2.7.3.1 Competition studies**

The specificity of binding of a nuclear protein (s) to the labelled DNA fragment can be assessed by attempting to compete out binding of the protein(s) to the labelled probe by an excess of unlabelled ('cold') probe. If the bandshift observed with labelled probe is specific it should disappear in a competition study with cold probe. Competition studies were carried out by pre-incubating the nuclear extract (in a total volume of 16μl made up with Buffer D) with an excess of cold probe (upto 100 X excess in a volume of 1.0μl) under similar binding reaction conditions as described in section 2.7.3 except with the addition of 'cold' instead of labeled probe, followed by the addition of labeled probe (in a volume of 1.0μl) and incubating the reaction for a further 30 min at room temperature. The reactions were terminated by the addition of sample buffer (DNA loading buffer lacking bromophenol blue).

#### **2.7.3.2 Supershift studies**

The identity of a transcription factor in a bandshift complex can be verified by incubating the nuclear extract with an antibody that recognises the factor prior to performing the binding reaction with the labeled probe. Depending on whether the antibody recognises

an epitope within the DNA-binding domain or elsewhere, the complex formation will either be prevented (observed as only a decrease in intensity of the shifted complex) or further retarded in mobility in the gel (termed a 'supershift'). 'Supershift' studies were conducted by incubating the nuclear extract (in a total volume of 15µl made up with Buffer D) with 1.0µl of an antibody that recognises the transcription factor of interest (p65, c-rel or p50) or an 'irrelevant' protein (Mcl-1) as a control on ice for 20 min. This was followed by the addition of 2µl of 10 x Binding reaction buffer, 1µl of poly dI.dC (2µg/ul) and 1µl of <sup>32</sup>P-labeled DNA probe and the reaction incubated for 30 min at room temperature. The reactions were terminated by the addition of sample buffer (DNA loading buffer lacking bromophenol blue).

#### **2.7.4 Nondenaturing polyacrylamide gel electrophoresis**

Size-fractionation of the 5' end-labeled DNA fragments that map to or within the -129 to +81 region of the *bfl-1* gene (section 2.7.2) and the resolution of the bandshift complexes were conducted by nondenaturing polyacrylamide gel electrophoresis using the Protean II cell system (Biorad).

##### **2.7.4.1 Preparation of the Protean II cell electrophoresis system**

The glass plates, spacers and combs were cleaned with detergent, rinsed thoroughly with tap water followed by upH<sub>2</sub>O, and wiped dry with clean dry tissue paper. 100% (v/v) ethanol was poured onto the glass plates and the plates wiped dry with tissue paper. The spacers and combs were wiped with tissue paper soaked in 100% (v/v) ethanol. The apparatus was then assembled and clamped together according to manufacturer's instructions (Biorad).

##### **2.7.4.2 Casting the gel**

An 8% gel was used for size-fractionation of the 5' end-labeled DNA fragments that map to or within the -129 to +81 region of the *bfl-1* gene while a 4% or 5% gel was used for

the resolution of the bandshift complexes. The composition of the gels is outlined in the Appendix. After addition of TEMED to the gel mix, the gel solution was poured immediately into the gel mould and the comb inserted. The gel was allowed to set for at least an hour, after which the gel mould was removed and placed in the electrophoresis tank. The lower chamber of the tank was filled with 1.2 litres of electrophoresis buffer (0.5 X TBE) whilst the upper chamber was filled with approximately 350 ml of 0.5 X TBE. The comb was carefully removed and the wells were washed thoroughly with electrophoresis buffer to remove any unpolymerised acrylamide that would otherwise impair the migration of the sample. The gel was pre-run for 30–40 min at a constant voltage of 140V before the samples were loaded and electrophoresis was carried out at the same voltage for approximately 2h at room temperature for EMSA analysis with the consensus NF- $\kappa$ B oligonucleotide and the oligonucleotide containing the NF- $\kappa$ B-like site at position –52 of *bfl-1*. For size-fractionation of the 5'-end-labeled fragments and EMSA with these fragments, electrophoresis was carried out at a constant voltage of 200V for 2.5 h. It should be pointed out that bromophenol blue was excluded from the loading buffer added to the samples as it has been reported to disrupt protein-DNA interactions in certain cases (Promega Technical Bulletin No. 110). It was therefore necessary to run just loading buffer containing bromophenol blue into one well of the gel to locate the leading edge of the gel. After electrophoresis, the gel was either exposed to X-ray film and processed for excision of end-labeled DNA fragments for use as probes in EMSA or vacuum dried and exposed to X-ray film to visualise bandshifts.

#### **2.7.4.3 Purification of 5'-end-labelled DNA fragments from polyacrylamide gels**

After polyacrylamide gel electrophoresis of the 5'-end-labeled fragments, the plates were disassembled. 'Orientation' markers were prepared by carefully streaking  $\gamma^{32}\text{P}$ -ATP-containing loading dye (containing bromophenol blue) across a piece of filter paper in a recognisable shape and wrapping the filter paper tightly with selotape. Two of such 'orientation' markers were placed directly on the gel at positions away from the lanes containing the samples of interest. The gel was then wrapped in cling film and exposed to X-ray film for a length of time (usually 1-2 min) that permitted visualisation of the bands

of interest and the orientation markers. Once the appropriate exposure of the gel was achieved, the X-ray film was placed on the gel and aligned using the 'orientation' markers. The DNA fragments of interest could thus be located in the gel and were excised using a clean sharp razor blade. After excision of the gel slices, the fragments were eluted by crushing the gel slices in TE buffer (pH 7.5) contained in a microfuge tube and allowing passive elution to occur overnight at room temperature. The gel pieces were removed from the eluate by centrifugation at 13,000 x g for 20 min at room temperature and the DNA fragments contained in the supernatant purified by phenol-chloroform extraction followed by ethanol precipitation. Following salt removal with 70% ethanol, the pellets were air-dried and dissolved in 20 µl TE buffer (pH 7.5). A 1 µl sample of each labeled probe was used to measure the level of incorporation of radiolabel in a scintillation counter.

#### **2.7.4.4 Gel drying and autoradiography**

After electrophoresis, the buffer was poured out of the electrophoresis tank and the plates disassembled. A piece of Whatmann 3MM filter paper (cut to size) was placed on top of the gel, avoiding air bubbles and the paper lifted gently with the gel attached to it. This was then covered with cling film and placed in a vacuum gel dryer, with the gel facing up. The gel was dried at 80°C for 2h. Once dry, the gel was placed in a cassette and exposed to X-ray film in the dark for at least 12 hours at -80 °C. The film was developed manually by successively immersing the film in developer for 3 min, water for 1 min, fixer for 3 min and water for 1 min. The film was then air-dried for approximately 1 hour. In some cases, development of x-ray film was also carried out using a manual developer.

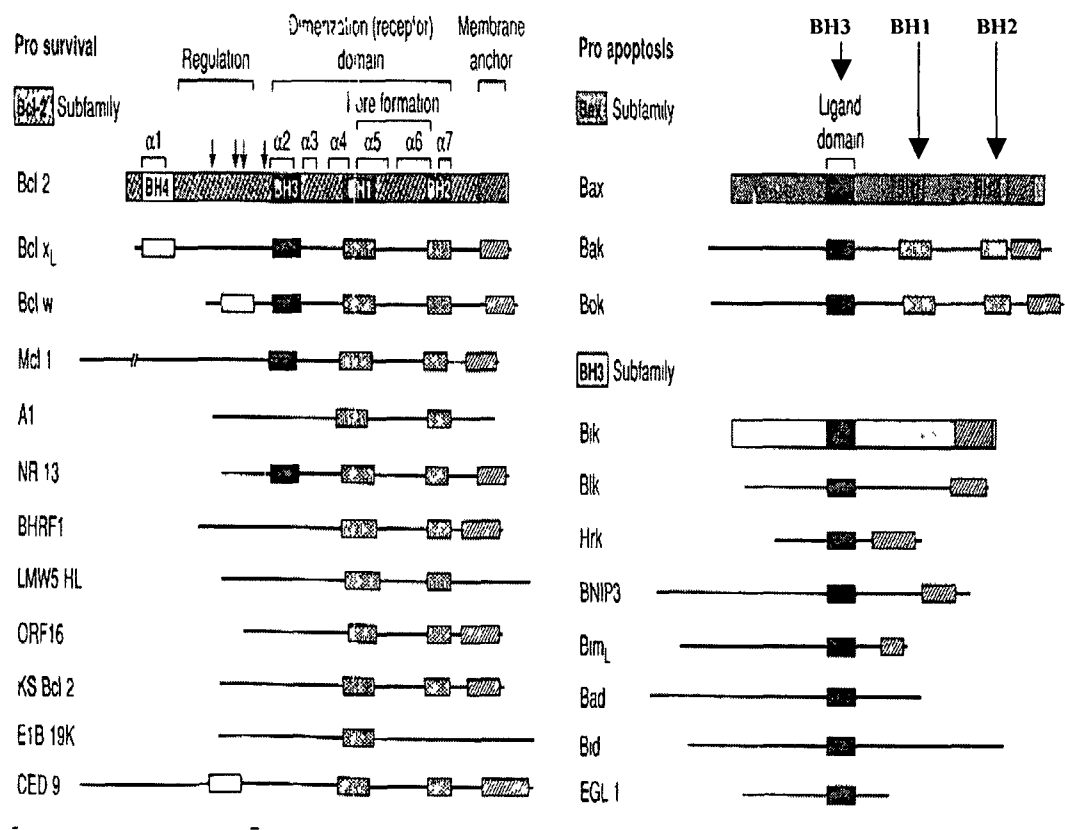
## **CHAPTER 3**

### **RESULTS**

### 3.1 INTRODUCTION

Bcl-2 is the prototype of a family of related proteins classified as either apoptotic death agonists or antagonists. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) (reviewed by Adams and Cory, 1998) (Figure 3.1). Most pro-survival members, contain at least BH1 and BH2, and those most similar to Bcl-2, such as Bcl-x<sub>L</sub>, have all four BH domains. There are two pro-apoptotic subfamilies and these differ markedly in their relatedness to Bcl-2. Bax, Bak and Bok, which contain BH1, BH2 and BH3, resemble Bcl-2. In contrast, the second subfamily of pro-apoptotic proteins, exemplified by B<sub>ik</sub>, Bad and B<sub>id</sub>, possess only the central short (9 to 16 residues) BH3 domain. The BH3 domain of the "BH3 domain" proteins is crucial to their pro-apoptotic activity. Pro- and anti-apoptotic family members can heterodimerise and seemingly titrate one another's function, suggesting that their relative concentration may act as a rheostat for the apoptotic death program (reviewed by Cory and Adams, 1998). Mutagenesis has established that the BH1, BH2, and BH3 domains strongly influence homo- and hetero- dimerisation and the three-dimensional structure of Bcl-x<sub>L</sub> has provided clues as to how this might occur (reviewed by Cory and Adams, 1998). Coalescence of the  $\alpha$  helices in its BH1, BH2 and BH3 regions creates an elongated hydrophobic cleft to which a BH3 amphipathic  $\alpha$ -helix can bind. BH3-cleft coupling may account for all dimerisation within this family. Hence, it is thought that Bax and its analogs may have alternate conformations, one like Bcl-x<sub>L</sub> and another with BH3 rotated outside to allow its insertion into the groove of pro-survival proteins. The BH4 region of Bcl-x<sub>L</sub> is required for pro-survival activity. Heterodimerisation does not appear to be required for pro-survival function but is essential for pro-apoptotic activity particularly for members of the BH3 domain group. Some death agonists may preferentially target subsets of the death repressors. Bok, for example, interacts with Mcl-1 and the Epstein-Barr viral protein BHRF1 but not with Bcl-2, Bcl-x<sub>L</sub> or Bcl-w. Within the BH3 group, B<sub>id</sub> is promiscuous, binding to Bax and Bak as well as to the anti-apoptotic proteins, but the other members bind only to certain of the death inhibitors (Bruckheimer, 1998).

Bcl-2 resides on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope and may register damage to these cellular compartments. Bcl-2 may thus affect the behaviour of these organelles by modifying the flux of small molecules or proteins (reviewed by Cory and Adams, 1998). The carboxy-terminal hydrophobic domain of Bcl-2 appears to be required for membrane docking. The pro-survival proteins seem to maintain organelle integrity. Bcl-2, for instance, prevents the release from mitochondria of cytochrome c. The structure of Bcl-x<sub>L</sub> (particularly its  $\alpha 5$  and  $\alpha 6$  helices) resembles the membrane insertion domains of bacterial toxins. This observation has prompted the hypothesis that members having the BH1 and BH2 domains function by forming pores in organelles such as mitochondria. Bcl-x<sub>L</sub>, Bcl-2 and Bax do form channels in lipid bilayers in vitro, and those created by Bax and Bcl-2 have distinct features including some ion selectivity (reviewed by Cory and Adams, 1998).



**Figure 3.1 The wider Bcl-2 family** Three subfamilies are indicated. The Bcl-2 cohort promotes cell survival, whereas the Bax and BH3 cohorts facilitate apoptosis. BH1 to BH4 are conserved sequence motifs. The functional domains of Bcl-2 are described in the text. The Bax subfamily resembles the Bcl-2

subfamily but lacks a functional BH4 domain. Except for the BH3 domain, the BH3 subfamily is unrelated to Bcl-2.  $\alpha 1$  to  $\alpha 7$  indicates helices identified in Bcl-xL, in which a core of two hydrophobic helices ( $\alpha 5$  and  $\alpha 6$ ) is flanked by five amphipathic helices, and a flexible (nonconserved) loop connects  $\alpha 1$  with  $\alpha 2$ . Small arrows indicate serine and threonine residues phosphorylated in Bcl-2. All proteins compared are mammalian (usually human), except for NR-13 (chicken), CED-9 and EGL-1 (*C. elegans*), and the viral proteins BHRF1, LMW5-HL, ORF-16, KS-Bcl-2, and E1B-19K. Adapted from Cory and Adams, 1998.

A central component of the overall EBV strategy and its role in the development of related malignant disease is the ability of the viral latent proteins to suppress the cellular apoptotic program (Allday, 1996, Klein, 1994). Group I BL cell lines and many EBV-negative BL lines can readily be triggered into apoptosis, whereas group III BL cell lines are relatively resistant to a variety of triggers of apoptosis including growth factor withdrawal,  $\text{Ca}^{2+}$  ionophore treatment and overexpression of the p53 tumour suppressor gene (Henderson *et al*, 1991 and Okan *et al*, 1995). EBV-negative BL cells converted to the type 3 latency state by infection with the B95-8 strain of EBV also display elevated thresholds of resistance to apoptotic stimuli (Gregory *et al*, 1991).

Transfection of individual EBV latent genes into EBV-negative BL cell lines has shown that upregulation of Bcl-2 expression correlates with the expression of LMP-1 (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, point to additional Bcl-2-independent survival mechanisms being important in BL cells and during B-cell differentiation within germinal centers: (i) hyperexpression of Bcl-2 protein in group I cells (well beyond the levels in group III cells) is necessary to attain the high levels of protection observed in group III cells (Milner *et al*, 1992), (ii) prolonged culture of group I lines *in vitro* has been shown to result in enhanced survival in the absence of Bcl-2-upregulation (Milner *et al*, 1992), (iii) 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) and (iv) The induction of Bcl-2 by LMP-1 is a delayed response with maximal levels detectable after 2 days of LMP-1 induction (Rowe *et al*, 1994). LMP-1 has been shown to also upregulate the expression of two other host stress-response proteins that

prevent apoptosis, A20 and Mcl-1 (Laherty *et al.*, 1992; Fries *et al.*, 1996; Wang *et al.*, 1996), with the latter probably serving as a rapidly inducible, transient and short-term effector of cell viability before Bcl-2 induction.

This study set out to investigate EBV-associated changes in the level of expression of a panel of *bcl-2*-related genes by comparing BL cell lines exhibiting a group I and group III phenotype as well as comparing an EBV-negative BL cell line with its EBV-infected counterpart (expressing all of the latent genes) and LCLs. As LMP-1 is a key effector of EBV-mediated transformation and of promotion of survival of B cells, the contribution of this viral protein to cell survival was investigated, based on findings of the preliminary RPA experiments of EBV-associated changes.

## 3.2 RESULTS

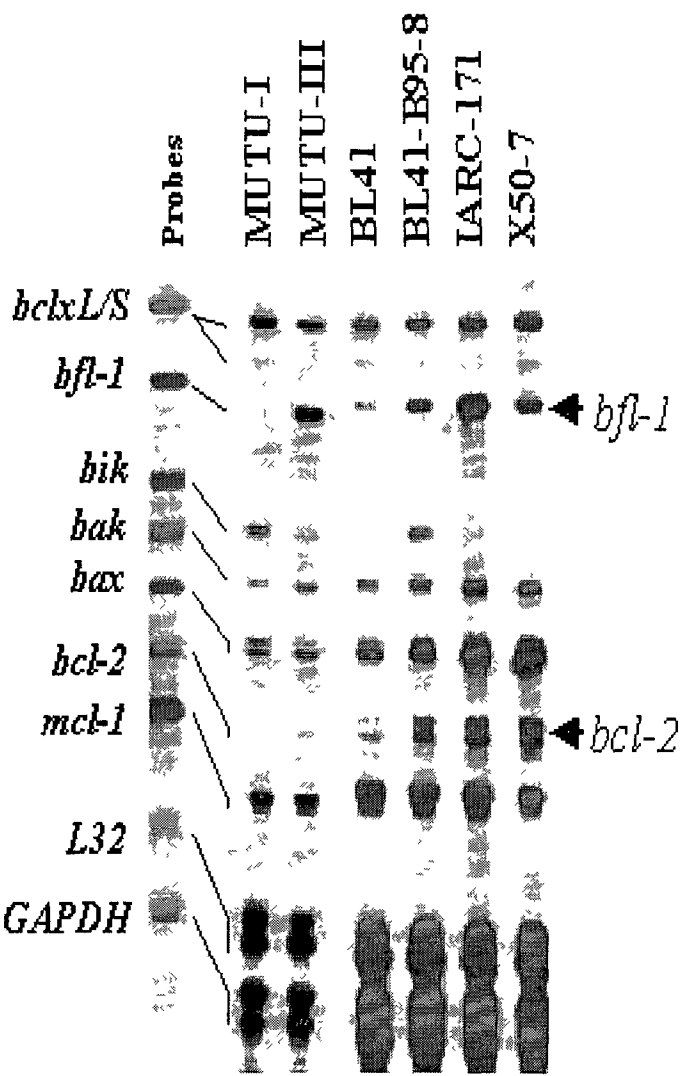
### 3.2.1 EBV-associated changes in the level of expression of *bcl-2*-related genes

As the apoptotic threshold of a cell is determined by the level of functional expression of a combination of pro- and anti- apoptotic proteins, changes in the relative level of expression of these proteins are important in determining the cellular response to apoptotic signals. Cytokine-mediated changes in the level of expression of several of these proteins is controlled at the level of transcription (Adams and Cory, 1998). This study set out to investigate EBV-associated changes in the expression of a panel of *bcl-2*-related genes in BL cells. In a preliminary experiment using two established isogenic EBV-positive BL cell lines, group I Mutu-BL (Mutu I) and group III Mutu-BL (Mutu III), the levels of mRNA transcribed from the *bclx<sub>LS</sub>*, *bfl-1*, *bik*, *bak*, *bax*, *bcl-2* and *mcl-1* genes were compared using multiprobe ribonuclease protection assay (RPA; hAPO-2 kit, Pharmingen). This approach permitted the simultaneous detection and quantitation of these mRNAs in a single sample and also enabled direct comparative analysis of RNA from these two cell lines by the inclusion of probes for transcripts from the two housekeeping genes *GAPDH* and *L32* (Figure 3.2). Group I BL cells (type I latency) express EBNA1 as the sole viral protein; when serially passaged *in vitro*, they 'drift' to

express all of the known latency-associated viral proteins (including LMP-1) and exhibit many of the phenotypic features characteristic of LCLs. In this state, the cells are said to exhibit type III latent infection and have become group III BL cells (Gregory *et al*, 1990). It can be seen from this experiment that significantly elevated steady state levels of *bfl-1* and *bcl-2* were present in Mutu III relative to Mutu I cells (Figure 3.2). The intensities of the bands corresponding to *bfl-1* were quantitated by densitometric scanning of the autoradiogram. GAPDH mRNA levels were similarly assessed and the values thus obtained were used to correct those for the bands of interest to account for any differences in the amounts of RNA analysed. The results indicated that an approximately twenty-fold higher level of *bfl-1* mRNA is present in Mutu III cells relative to Mutu I. The observation of a higher level of *bcl-2* mRNA in Mutu III relative to Mutu I cells is in keeping with the findings of others (Henderson *et al*, 1991).

In order to investigate if upregulated *bfl-1* expression is a general feature of EBV-infected B cells, *bfl-1* mRNA levels in the EBV-negative BL cell line BL41 were compared to those in its EBV-superinfected derivative, BL41-B95.8, an EBV-transformed LCL (IARC171) derived by infection of peripheral B lymphocytes from the same patient from whom BL41 with the B95.8 strain and a reference LCL, X50-7 (Figure 3.2). There were easily detectable levels of *bfl-1* mRNA in BL41, which were significantly elevated in BL41-B95.8. Higher levels of *bfl-1* mRNA were also detected in the two LCLs IARC171 and X50-7 relative to BL41. Comparison of the level of expression of *bcl-2* in BL41-B95.8 and the LCLs IARC171 and X50-7 relative to BL41 also showed a similar trend to *bfl-1* expression. Thus, elevated expression of *bfl-1* and *bcl-2* mRNA appear to be a general feature of LCLs and BL cells expressing all of the viral latency-associated proteins. Transcript from the gene encoding the pro-apoptotic protein Bcl-2 is not detectable in the two LCLs examined in this experiment, an interesting observation in that it is also downregulated in Mutu-BL upon drift to the group III phenotype. This does not however appear to be a general feature of EBV-positive BL cells exhibiting a group III phenotype in that the level of *bcl-2* mRNA is significantly greater in BL41-B95.8 cells relative to BL41. Additionally, although elevated levels of

*bak* mRNA were observed in Mutu III relative to Mutu I, this effect is also inconsistent in that it is not observed in the BL41/B95 8 pair



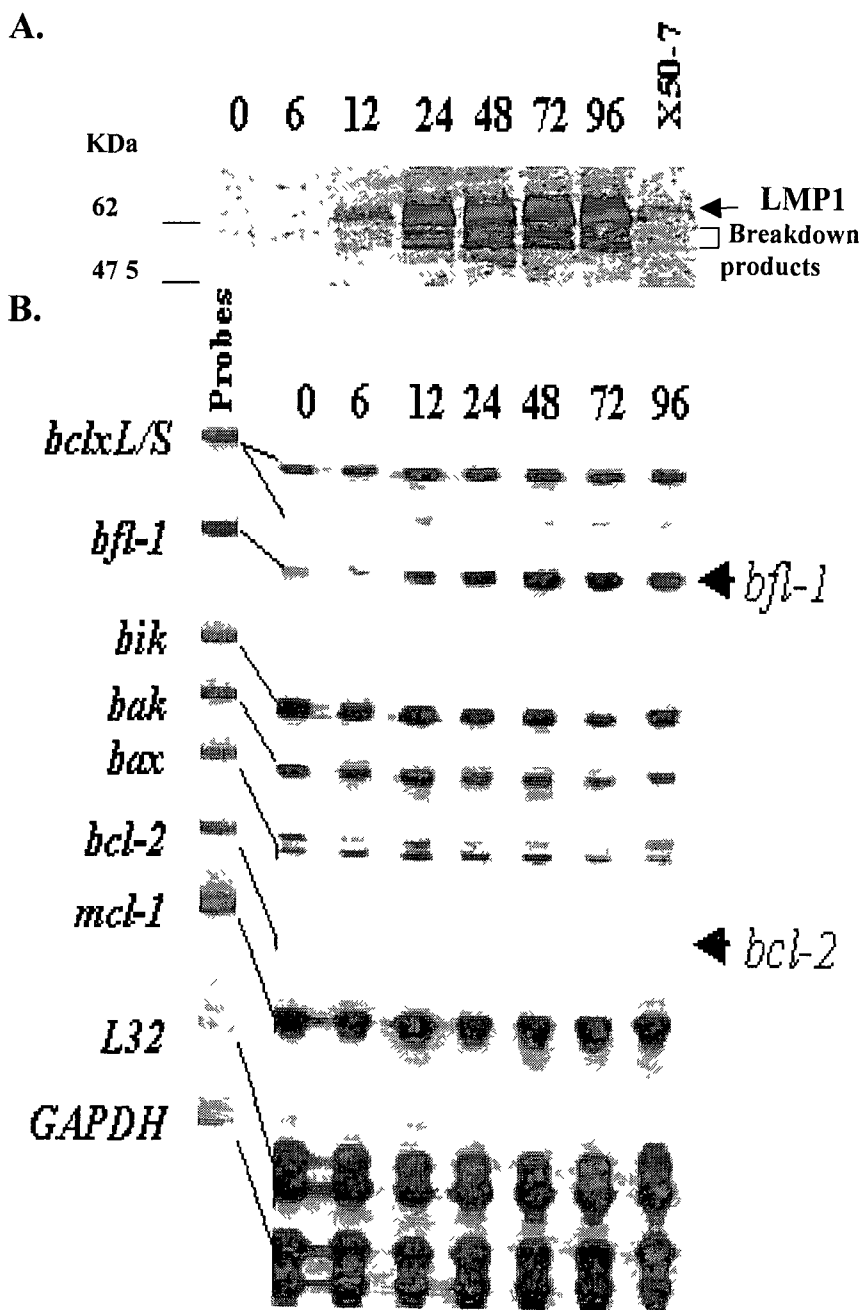
**Figure 3 2 EBV-associated modulation of the steady-state levels of mRNAs corresponding to *bcl-2* related genes in B cells** EBV-infected LMP-1 expressing B cell lines exhibit increased steady-state levels of expression of *bfl-1* and *bcl-2* mRNAs RPA autoradiogram (18 h exposure) in which mRNA levels from the apoptosis-related genes *bclx<sub>L/S</sub>*, *bfl-1*, *bik*, *bak*, *bax*, *bcl-2* and *mcl-1* were analysed in a range of BL cell lines and LCLs Unprotected [<sup>32</sup>P]-labeled antisense riboprobes (5000 cpm, indicated on the figure as ‘Probes’) were loaded alongside RPA-processed samples and are shown linked to their smaller RNase-protected fragments which correspond to the steady-state levels of the corresponding mRNA in the sample The names of the cell lines used are given above each track The locations of

protected fragments derived from *bcl-2* and *bfl-1* mRNAs are highlighted with arrows. The Mutu I and Mutu III tracks are from a separate RPA to the other four tracks.

### 3.2.2 LMP-1 upregulates *bfl-1* expression in the EBV-negative Burkitt's lymphoma cell line DG75

Of all of the EBV latent proteins, LMP-1 has been shown to make a major contribution to promotion of survival of EBV-infected cells (Gregory, 1995). It was therefore of interest to investigate if expression of this viral protein alone could contribute to the upregulation of *bfl-1* by EBV. To this end, an established tightly-regulatable expression system was employed to induce LMP-1 expression in the EBV-negative BL cell line DG75-tTA-LMP-1 (Floettmann *et al*, 1996). In this system, the inducible promoter driving LMP-1 expression contains binding sites for a hybrid tetracycline-regulated transactivator (tTA) that is constitutively expressed in the parental clone DG75-tTA. Removal of tetracycline from the growth medium leads to tTA binding to the promoter and the expression of LMP-1. The level of induced LMP-1 was monitored by Western blot analysis using the anti-LMP-1 mouse monoclonal antibody cocktail CS 1-4 (Rowe *et al*, 1992). In this experiment, LMP-1 was detectable in DG75-tTA-LMP-1 cells at 12 hours post-induction and its level rose to several times that observed in a reference LCL (X50-7) by 96 hours (Figure 3.3 A). The lower molecular weight proteins detected below the major LMP1 band most likely correspond to breakdown products of LMP1 and have been detected in LMP1 expressing cells by anti-LMP1 antibodies in Western blots (Young *et al*, 1988). Total RNA prepared from cells harvested from the same pool and at the same time points as used for monitoring the induction of LMP-1 were used in RPA analysis with the same set of probes as before. The result of this experiment is shown in Figure 3.3 B. The steady-state level of mRNA from the *bfl-1* gene rose significantly in response to induction of LMP-1 in DG75-tTA-LMP-1 cells. Upregulation of *bfl-1* mRNA was dramatic and was detectable at 12 hours post-induction (when the levels of LMP-1 were comparable to those observed in X50-7). Densitometric scanning indicated that the degree of induction of *bfl-1* was maximal by 48 hours at which time a 15-fold induction was observed (after normalisation of values to those for the *GAPDH* internal control mRNA). Although high levels of induction of *bfl-1* mRNA were maintained thereafter, a decrease in the extent of induction to approximately 11-fold was observed by 96 hours.

post-induction In the same experiment, the *bcl-2* mRNA level also rose, although in this case much lower levels of transcript were detected in the cell and maximal upregulation was only detected at 96 hours post induction of LMP-1 upon prolonged exposure of the autoradiogram to film (result not shown) This result of the kinetics of induction of *bcl-2* mRNA by LMP1 was supported by Northern analysis (Figure 3 60)

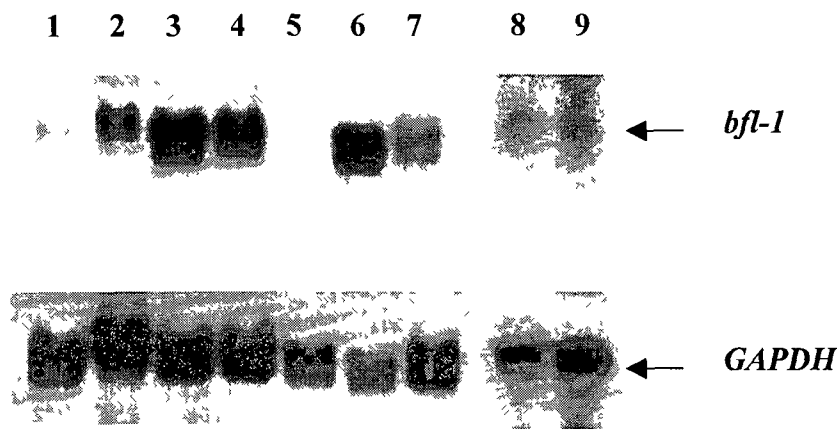


**Figure 3.3 Induction of LMP-1 in DG75-tTA –LMP-1 cells correlates with an increase in the steady-state levels of *bfl-1* and *bcl-2* mRNAs.** **A)** Western blot of DG75-tTA-LMP-1 cells induced to express LMP-1 by reculturing cells in the absence of tetracycline. Cells were harvested at various time points (indicated above each lane) after removal of tetracycline from the growth medium and analysed for LMP-1 expression; also included was the reference LCL X50-7. **B)** RPA performed using RNA samples from the same experiment as that in panel A. Exposure to film was for 18 hours. The numbers above the lanes correspond to the times (in hours) after LMP-1 induction at which RNA was harvested.

### **3.2.3 Northern blot analysis confirms that LMP-1 contributes to EBV-mediated upregulation of *bfl-1* mRNA expression**

One drawback of RPA is that it provides no indication of the size of a particular mRNA species. Furthermore, owing to the nature of the RPA assay, the identification of alternatively spliced transcripts may be obscured if the probe spans a common region within the different transcripts. Northern blot analysis can often compensate for these deficiencies in RPA and thus allow for a more informative approach in RNA studies. For Northern analysis, the same *bfl-1* riboprobe used in RPA analysis was used to probe nitrocellulose filters onto which the same RNA samples from DG75-tTA-LMP-1 cells induced to express LMP-1 were blotted. Also included in this Northern blotting experiment were RNA samples from Mutu I and Mutu III cells and a non-LMP-1-expressing variant of Mutu III (Mutu III LMP<sup>-</sup>). The result of this experiment is shown in Figure 3.4 and leads to the following conclusions: (a) only a single *bfl-1* mRNA transcript of 0.8–0.85 kilobases is expressed in all cases, this being in agreement with the previously reported size of the transcript from this gene (Karsan *et al.*, 1996a, Kenny *et al.*, 1997) (b) significantly higher levels of *bfl-1* mRNA are observed in Mutu III cells relative to Mutu I BL and upon induction of LMP-1 expression in DG75-tTA-LMP-1 cells, confirming the result obtained by RPA; (c) the level of *bfl-1* mRNA in the non-LMP-1 expressing variant of Mutu III is significantly lower than in the wild-type Mutu III cells (compare lanes 6 and 7, upper panel), implicating a role for other EBV latent proteins in upregulating *bfl-1* mRNA levels. (d) The effect on *bfl-1* mRNA expression in the DG75-tTA-LMP-1 cell system is specific to LMP-1, since the level of expression of

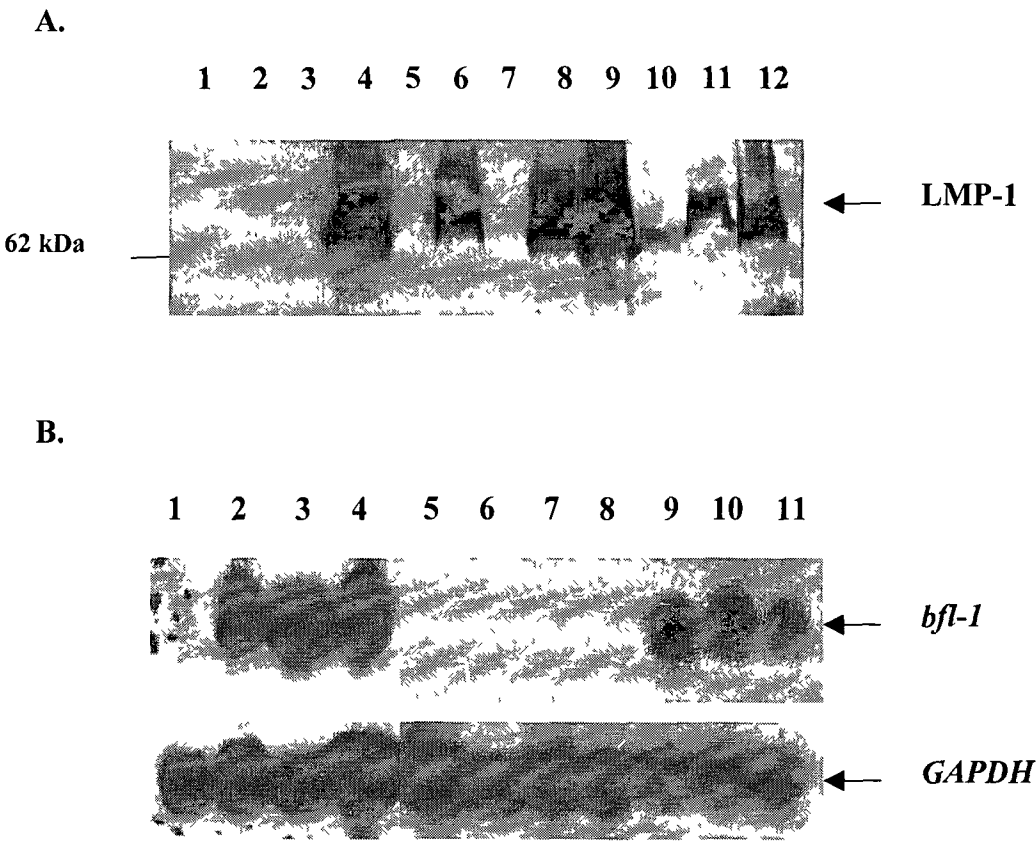
this gene remained unchanged in the parental DG75-tTA cells on removal of tetracycline from the growth medium (compare lanes 8 and 9)



**Fig 3 4 Northern blot analysis of *bfl-1* mRNA levels upon induction of LMP-1 in DG75-tTA-LMP-1 cells** Total RNA samples (30µg) from DG75-tTA-LMP-1 cells grown in the presence of tetracycline (lane 1) or recultured in the absence of tetracycline for 12 (lane 2), 48 (lane 3) or 96 (lane 4) hours as well as Mutu I (lane 5), Mutu III (lane 6) and Mutu III LMP (lane 7) were subjected to Northern analysis with an antisense *bfl-1* riboprobe (upper panel) As a control, RNA from DG75-tTA cells grown either in the presence of tetracycline (lane 8) or in the absence of tetracycline for 48 hours (lane 9) were similarly analysed The blots were exposed to film for 20 hours (lanes 1-7) or 18 hours (lanes 8 and 9) The lower panel shows the same blots stripped and reprobed with a *GAPDH* antisense riboprobe (exposure to film was for 12 h)

To strengthen the observation that upregulated *bfl-1* expression is a feature of EBV-infected LMP-1-expressing B cells, RNA samples from a series of EBV-negative cell lines, LCLs as well as EBV – positive BL cell lines exhibiting either a group I or group III phenotype were used in Northern blots with the <sup>32</sup>P-labelled antisense *bfl-1* riboprobe Indeed, Northern analysis (Figure 3 5 B) revealed that the levels of *bfl-1* mRNA were much higher in LCLs (IARC 171, X50-7, OKU LCL, IARC 290B) and EBV - positive BL cell lines with a group III phenotype (BL41-B95 8, Ag 876) than in EBV - negative BL cell lines (BL41, DG75) and EBV-positive cell lines with a group I phenotype (Mutu I, Rael, OKU BL) Furthermore, Western blot analysis with anti-LMP-1 antibodies,

revealed that the cell lines exhibiting upregulated *bfl-1* expression also expressed the latent viral protein LMP-1 (Figure 3 5 A) Thus, RPA and Northern analysis suggest that LMP-1 can contribute to the EBV-mediated upregulation of *bfl-1* mRNA in B lymphocytes

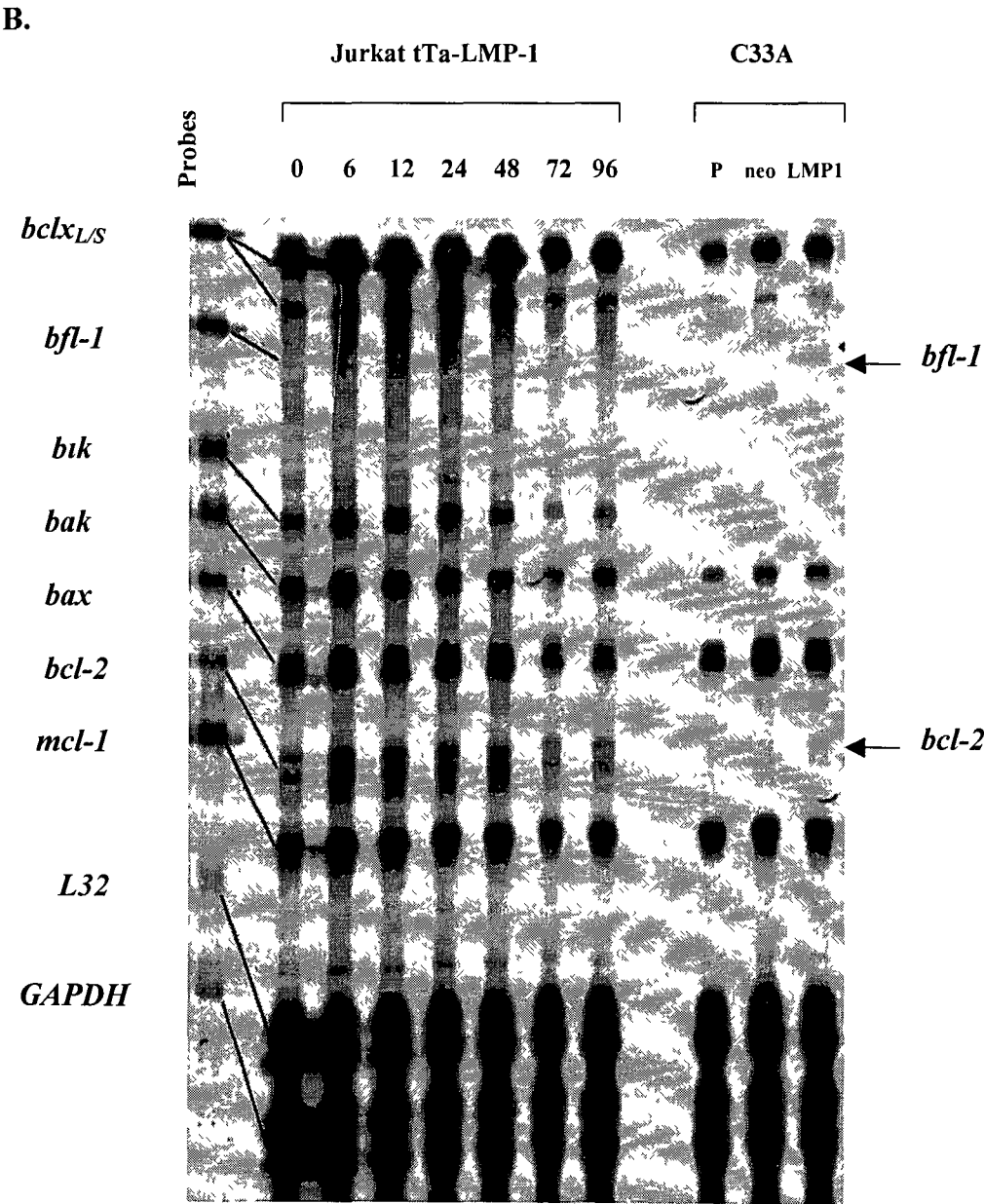
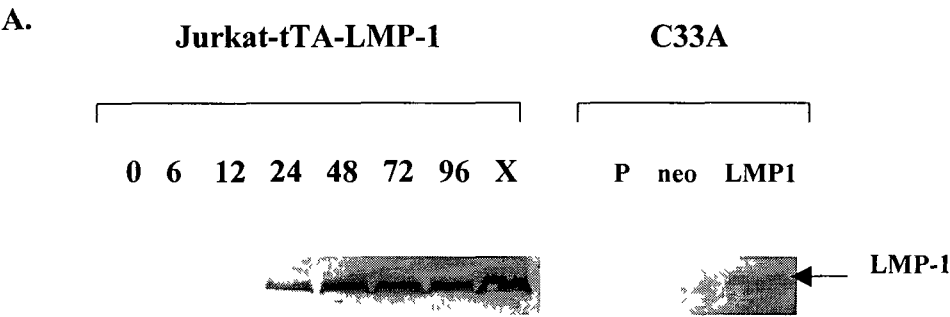


**Figure 3 5 Northern blot analysis of *bfl-1* expression in a series of EBV-negative and –positive BL cell lines and LCLs.** Upregulated *bfl-1* mRNA levels is a feature of EBV-positive LMP-1 expressing B lymphocytes **A)** Western blot analysis of LMP-1 expression in a series of EBV-negative BL cell lines [ DG75 (lane 1), BL41 (lane 7)], EBV- positive BL lines with either a group I phenotype [ Rael (lane 2), Mutu I (lane 3), OKU BL (lane 5)] or a group III phenotype [ Mutu III (lane 4), BL41-B95 8 (lane 8), Ag876 (lane 12)] and LCLs [ OKU LCL (lane 6), IARC 171 (lane 9), X50-7 (lane 10), IARC 290B (lane 11)] The position of the 62 kDa Protein molecular weight marker is indicated on the left hand side of the photograph **B)** Northern blot analyses of *bfl-1* expression (upper panel) in EBV-negative BL cell lines [ BL41 (lane 1), DG75 (lane 5)], EBV- positive BL lines with either a group I phenotype [ Rael (lane 6), Mutu I (lane 7), OKU BL (lane 8)] or a group III phenotype [ BL41-B95 8 (lane 2), Ag876 (lane 11)] and LCLs [ IARC 171 (lane 3), X50-7 (lane 4), OKU LCL (lane 9), IARC 290B (lane 10)] The blots were

exposed to film for 70 h (lanes 1-4) or 48 h (lanes 5 –11) The lower panel shows the same blots stripped and reprobed with *GAPDH* riboprobe (exposure of the blots was for 18 h)

### 3.2.4 Cell type-specific effect of LMP-1 on *bfl-1* expression

Although many EBV-associated lymphomas (of B- and T-cell origin) and NPCs exhibit expression of LMP-1, there are tissue-specific differences to be seen in the effects of LMP-1 on gene expression For example, the upregulation of Bcl-2 by LMP-1 is a B-lymphocyte-specific effect whereas LMP-1-mediated induction of A20 is observed in both lymphocytic and epithelial cell contexts (Henderson *et al* , 1991, Rowe *et al* , 1994, Laherty *et al* , 1992, Miller *et al* , 1995, 1997, Fries *et al* , 1996) It was therefore of interest to investigate whether LMP-1-induced upregulation of *bfl-1* occurred in a T cell or an epithelial cell background, the two other principal cell types that are relevant to EBV The following cell lines were used to address this question (a) a tetracycline-regulated LMP-1-expressing clone derived from the Jurkat T cell line, Jurkat tTA-LMP-1 and (b) a stably transfected LMP-1-expressing clone of the epithelial cell line C33A *bfl-1* transcript was not detected in either of these cell lines irrespective of their LMP-1 status (Figure 3 6 A and B) indicating that *bfl-1* is not expressed in either cell type nor is it upregulated by LMP-1 in either cell context Additionally, no change was observed in *bcl-2* mRNA levels in either cell context on induction of LMP-1 expression The absence of *bfl-1* upregulation was not due to an insufficient level of expression of LMP-1 since, at least in the case of the Jurkat tTA-LMP-1 cells, levels similar to that expressed in the LCL X50-7 were observed It thus appears that LMP-1 does not upregulate *bfl-1* expression in T cells or epithelial cells However, this conclusion is subject to demonstrating known LMP-1 induced effects on parameters relevant to these cellular contexts such as NF- $\kappa$ B activation and expression of the anti-apoptotic protein A20



**Figure 3.6 LMP-1 does not induce *bfl-1* expression in a T-cell or an epithelial cell context. A)**

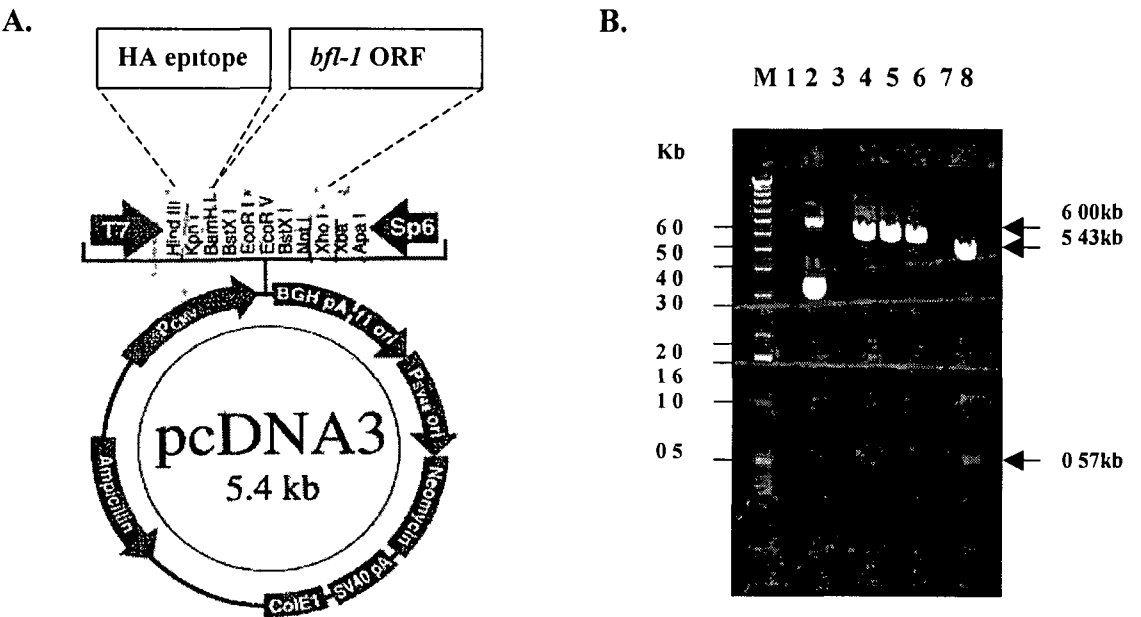
Western blot analysis of expression of LMP-1 in Jurkat tTA-LMP-1 cells and the stable transfectant C33ALMP-1. Jurkat tTA-LMP-1 cells were induced to express LMP-1 by reculturing cells in the absence of tetracycline (time after tetracycline-removal is indicated above each lane). Also included was the reference LCL X50-7 (X) and the parental C33A (P), the neomycin-resistant control transfectant C33Aneo (neo) and the LMP-1 transfectant C33ALMP1 (LMP1). **B)** RPA performed using RNA samples from the same experiment as that in panel A using the hAPO-2 set of riboprobes. The location of protected fragments derived from the *bfl-1* (expected location) and *bcl-2* probes are indicated by arrows. Exposure to film was for 18 hours.

**3.2.5 Functional analysis of Bfl-1 expression in a type I Burkitt's Lymphoma cell line**

*bfl-1* is one of the relatively newer members of the *bcl-2* family of genes. This gene bears closest homology to the mouse *A1* gene since the proteins coded by these genes share about 72% amino acid identity. *A1* has been shown to extend the survival of myeloid precursor cells after growth factor withdrawal (Lin *et al.*, 1996) and can also protect B lymphocytes from antigen-receptor ligation-induced apoptosis (Grumont *et al.*, 1999). Like *A1*, Bfl-1 can also protect cells from a number of apoptotic stimuli. For example, Bfl-1 preferentially suppresses chemotherapy-induced apoptosis in a fibrosarcoma cell line (Wang *et al.*, 1999) and protects endothelial cells from TNF- $\alpha$ -induced cell death (Karsan *et al.*, 1996). Additionally, Bfl-1 suppresses p53-induced apoptosis, exhibits cell proliferative properties and cooperates efficiently with the *Ela* oncogene in the transformation of rodent epithelial cells (D'Sa-Eipper *et al.*, 1996). It was therefore of interest to examine the functional consequence of the upregulation of *bfl-1* expression by LMP-1 in BL cells. To this end, it was necessary to take two factors into consideration. The first factor relates to the detection of human Bfl-1 protein. Owing to the lack of appropriate antibodies to detect human Bfl-1 protein, attempts to study the functional role of Bfl-1 protein in different cell types have been based on expressing it as a fusion protein with an 'irrelevant' peptide (such as included the influenza virus hemagglutinin (HA) epitope and FLAG-octapeptide) that contains an epitope to which reactive antibodies are available. The second factor relates to the choice of the appropriate target cell line to achieve this purpose. Although the effects of LMP-1 on *bfl-*

*l* expression as reported in this thesis were conducted in the context of the DG75 cell line, these cells are not as sensitive to apoptotic death induced by various stimuli including growth factor-deprivation compared to many other EBV-negative and group I EBV-positive BL cell lines (result not shown) On the other hand, Mutu I cells, which exhibit a type I latent infection, are extremely sensitive to apoptotic death (Gregory *et al* , 1991, Henderson *et al* , 1991, Milner *et al* , 1992) and express extremely low levels of *bfl-1* and *bcl-2* mRNA (Figure 3 1) Furthermore, these cells have also been shown to be easy to transfect by electoporation (Milner *et al* , 1992) Hence, Mutu I cells were chosen as the target cell line to study the functional effects of ectopic expression of Bfl-1

An expression vector, pcDNA3-HA-Bfl-1, which has been shown to ensure high levels of expression of HA-tagged Bfl-1 protein in mammalian systems was employed for the purpose (D'Sa-Eipper *et al* , 1996) Figure 3 7A shows the map of the vector pcDNA3 and the restriction sites in the polylinker region into which *HA-bfl-1* was cloned and figure 3 7B shows the results of the restriction enzyme analysis used to confirm the organisation of the DNA fragment encoding HA-Bfl-1 in the vector pcDNA3-HA-Bfl-1

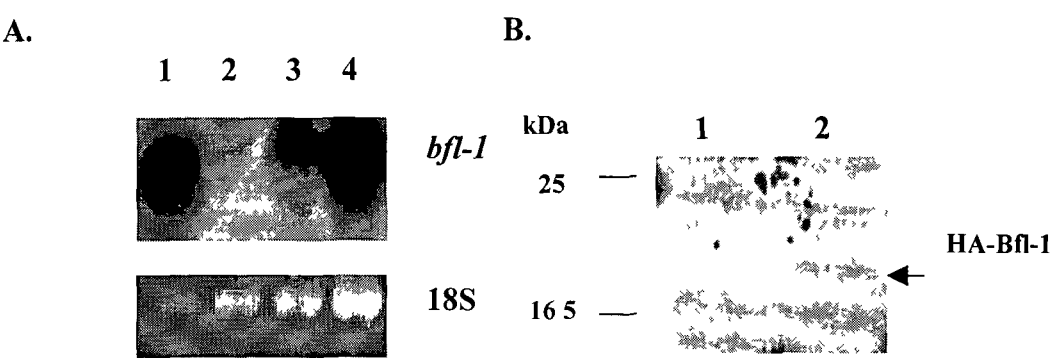


**Figure 3 7 A Map of the expression vector pcDNA3** The polylinker containing multiple cloning restriction sites is flanked by the T7 and Sp6 promoters The HA epitope was cloned between the *Hind* III and *Bam* HI sites and the *bfl-1* ORF was cloned between the *Bam* HI and *Xho* I sites in the polyhnr region pCMV = CMV promoter directing strong expression of HA-Bfl-1 BGHpA = polyadenylation signal from the bovine growth hormone **B Restriction enzyme analysis of the expression vector pcDNA3-HA-Bfl-1** Digestion with either *Hind* III (lane 4) , *Bam* HI (lane 5) or *Xho* I (lane 6) linearises the plasmid (approximately 6 00 kb ) Digestion of the plasmid with *Bam* HI and *Xho* I excises the 570 bp BFL-1 ORF encoding DNA fragment (lane 8) Lane 2 = undigested plasmid M = DNA molecular weight markers (sizes in kb indicated on the left hand side of the photograph) Lanes 1, 3 and 7 were unused A 0 8% agarose gel in 1 x TAE was used and electrophoresis was carried out for at 100 V for 1 h 20 min in 1 x TAE

As a control for the generation of cells transfected with pcDNA3-HA-Bfl-1 it was necessary to derive the corresponding vector expressing only the HA epitope The strategy used to construct pcDNA3HA involved excision of the *bfl-1* cDNA from pcDNA3-HA-Bfl-1 with *Bam* HI and *Xho* I (Figure 3 7A), conversion of the restricted sites to blunt ends and subsequent religation of the vector (not shown)

The pcDNA3 vector carries the neomycin-resistance gene (Figure 3 7A), and hence selection of transfectants transduced with pcDNA3-HA-Bfl-1 or pcDNA3-HA was based on resistance to G418 when included in the growth medium Prior to transfection, the sensitivity of the target cell line, Mutu I, to G418 was determined as described in section 2 4 9 1 It was found that G418 when added to the growth medium at an 'active' concentration of 1400 µg/ml was sufficient to kill >95% of Mutu I cells by 12 days Mutu I cells were transfected with either pcDNA3-HA-Bfl-1 or pcDNA3-HA by electroporation using conditions described in section 2 4 8 1. G418-selection was initiated 48 hours post-electroporation and continued for the next 21 days before analysing the respective transfected pools of cells for expression of HA-Bfl-1 or HA and sensitivity to apoptosis Northern Blot analysis with a *bfl-1* antisense riboprobe showed that *bfl-1* mRNA was expressed in only the pcDNA3-HA-Bfl-1-transfected pool and not the pcDNA3-HA transfected pool (Figure3 8A) The level of *bfl-1* mRNA expression in

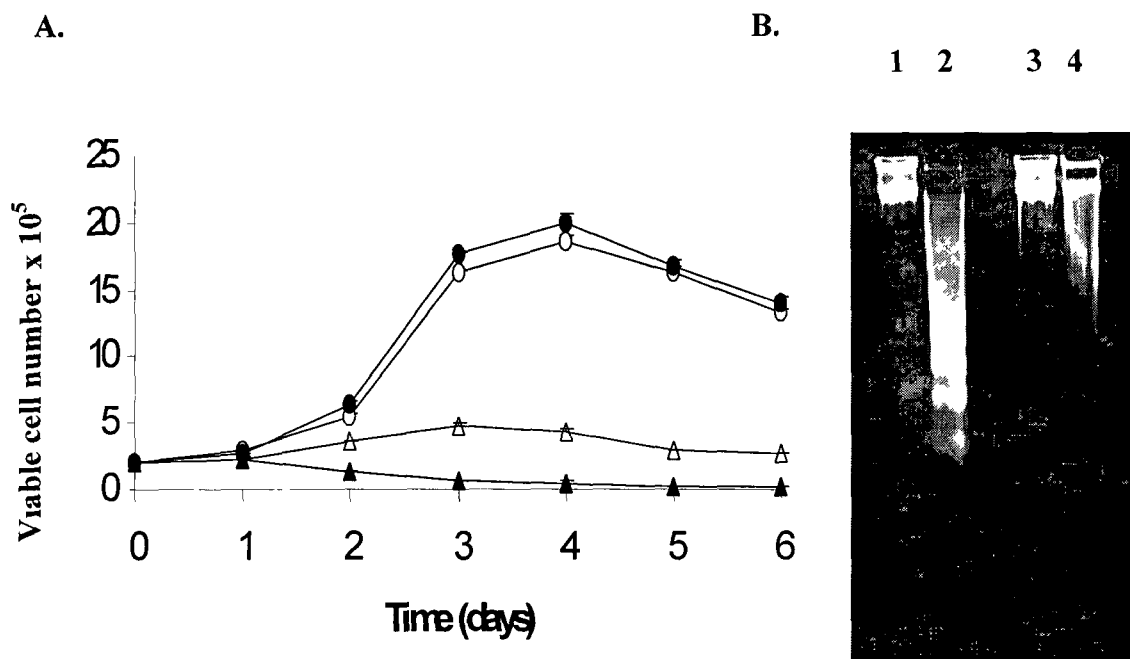
the pcDNA3-HA-Bfl-1-transfected pool was lower than that expressed in either Mutu III or the LCL IARC171. Western blot analysis with anti-HA polyclonal antibodies revealed the presence of a novel band at 20 kDa, corresponding to the molecular weight expected for HA-Bfl-1, with lysate from only the pool transfected with pcDNA3-HA-Bfl-1 (Figure 3 8B)



**Figure 3 8** Generation of a pool of transfectants of Mutu I cells expressing HA-tagged Bfl-1. Mutu I cells were transfected with either pcDNA3-HA-Bfl-1 expressing HA-tagged Bfl-1 or control vector (pcDNA3-HA) and subjected to selection for 21 days with G418. Total RNA and protein were then prepared for analysis. **A)** The upper panel shows a Northern blot analysis of *bfl-1* mRNA in Mutu III (lane 1), cell pools transfected with either pcDNA3-HA (lane 2) or pcDNA3-HA-Bfl-1 (lane 3) and the LCL IARC171 (lane 4). The lower panel is a photograph of the 18S rRNA band from the same ethidium bromide-stained gel used for blotting. **B)** Western blot analysis using polyclonal anti-HA antibodies to detect HA-Bfl-1 expression in cell extracts from the same transfected Mutu I cell pools (lane 1, pcDNA3-HA, lane 2, pcDNA3-HA-Bfl-1) as shown in panel A. The position of HA-Bfl-1 is indicated.

The growth kinetics of the Bfl-1 transfected and control-transfected pools were then compared under optimal (10% FBS) and sub-optimal culture conditions (0.1% FBS). The results of this experiment are represented in Figure 3 9 A. In 10% FBS, after an initial lag phase, both pools of transfected cells exhibited rapid growth to high saturation density followed by a slight fall in viability. However, in 0.1% FBS, the behaviour of the two pools differed significantly in that while the control cells showed a rapid decline in viability with time, the Bfl-1 expressing cells exhibited a slight increase in proliferation.

over the first 72h in culture followed by a gradual decline in viability. To assess if the decline in viability of the control cells cultured in the presence of 0.1% FBS was due to apoptotic death two assays were employed. The first, the 'DNA ladder assay', is based on the usage of agarose gel electrophoresis to detect internucleosomal DNA fragmentation (observed as a ladder of DNA fragments after ethidium bromide-staining of the gel). The second, is based on the detection of chromatin condensation by acridine orange staining of cells followed by fluorescence microscopy. A marked increase in DNA fragmentation was detected when the control cells were grown under low-serum conditions (Figure 3.9B, compare lanes 1 and 2). In contrast, the HA-Bfl-1 transfected cells exhibited much less DNA fragmentation when grown under similar sub-optimal conditions (Figure 3.9B, compare lanes 2 and 4). The ability of Bfl-1 to promote cell survival during growth factor deprivation was further supported by the observation that while an average of 82% of the control cells exhibited chromatin condensation on staining with acridine orange after 72h of culture in 0.1% FBS, 35% of HA-Bfl-1-transfected cells contained condensed chromatin under similar culture conditions. In 10% FBS-containing medium, approximately 7% of the cells in both pools of transfectants exhibited chromatin condensation. Based on these observations, it is clear that Bfl-1 can protect BL cells from apoptotic death induced by serum-deprivation. The fact that a minority of cells in the HA-Bfl-1 - expressing pool continue to undergo apoptotic death in response to serum deprivation is likely to reflect heterogeneity in HA-Bfl-1 levels between individual transfected cells, some of which may express this protein at very low levels. Indeed, the overall *bfl-1* mRNA level in the transfected pool was lower than that in Mutu III or IARC-171 cells (Figure 3.8A).

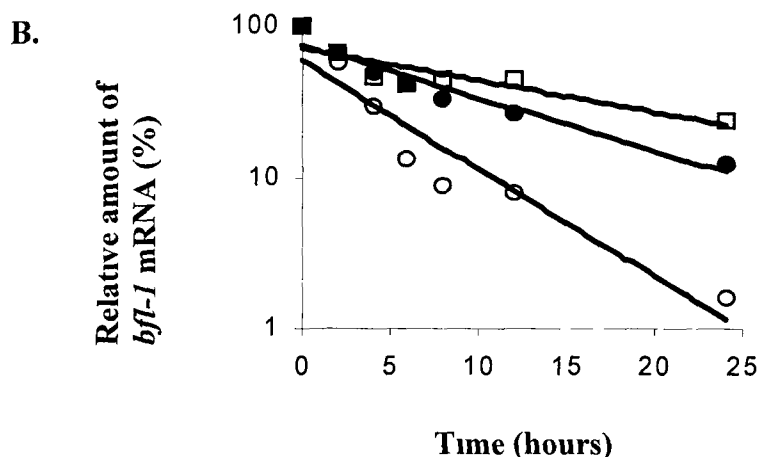
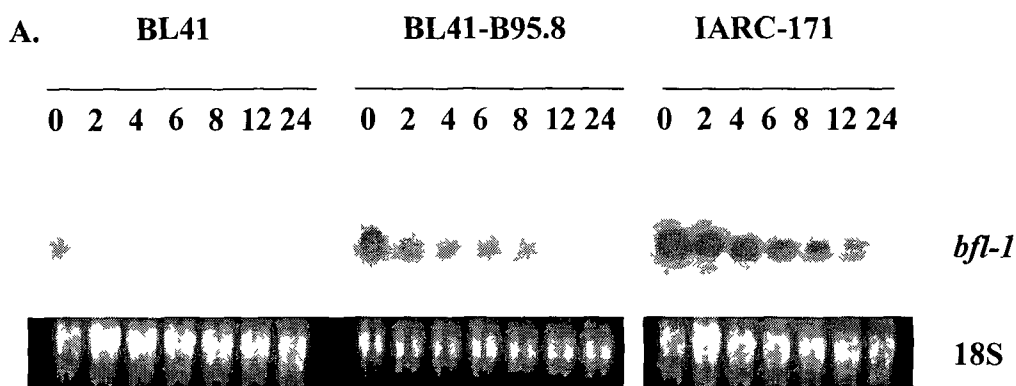


**Figure 3.9 Functional analysis of Bfl-1 expression in Mutu I BL cells** **A)** Growth curves of Mutu I cells transfected with either control [pcDNA3-HA (solid symbols)] or HA-Bfl-1 expression vector [pcDNA3-HA-Bfl-1 (open symbols)] Cells from exponentially growing cultures of the pools of transfected cells were seeded at  $2 \times 10^5$  cells per ml of growth medium containing either 10% FBS (circles) or 0.1% FBS (triangles) and assessed at daily intervals for viable-cell numbers by trypan blue exclusion **B)** DNA fragmentation assay was carried out on the transfected Mutu I cells described in panel A cultured in medium containing either 10% FBS (lanes 1 and 3) or 0.1% FBS (lanes 2 and 4) for 72h as described in Materials and Methods section 2.4.10.1 Lanes 1 and 2 contain DNA from cells transfected with control plasmid (pcDNA3-HA), and lanes 3 and 4 contain DNA from cells transfected with pcDNA3-HA-Bfl-1

### 3.2.6 LMP-1 increases the half-life of *bfl-1* mRNA

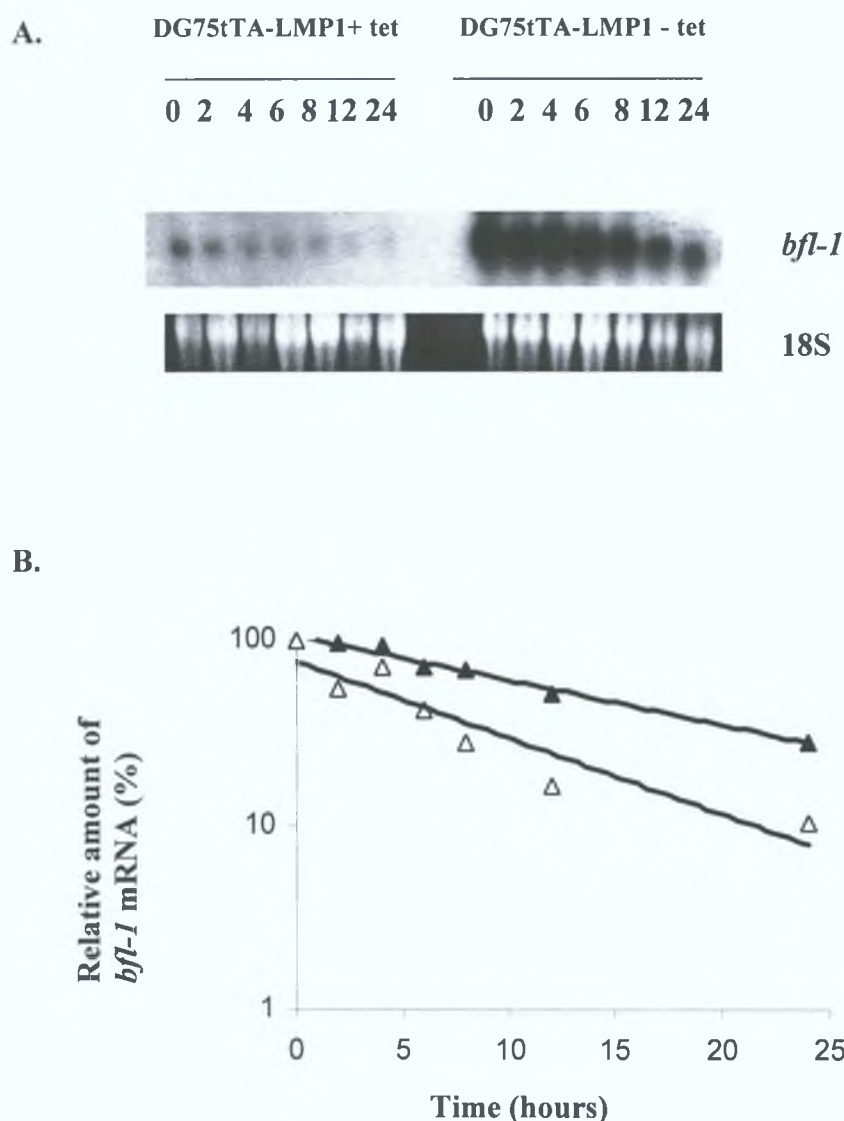
The increase in the steady-state levels of *bfl-1* mRNA could be due to an increase in either the rate of transcription of the *bfl-1* gene or stability of previously transcribed mRNA or a combination of both mechanisms *bfl-1* has already been classified as an immediate-early response gene (Karsan *et al*, 1996; Lin *et al*, 1996, Moreb and

Schweder, 1997) Since many early response genes are regulated at the level of mRNA stability it was of interest to investigate if this was a factor in regulating *bfl-1* expression in BL cells with a latency group III phenotype and in response to LMP-1 expression The rate of decay of *bfl-1* mRNA was monitored by Northern blotting after treatment of cells with an inhibitor of RNA polymerase II, actinomycin D (5µg/ml) (Figures 3 10 and 3 11, A and B upper panel) *bfl-1* mRNA levels in the untreated and treated cells were determined after Northern blotting by densitometric analysis of autoradiograms and values were corrected for variations due to loading after estimating 18S rRNA levels in each lane (as determined by densitometric analysis of ethidium bromide stained gels) Values thus obtained were expressed as a percentage of the *bfl-1* mRNA level seen in untreated cells (taken as 100%) The half-life of *bfl-1* mRNA was then determined from the best-fit semi-logarithmic line of the graph of these values plotted against time (hours) of exposure to actinomycin D (Figures 3 10 and 3 11, A and B, lower panel) generated from the relative amounts of *bfl-1* mRNA as determined by densitometric scanning of the Northern blots The half-life of *bfl-1* mRNA in BL41 cells was found to be approximately 1 hour, which was increased to 7.4h in BL41-B95.8 cells and 5.2h in the corresponding LCL IARC-171 (Figure 3 10 B) Under similar experimental conditions, the half-life of *bfl-1* mRNA in uninduced DG75-tTA-LMP-1 cells was 4.25h and this subsequently increased to 11.5h on induction of LMP-1 (Figure 3 11 B) It is not clear why the stability of *bfl-1* mRNA in uninduced DG75-tTA-LMP-1 cells was higher than that in BL41 cells, although both are EBV-negative BL cell lines It is possible that the rate of inhibition of RNA synthesis by actinomycin D differs between the two cell lines Alternatively, the uninduced DG75-tTA-LMP-1 cells may exhibit 'leaky' expression of LMP-1 below the level of sensitivity of detection of LMP-1 expression by western blotting which may account for the higher stability of the *bfl-1* transcript Nevertheless, against a similar cellular background, EBV infection or LMP-1 expression resulted in significant increases in the stability of *bfl-1* mRNA



**Figure 3 10 EBV infection increases the stability of *bfl-1* mRNA in BL cells** Exponentially growing cultures of the EBV-negative BL cell line BL41, its EBV (B95 8 strain) –infected derivative BL41-B95 8 and the corresponding LCL IARC-171 were treated with 5µg/ml actinomycin D to block RNA synthesis. The cells were then further incubated for the 2, 4, 6, 8, 12 and 24 hours before total cellular RNA was extracted and the rate of decay of *bfl-1* mRNA monitored by Northern analysis. **A)** The upper panel shows the Northern blot analysis of the decay of *bfl-1* mRNA over time (indicated in hours above each lane) after treatment with actinomycin D in BL41, BL41-B95 8 and IARC-171 cell lines. The lower panel is a photograph of the 18S rRNA band from the same ethidium bromide-stained gel used for blotting. **B)** The graphs show the best-fit semilogarithmic lines generated from the relative amounts of *bfl-1* mRNA as determined from densitometric scanning of the autoradiograms of the Northern blots in panel A above.

plotted against time of exposure to actinomycin D. All values were normalised for loading based on the intensity of the 18S rRNA bands on the corresponding ethidium bromide-stained gels. Relative levels of *bfl-1* mRNA as determined in BL41 (○), BL41-B95.8 (□) and IARC-171 (●).



**Figure 3.11 Induction of LMP-1 in DG75-tTA-LMP-1 cells increases the stability of *bfl-1* mRNA.** DG75-tTA-LMP-1 cells were grown in the presence (+ tet) or absence (-tet) of tetracycline for 36 hours before addition of 5µg/ml actinomycin D. The cells were then further incubated for the 2, 4, 6, 8, 12 and 24 hours before total cellular RNA was extracted and the rate of decay of *bfl-1* mRNA monitored by Northern analysis. **A)** The upper panel shows the Northern blot analysis of the decay of *bfl-1* mRNA over time (indicated in hours above each lane) after treatment with actinomycin D in uninduced DG75-tTA-LMP-1 cells (+ tet) and 36 hours after LMP-1-induction (-tet). The lower panel is a photograph of the 18S rRNA band from the same ethidium bromide-stained gel used for blotting. **B)** The graphs show the best-fit

semilogarithmic lines generated from the relative amounts of *bfl-1* mRNA as determined from densitometric scanning of the autoradiograms of the Northern blots in panel A above plotted against time of exposure to actinomycin D Relative levels of *bfl-1* mRNA as determined in uninduced DG75-tTA-LMP-1 cells ( $\Delta$ ) and at 36h after LMP-1 induction ( $\blacktriangle$ )

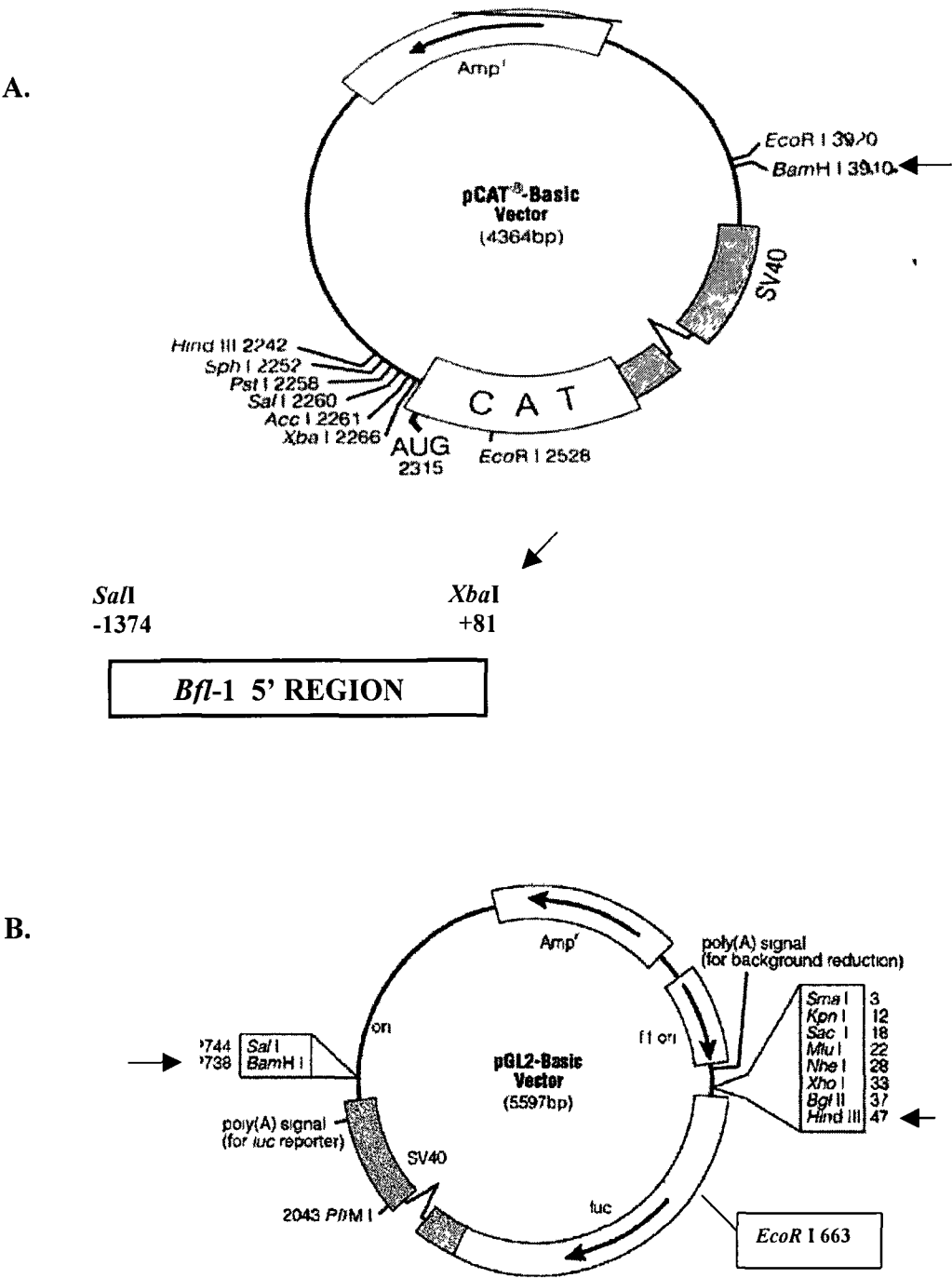
### 3.2.7 Effect of LMP-1 on *bfl-1* promoter activity

To investigate if the increase in the steady-state levels of *bfl-1* mRNA induced by LMP-1 expression could also be due to an increase in the rate of transcription of the *bfl-1* gene, the effect of expression of this EBV gene on the promoter activity of *bfl-1* as an indirect parameter was analysed

Promoter-reporter assays are one of the most common and convenient ways of assessing transcriptional activity. The activity of the promoter of interest is assessed by transfection of a construct containing the promoter sequence fused to a reporter gene into an appropriate cell line. Promoter activity in response to a chemical stimulus or a protein expressed from another plasmid that is cotransfected with the promoter-reporter construct is then measured by assaying for reporter activity. Two of the commonly used reporter genes in promoter-reporter constructs encode the enzymes chloramphenicol acetyl transferase (CAT) and luciferase (luc). However, the greater sensitivity and rapidity of luciferase assays has resulted in the popular usage of *luc*-based rather than *CAT*-based promoter-reporter constructs for evaluating transcriptional activity in transfected cells. It was therefore decided to investigate the regulation of *bfl-1* promoter activity by LMP-1 using a luciferase-based system.

The promoter region of the human *bfl-1* gene has been cloned (Zong *et al*, 1999, Edelstein and Gelinas, unpublished observations) and increased activity of a 1.4 kb region upstream of *bfl-1* has been demonstrated in response to TNF- $\alpha$  in promoter-reporter assays (Zong *et al*, 1999). The 1.4 kb DNA sequence from the 5' regulatory region (-1374 / +81) of the *bfl-1* gene was obtained as a fragment cloned into the promoterless pCAT-basic plasmid (Zong *et al*, 1999). In order to change the reporter-context of the p-1374/+81Bfl-1-CAT construct to a luciferase-based system, a cloning strategy based on replacing the CAT gene with the luciferase gene from pGL2-basic vector (Promega) in the pCAT basic background was employed. The circle maps of pCAT-basic and pGL2-basic are shown in Figure 3.12. The sites in the polylinker region

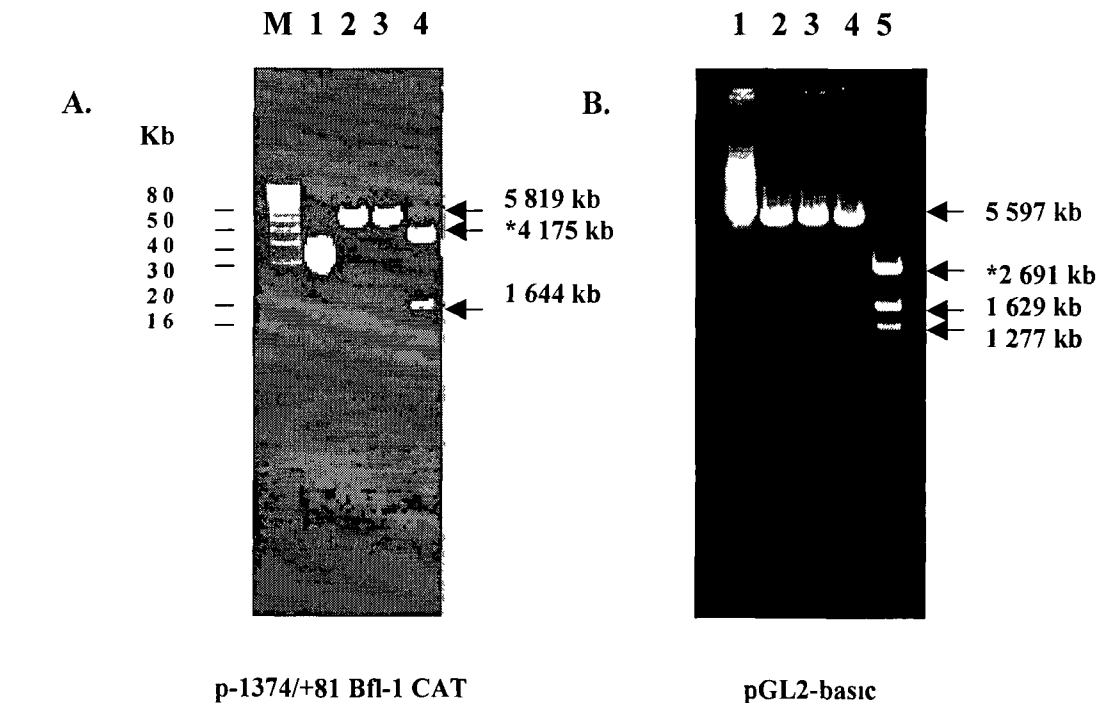
of pCAT-basic into which the -1374/+81 region of the *bfl-1* gene was cloned is also indicated in Figure 3 12



**Figure 3.12** A) Schematic circular map of pCAT Basic vector Indicated on the map are the sites (*Sal* I, *Xba* I) in the polylinker into which the -1374/+81 region of *bfl-1* was cloned by Zong *et al* (1999) to create p-1374/+81 Bfl-1-CAT The *Bam* HI and *Xba* I restriction enzymes (sites indicated by arrows) were used to

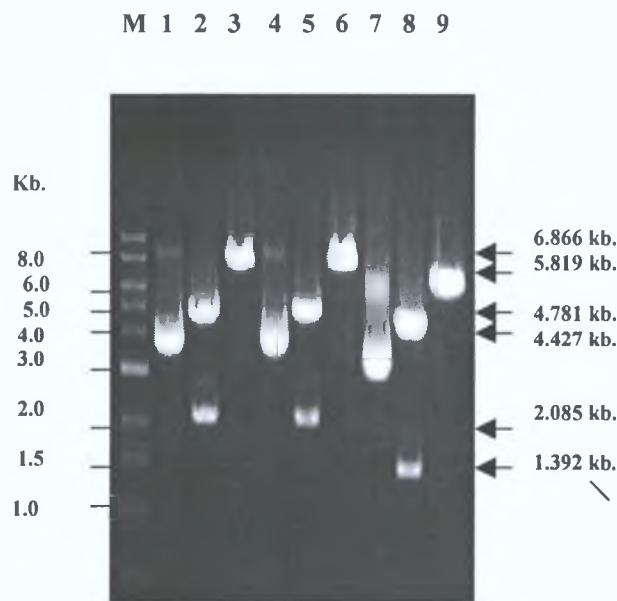
excise a 4 175 kb fragment containing the *bfl-1* promoter **B) Schematic circular map of pGL2-Basic vector** The *BamH* I and *Hind* III restriction enzymes (sites indicated by arrows) were used to excise a 2 691 kb fragment containing the luciferase reporter gene The *EcoR* I site in the luciferase gene (position 663) is also indicated

The *bfl-1* promoter region (-1374/+81) had previously been inserted by directional cloning into the *Sal* I and *Xba* I sites of pCAT-basic to create the p-1374/+81Bfl-1-CAT plasmid (Zong *et al* , 1999) To construct the p-1374/+81Bfl-1-luc plasmid containing the -1374/+81 region of *bfl-1* cloned just upstream of the *luc* gene, the 4 175 kb *BamH* I-*Xba* I DNA fragment (containing the *bfl-1* promoter region) from p-1374/+81Bfl-1-CAT was ligated to the 2 691 kb *BamH* I-*Hind* III fragment (containing the *luc* gene) of the pGL-2 basic plasmid In summary, the ligation reaction involved blunt-end ligation between the ‘filled-in’ *Xba* I and *Hind* III ends and ‘sticky-end’ ligation between the *BamH* I ends Figure 3 13 shows the restriction enzyme analysis of p-1374/+81Bfl-1-CAT and pGL-2 basic to excise the DNA fragments of interest for use in ligation to create the p-1374/+81Bfl-1-luc plasmid



**Figure 3.13 Restriction enzyme analysis of p-1374/+81 Bfl-CAT and pGL2-basic .** A) The 5.819 kb. p-1374/+81Bfl-1-CAT plasmid was digested with *BamH* I and *Xba* I to generate the *bfl-1* promoter containing DNA fragment of 4.175 kb. (marked by an asterik \*). lane 1: undigested plasmid, lane 2: *BamH* I-digested plasmid, lane 3: *Xba* I-digested plasmid, lane 4: plasmid digested with *BamH* I and *Xba* I. M= DNA molecular weight markers **B)** The 5.597 kb. pGL2-basic plasmid was digested with *Hind* III, *BamH* I and *Sca* I to yield a 2.691 kb *Hind* III-*BamH* I fragment (marked by an asterisk \*) containing the *luc* gene. Lane 1: undigested plasmid, lane 2: *Hind* III-digested plasmid, lane 3: *BamH* I- digested plasmid, lane 4: *Sca* I-digested plasmid, lane 5: plasmid digested with *Hind* III, *BamH* I and *Sca* I. A 1% agarose-1 x TAE gel was used and electrophoresis was carried out at 100 V for 1 h.

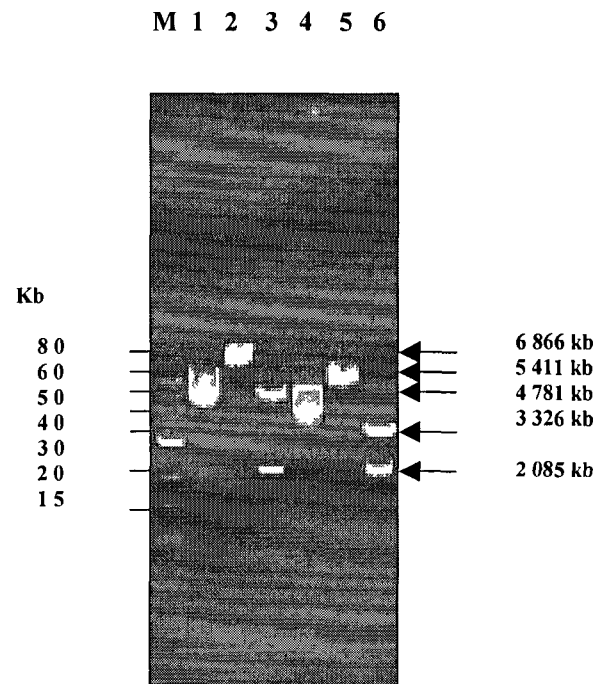
The p-1374/+81 Bfl-1-luc plasmid created by ligating the 4.175 kb. *BamH* I-*Xba* I fragment from p-1374/+81Bfl-1-CAT to the 2.691 kb. *Hind* III-*BamH* I fragment from pGL2-basic was confirmed by restriction enzyme analysis (Figure 3.14). The *EcoR* I enzyme has two recognition sites in the p-1374/+81 Bfl-1-luc plasmid, one within the luciferase gene (figure 3.12B) and the other within the vector backbone derived from the p-1374/+81Bfl-1-CAT plasmid (figure 3.12A) and was thus useful in confirming that the ligation worked.



**Figure 3.14 Restriction enzyme analysis of p-1374/+81 Bfl-1-luc.** The *EcoR* I restriction digestion pattern of p-1374/+81 Bfl-1-luc plasmid confirms the expected organisation of the plasmid. Lanes 1-3 : p-1374/+81 Bfl-1-luc preparation 1 and lanes 4-6: p-1374/+81 Bfl-1-luc preparation 2. Lanes 7-9: p-

1374/+81 Bfl-1-CAT Lanes 1, 4 and 7 Undigested plasmid, lanes 2, 5 and 8 *EcoR* I digestion pattern, Lanes 3, 6 and 9 *BamH* I digestion pattern Restriction digestion with *BamH* I linearises p-1374/+81 Bfl-1-luc (6 866 kb) and p-1374/+81 Bfl-1-CAT (5 819 kb) Restriction digestion of p-1374/+81 Bfl-1-luc with *EcoR* I yields two fragments of 4 781kb and 2 085 kb as expected of the ligation of the the 4 175 kb *BamH* I-*Xba* I fragment from p-1374/+81Bfl-1-CAT to the 2 691 kb *Hind* III-*BamH* I fragment from pGL2-basic

The promoterless reporter vector (basal/basic vector) was constructed by ligating the 2 720 kb *BamH* I-*Sal* I fragment from p-1374/+81-Bfl-1-CAT to the 2 691 kb *BamH* I-*Hind* III fragment from pGL2-basic (Figure 3 12B) and involved prior conversion of the *Sal* I and *Hind* III ends to blunt ends Restriction enzyme analysis confirmed the organisation of the derived basal/basic luciferase reporter plasmid (Figure 3 15)



**Figure 3 15 Restriction enzyme analysis of the promoterless (basic/basal) luciferase vector** The *EcoR* I restriction digestion pattern of the promoterless luciferase plasmid confirms the expected organisation of the plasmid Lanes 1-3 p-1374/+81 Bfl-1 luc and lanes 4-6 promoterless luciferase plasmid Lanes 1, 4 Undigested plasmids Lanes 2, 5 *BamH* I digestion pattern Lanes 3, 6 *EcoR* I digestion pattern Restriction digestion with *BamH* I linearises p-1374/+81 Bfl-1-luc (6 866 kb ) and the promoterless luciferase plasmid (5 411 kb) Restriction digestion of the promoterless luciferase plasmid with *EcoR* I yields two fragments of 3 326 kb and 2 085 kb as expected A 1% agarose-1 x TAE gel was used and electrophoresis was carried out at 100 V for 1h in 1 x TAE

The LMP-1 expression plasmid, pEFCXLMP-1, has been described previously (Liljeholm *et al.*, 1998) and was constructed by cloning the LMP-1 cDNA into the 'empty' vector, pEFCX. The expression of LMP-1 from pEFCXLMP-1 is driven by the strong polypeptide chain elongation factor 1 $\alpha$  promoter. A key feature of this promoter is that its activity is not NF- $\kappa$ B-dependent and thus allows the usage of NF- $\kappa$ B inhibitors to study any NF- $\kappa$ B-dependent effects of LMP-1 without affecting LMP-1 expression.

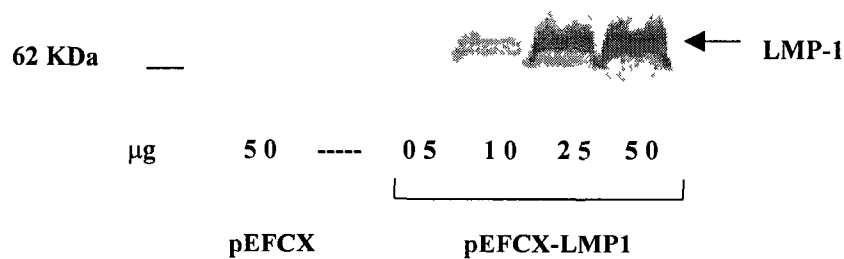
The upregulation of *bfl-1* mRNA by LMP-1 was observed in the context of the EBV-negative BL cell line, DG75. This cell line also exhibits a high efficiency of transfection by the DEAE-dextran method and by electroporation (Ricksten *et al.*, 1988; Liljeholm *et al.*, 1998) and was thus a convenient and relevant target cell line for investigating the effect of transient expression of LMP-1 on *bfl-1* promoter activity. Although the majority of the promoter studies were conducted using this cell line, some of the promoter studies were also repeated in another EBV-negative BL cell line, BL41, and in the T-cell line, Jurkat. Although the induction of LMP-1 in Jurkat-tTA-LMP-1 cells failed to induce *bfl-1* mRNA, the lack of an effect may be explained by the functional deficiency of the necessary components in the transduction pathway in this clone. Indeed, clonal variants with widely different characteristics have been isolated in case of Jurkat-T cells (Martinez-Lorenzo *et al.*, 2000) and the Jurkat-tTA-LMP-1 cells may represent a clonal variant of the original population of cells. Hence, promoter studies of *bfl-1* gene were extended to the parental Jurkat T cell population. This cell line has been shown to exhibit many of the LMP-1-induced changes in gene expression observed with B cell lines in transient transfection studies (Huen *et al.*, 1995).

#### **3.2.7.1 LMP-1 increases *bfl-1* promoter activity in the BL cell Line DG75**

Transfection of increasing amounts (0.5, 1.0, 2.5 or 5.0  $\mu$ g) of the LMP-1-expression plasmid, pEFCXLMP-1, into DG75 cells led to a dose-dependent increase in the levels of expression of LMP-1 protein as assessed by Western blot analysis (Figure 3.16 A). As expected, transfection with the empty vector, pEFCX, did not result in the expression of a

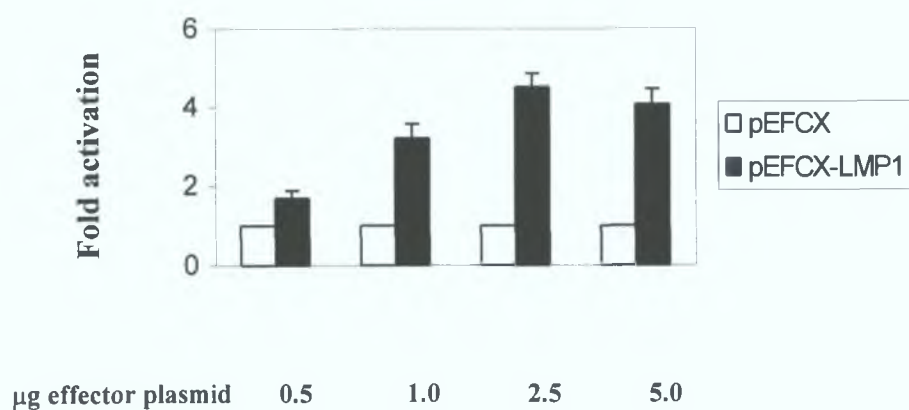
protein that reacted specifically with the anti-LMP-1 antibodies. Since LMP-1 has been demonstrated to activate the transcription factor NF- $\kappa$ B, the functionality of LMP-1 expressed from pEFCXLMP-1 in DG75 cells was assessed by cotransfection of an NF- $\kappa$ B-dependent luciferase reporter plasmid, 3 x enh NF- $\kappa$ B-luc (containing three tandem repeats of the Ig $\kappa$ -light chain NF- $\kappa$ B element fused to the minimal conalbumin promoter) and measuring luciferase activity at 24h post-transfection. As shown in Figure 3.16 (A and B) increased levels of LMP-1 expression correlated with increased activation of NF- $\kappa$ B with peak activation of approximately 4.5-fold measured with transfection of 2.5  $\mu$ g of pEFCXLMP-1. Transfection of the higher amount of 5.0  $\mu$ g of the expression vector for LMP-1 did not result in a further increase in luciferase activity. A similar dose-dependent effect was observed when the effect of expression of increasing amounts of LMP-1 on *bfl-1* promoter activity was assessed on cotransfection of the *bfl-1*-promoter-luciferase construct, p-1374/+81-Bfl-1-luc. Indeed, maximal activation of 7.8-fold was detected with transfection of 2.5  $\mu$ g of pEFCXLMP-1 and transfection of a higher amount of the LMP-1 expression plasmid did not result in a further increase in this level of activation (Figure 3.16 C).

A



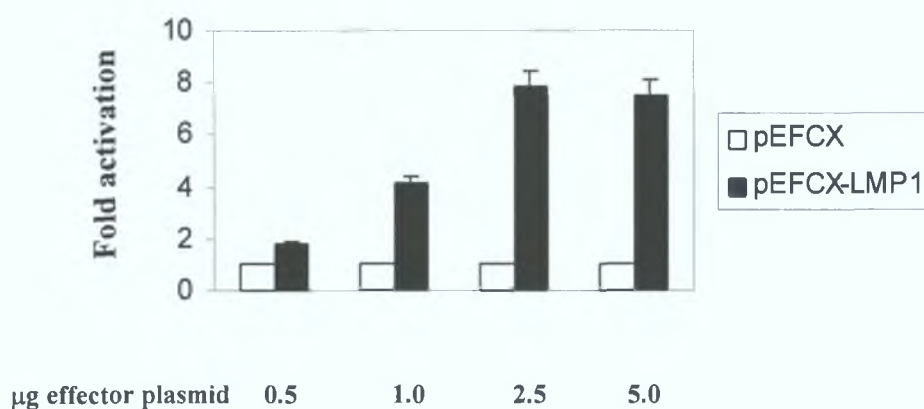
B.

### 3 x enh kB luc



C.

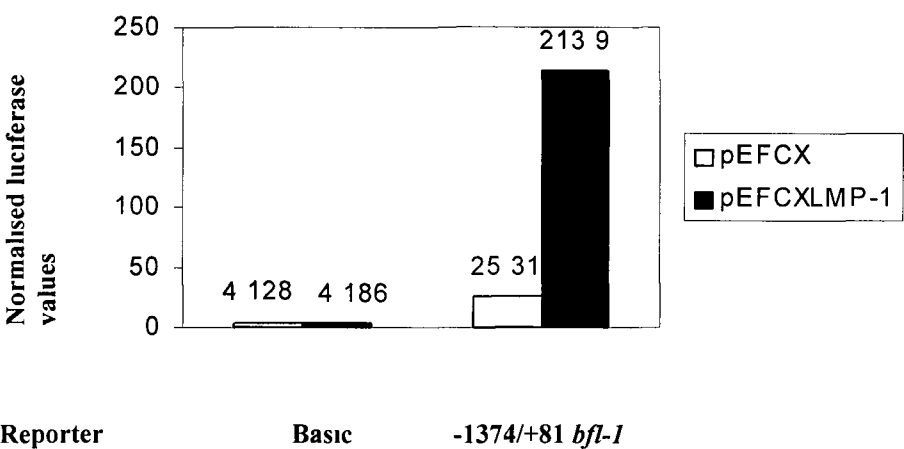
### -1374/+81 Bfl-1-luc



**Figure 3.16 Dose-dependent effect of LMP-1 expression on *bfl-1* promoter activity.** DG75 cells were cotransfected with increasing amounts (0.5, 1.0, 2.5, 5.0 µg) of either pEFCX or pEFCX-LMP-1 and either 2.5 µg of 3 x enh κB luc (panel B) or p-1374/+81 Bfl-1-luc (panel B) or 3xenh κB luc (panel C). Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (A) and luciferase activity produced from either the NF- κB -luc construct (B) or the *bfl-1* promoter - luc construct (C) was measured. Fold-activation represent the normalised (for transfection efficiency based on β-galactosidase activity measured from cotransfected pCMVlacZ reporter included in all transfections) luciferase activity due to transfection of the LMP-1 expression plasmid, pEFCXLMP-1, relative to that due to transfection of the empty vector pEFCX (luciferase activity of reporter obtained on transfection with

empty vector was arbitrarily assigned a value of 1.0) and depict the mean  $\pm$  s.d. of two independent experiments

The effect of LMP-1 was specific to the DNA sequence of p-1374/+81Bfl-1luc spanning the promoter region of *bfl-1* since LMP-1 expression did not affect luciferase activity from the cotransfected basic vector (Figure 3.17). In the same transfection set and under similar experimental conditions, LMP-1 expression stimulated luciferase synthesis from the p-1374/+81Bfl-1-luc reporter approximately 8.5-fold relative to empty vector. These results indicate that LMP-1 upregulates *bfl-1* promoter activity in the Burkitt's lymphoma cell line DG75.



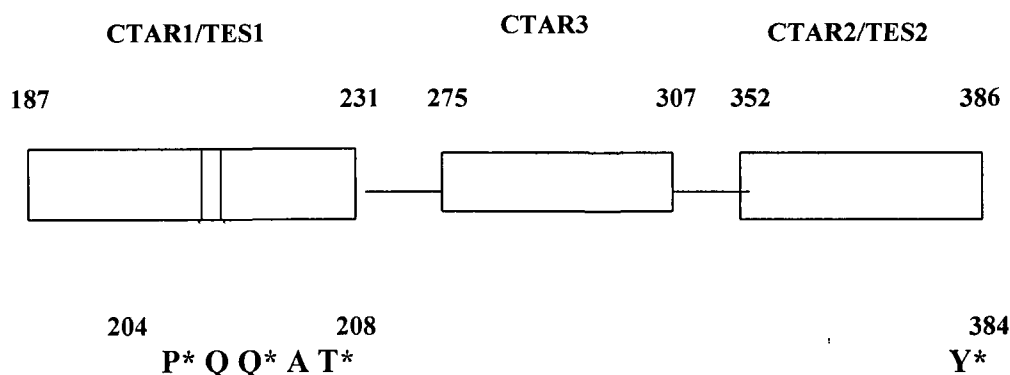
**Figure 3.17 Expression of LMP-1 does not affect the activity of the basic/ promoterless luciferase reporter** DG75 cells were cotransfected with 2.5  $\mu$ g of LMP-1 expression vector pEFCXLMP-1 or control plasmid pEFCX and an equal amount of the basic/promoterless reporter or the *bfl-1*-promoter- luciferase construct, p-1374/+81 Bfl-1-luc and luciferase activity was analysed 24 h post-transfection. Normalised luciferase readings from one representative experiment are plotted and the values are indicated above each column in the graph.

**3.2.7.2 Identification of LMP-1 signaling domains required for induction of *bfl-1* promoter activity**

The LMP-1 molecule comprises a short N-terminal cytoplasmic domain, six membrane spanning helices that facilitate aggregation and a long cytoplasmic carboxy-terminus

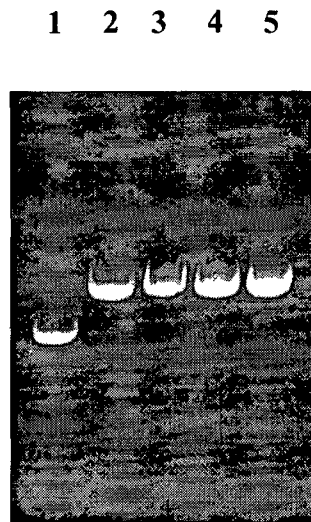
(Figure 1 7) As described in Section 1 6 5, the majority of the signaling functions of LMP-1 have been assigned to two domains, CTAR1/TES1 and CTAR2/TES2, within the carboxy-terminus of the cytoplasmic portion of the protein (Huen *et al* , 1995, Mitchell and Sugden, 1995) (Figure 3 18) Although both of these domains contribute independently to activation of NF- $\kappa$ B by LMP-1 in a TRAF-dependent mechanism, CTAR2 exerts a predominant effect in most cell types, accounting for 70 – 80 % of the activation of this transcription factor by LMP1 CTAR2 alone is responsible for the activation of AP-1 by wild-type LMP-1 LMP-1 can also activate the p38 MAPK (mitogen-activated protein kinase) pathway and both CTAR-1 and CTAR-2 are responsible for mediating this effect in a TRAF2-dependent manner (Ehopoulos *et al* , 1999) A third functional domain of LMP-1, CTAR3, has recently been identified which localises to a region of the carboxy terminus of LMP-1 located between CTAR-1 and CTAR-2 (Figure 3 18) This domain is responsible for the interaction of LMP-1 with Janus kinase (JAK)-3 which in turn activates STAT (signal transducers and activators of transcription) proteins (Gires *et al* , 1999)

To identify the domain(s) of LMP-1 that are responsible for the effect on *bfl-1* promoter activity, plasmids expressing LMP-1 mutants containing point mutations of critical residues in CTAR-1 and CTAR-2 were used in comparative analysis with wild-type LMP-1 in promoter-luciferase assays Three different LMP-1 mutants were used (Figure 3 18) The first, LMP-AAA, contains a triple mutation in the central core of the TRAF interaction domain of CTAR-1 (P<sup>204</sup>xQ<sup>206</sup>xT<sup>208</sup> → AxAxA) This triple mutation has been previously shown to block CTAR-1-mediated NF- $\kappa$ B activation by abrogating TRAF binding to this domain (Devergne *et al* , 1996, Ehopoulos *et al* , 1997, 1999) The second, LMP G, contains a single point mutation in CTAR-2 such that the tyrosine residue at position 384 is mutated to glycine This mutation has been shown to potently inhibit activation of NF- $\kappa$ B and AP-1 by LMP-1 by inhibiting TRADD binding (Ehopoulos *et al* , 1999, Floettmann *et al* , 1997) The third, LMP AAAG, encompasses the mutations present in LMP AAA and LMP G and is unable to activate NF- $\kappa$ B or AP-1 (Brennan *et al.*, 2001).



**Figure 3 18 Schematic representation of the carboxy-terminal cytoplasmic region of LMP1** The three signaling domains, CTAR1/TES1 (aa 187 to 231) and CTAR2/TES2 (aa 352 to 386) and CTAR3 (aa 275 to 307) are represented by empty boxes The amino acid residues within CTAR1 and CTAR2 that are directly implicated in TRAF and TRADD binding respectively are indicated The CTAR3 domain is implicated in JAK3 activation Residues within the TRAF binding core of CTAR1 that are marked with an asterik were mutated to alanine in the LMP1 mutant LMPAAA The Y384 residue in CTAR2 that is important for TRADD binding was mutated to glutamate in the LMP1 mutant LMP G LMP AAAG is a double mutant containing the mutations present in both LMP AAA and LMP G

The constructs from which wild-type and mutant LMP-1 molecules were expressed were all based on the pSG5 vector (Stratagene) from which expression is driven by the SV40 early gene promoter A comparison of the plasmids by agarose gel electrophoresis suggested that the preparations were of similar quality suitable for use in transfections (Figure 3 19)



**Figure 3 19 Analysis of the quality of the expression constructs for wild-type (WT) and mutant LMP-1 proteins** Lane 1 pSG5 (empty vector), lane 2 pSG5-LMP WT, lane 3 pSG5-LMP AAA, lane 4 pSG5-LMP G, lane 5 pSG5-LMP AAAG A 1% agarose-1 x TAE gel was used and electrophoresis was carried out for 1h at 100 V in 1 x TAE

The effect of expression of wild-type and mutant LMP-1 proteins on the activity of the *bfl-1* promoter was compared by cotransfection of expression vectors for the effector proteins together with the *bfl-1* promoter-luciferase construct p-1374/+81 Bfl-1-luc into DG75 and Jurkat cells and assaying for luciferase activity 24 h post-transfection The effect of expression of the wild-type and mutant LMP-1 proteins on NF- $\kappa$ B activity was investigated in a similar experimental strategy but involving cotransfection of the 3 x enh  $\kappa$ B-luc construct Expression of wild-type LMP-1 activated *bfl-1* promoter activity 6 4-fold (Figure 3 20 A middle panel) and induced luciferase synthesis from the 3 x enh  $\kappa$ B-luc construct approximately 3 6-fold in DG75 cells (Figure 3 20 A lower panel) Although not formally investigated, the slightly lower extent of activation of *bfl-1* promoter activity and NF- $\kappa$ B activation by LMP-1 expressed from pSG5LMP-1 relative to that observed with pEFCXLMP1 in DG75 cells is likely to be due to differences in the level of expression of LMP-1 from the two expression vectors Expression of LMP AAA

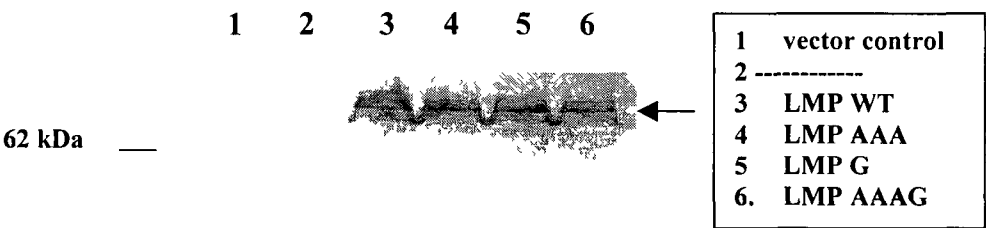
induced *bfl-1* promoter activity that was approximately 76% of wild-type LMP-1 activity and induced approximately 73% of the NF- $\kappa$ B activity induced by wild-type LMP-1. In comparison, expression of LMP G induced *bfl-1* promoter activity to approximately 20% of wild-type LMP-1 activity and NF- $\kappa$ B activity to approximately 25% of wild-type LMP-1 activity. LMP AAAG was unable to activate NF- $\kappa$ B or induce *bfl-1* promoter activity when ectopically expressed in DG75 cells (Figure 3 20 A middle and lower panels). The effects of the LMP mutants on *bfl-1* promoter activity and NF- $\kappa$ B activation did not seem to be due to differential levels of expression of these proteins since Western blot analysis revealed similar levels of expression of wild-type and mutant LMP1 proteins when the corresponding expression vectors were transfected (Figure 3 20 A, upper panel). These results indicate that in DG75 cells, the CTAR-1 and CTAR-2 domains of LMP-1 can independently contribute to activation of *bfl-1* promoter activity, with CTAR-2 being the predominant contributor in this effect. Furthermore, the functional activity of both domains is required for maximal activation of the *bfl-1* promoter and NF- $\kappa$ B activity. Also, the relative contribution of the individual domains of LMP-1 to inducing *bfl-1* promoter activity correlates with the effect on NF- $\kappa$ B activation, an observation that suggests that NF- $\kappa$ B activation may be important in mediating the effect of LMP-1 on activation of *bfl-1*. The observation that the double mutation in CTAR-1 and CTAR-2 completely abrogates LMP-1-mediated activation of the *bfl-1* promoter does not necessarily exclude the functional involvement of signaling domains mapping to other regions of LMP-1 such as CTAR3 since the function of CTAR3 may be dependent on CTAR1 and/or CTAR2 (Brennan *et al* , 2001).

In Jurkat cells, expression of wild-type LMP-1 induced a 3.4-fold activation of the *bfl-1* promoter and activated NF- $\kappa$ B 104-fold. Expression of LMP AAA (which contains an intact CTAR2 domain) resulted in retention of 40% and 32% of wild-type LMP-1 transactivation of the *bfl-1* promoter and NF- $\kappa$ B respectively (Figure 3 20 B, middle and lower panels) suggesting that in Jurkat cells CTAR-2 makes a lower contribution to induction of *bfl-1* promoter activity and NF- $\kappa$ B activation compared to the BL cell line DG75. Expression of LMP G (which contains an intact CTAR1 domain) resulted in

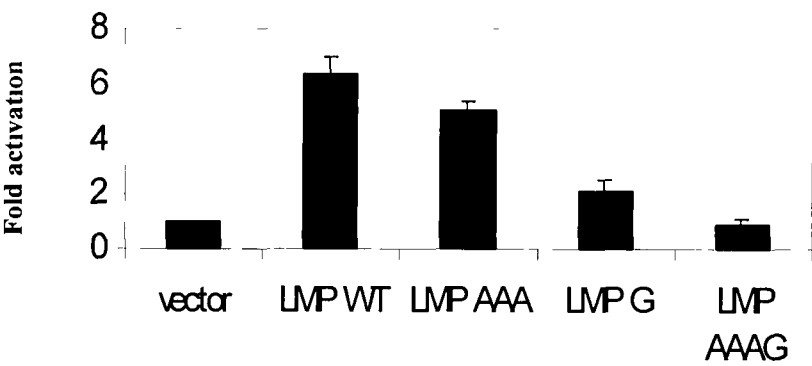
retention of even lower levels of *bfl-1* promoter activation and NF- $\kappa$ B activity, retaining approximately 5% of wild-type LMP-1 activity in both cases. This result suggests that in Jurkat cells, the function of CTAR1 is more severely impaired than CTAR2. LMP AAAG did not activate the *bfl-1* promoter and barely activated NF- $\kappa$ B in Jurkat cells. Thus, the pattern of contribution of the CTAR-1 and CTAR-2 of LMP-1 to *bfl-1* promoter activity and NF- $\kappa$ B activation in Jurkat cells would appear to differ to that in B cells, in that the independent functions of these domains is less efficient in Jurkat cells than in DG75 with CTAR-1 function being the more severely impaired. The finding that the extent of CTAR-1- and CTAR-2- mediated signaling in LMP-1 mutants can vary with the cellular background is consistent with previous reports (Huen *et al* , 1995, Floettmann *et al* , 1998)

A.

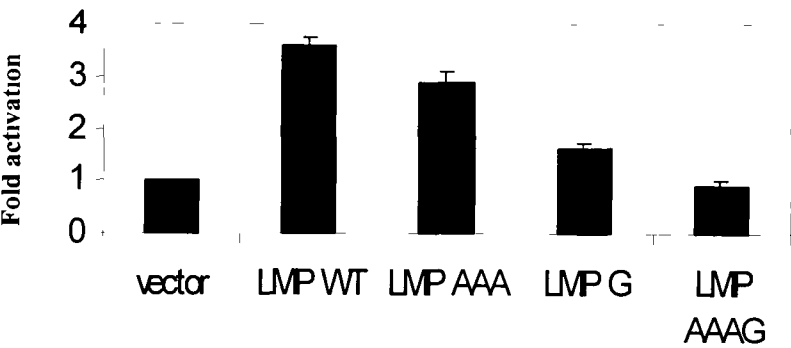
DG75



-1374/+81Bfl-1-luc

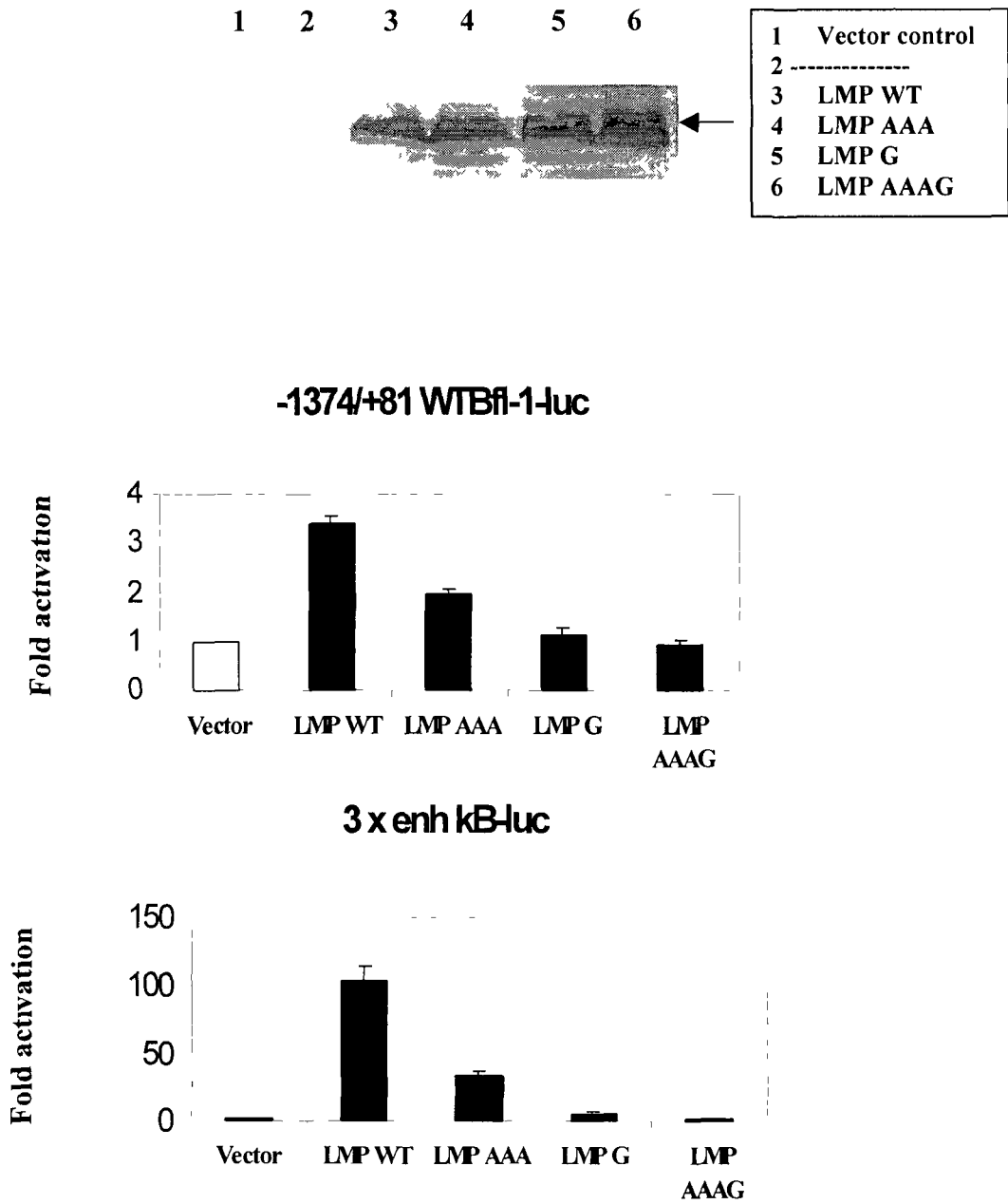


3 x enh kB-luc



B.

Jurkat



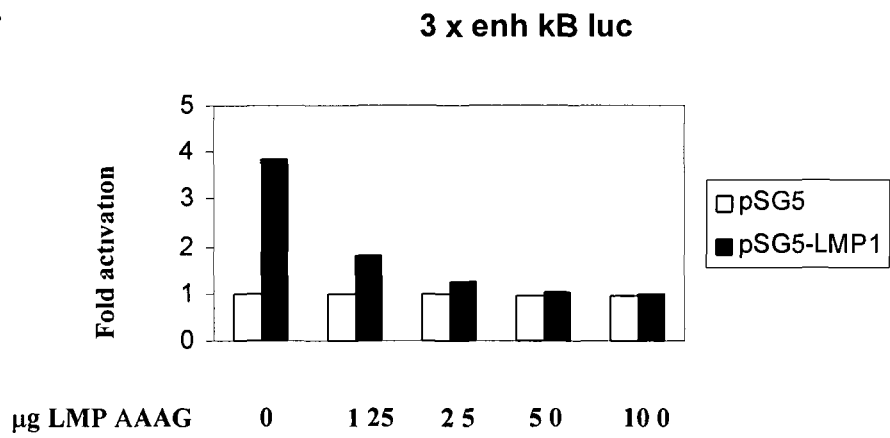
**Figure 3 20** Analysis of the function of CTAR-1 and CTAR-2 domains of LMP-1 in upregulating *bfl-1* promoter activity (middle panel) and NF- $\kappa$ B activity (lower panel) in DG75 (A) and Jurkat T (B) cells. Cells were cotransfected with 2.5  $\mu$ g of either pSG5 (vector control), pSG5 LMP wt (expressing wild-type LMP-1), pSG5LMP AAA (expressing LMP-1 containing a non-functional CTAR-1 domain),

pSG5LMP G (expressing LMP-1 containing a non-functional CTAR-2 domain) or pSG5 LMP AAAG (expressing LMP-1 mutant that is non-functional in both CTAR-1 and CTAR-2) and 2.5 µg of either p-1374/+81-Bfl-1-luc (middle panel) or 3 x enh κB-luc (lower panel). Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (upper panel) and luciferase activity produced from either the *bfl-1* promoter – luc construct (middle panel) or the NF-κB–luc construct (lower panel) was measured. Normalised luciferase activities were expressed as fold activation over control (empty-vector) as before.

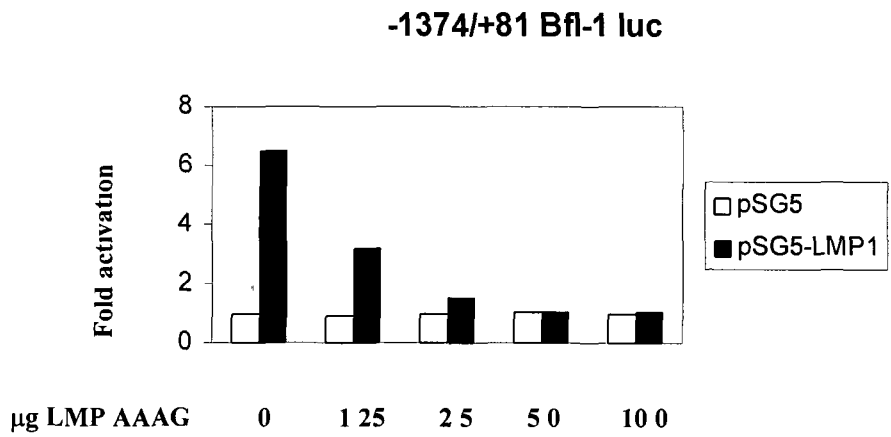
### 3.2.7.3 Dominant negative action of the double mutant of LMP1, LMP AAAG

Although LMP-1 lacks an extracellular ligand-binding domain it mimics a constitutively activated receptor and oligomerisation is required for its functional activation (Gires *et al*, 1997). Furthermore, cooperation between CTAR1 and CTAR2 has to occur within a hetero-oligomeric complex for a fully functional signal to be generated (Floettmann *et al*, 1998). The requirement of physical cooperation between CTAR1 and CTAR2 domains present on different LMP-1 molecules within the same complex for functional activity, forms the basis of the dominant-negative action of LMP AAAG. Thus, coexpression of an equimolar amount of LMP AAAG was shown to result in up to 90% inhibition of the NF-κB activity of wild-type LMP-1 in Jurkat cells (Brennan *et al*, 2001). In a preliminary experiment, the dominant negative action of LMP AAAG was tested in DG75 cells coexpressing wild-type LMP-1 in transient transfection assays with a NF-κB-dependent luciferase reporter or the-1374/+81 *bfl-1*-luciferase reporter. It can be seen from Figure 3.21 that coexpression of LMP AAAG potentially inhibited wild-type LMP-1-induced NF-κB activation and *bfl-1* promoter activity in a dose-dependent fashion. Thus, cotransfection of 1.25 µg and 2.5 µg of pSG5-LMP AAAG resulted in a 72% and 91% inhibition of NF-κB activation induced by expression of wild-type LMP-1 from 2.5 µg of the corresponding plasmid (Figure 3.21 A). Transfection of higher amounts of pSG5-LMP AAAG completely blocked the LMP-1-mediated effect. The effect of expression of LMP AAAG on induction of *bfl-1* promoter activity by LMP-1 paralleled its effect on activation of NF-κB with transfection of 1.25 µg and 2.5 µg of pSG5-LMPAAAG resulting in approximately 60% and 90% inhibition of wild-type LMP-1 activity of the promoter (Figure 3.21 B).

A.



B.



**Figure 3 21 Dominant negative effect of the LMP-1 mutant LMP AAAG** DG75 cells were cotransfected with 2.5 µg of pSG5-LMP-1 or empty vector pSG5 and 2.5 µg of either 3 x enh κB-luc (panel A) or p-1374/+81-Bfl-1-luc (panel B) together with various amounts of (0, 1.25, 2.5, 5.0 or 10.0 µg) of a plasmid expressing dominant negative LMP-1 mutant, pSG5-LMP-AAAG. Cells were harvested 24h post-transfection and analysed for luciferase activity as before. Normalised luciferase activities were expressed as fold activation over control (empty vector in the absence of expression of LMP-AAAG = 1) as before.

#### 3.2.7.4 LMP-1 induces *bfl-1* promoter activation via a mechanism involving TRAF2

TRAF2 has been shown to bind to the cytoplasmic tail of LMP-1 and is an important mediator of NF- $\kappa$ B activation by LMP-1 (Kaye *et al.*, 1996). TRAF2 binds directly to the CTAR1 domain of LMP-1 and via the adaptor protein TRADD to CTAR2. The carboxy-terminal domain of TRAF-2 mediates TRAF-2-binding to CTAR1 and TRADD whereas the amino-terminal RING-finger containing domain is involved in homo- and heterodimerisation of TRAF family members, binding to c-IAPs and is essential for TRAF-2-mediated activation of NF- $\kappa$ B and JNK (Kaye *et al.*, 1996; Rothe *et al.*, 1995; Hsu *et al.*, 1996). A dominant negative mutant of TRAF-2, TRAF2 $\Delta$ (6-86), that is deleted for aminoacids 6-86 in the N-terminal region, has been demonstrated to inhibit activation of NF- $\kappa$ B and JNK by LMP-1 (Eliopoulos *et al.*, 1997; 1999).

In order to examine a direct role for TRAF-2 in mediating LMP-1-induced activation of *bfl-1* promoter activity, DG75 cells were transiently co-transfected with 2.5  $\mu$ g of pEFCXLMP-1 or control vector (pEFCX) and 2.5  $\mu$ g of the *bfl-1*-luc construct (p-1374/+81 Bfl-1-luc) in the presence or absence of increasing amounts of a plasmid expressing TRAF2 $\Delta$ (6-86). Expression of TRAF2 $\Delta$ (6-86) was found to significantly reduce *bfl-1* promoter activation by LMP-1 in a dose-dependent manner, such that transfection of 5.0  $\mu$ g of pSG5-TRAF2 $\Delta$ (6-86) caused a 25% decrease in LMP-1 – mediated transactivation of *bfl-1* and transfection of 10.0  $\mu$ g of the TRAF2 $\Delta$ (6-86) resulted in a further decrease (by 48%) in LMP-1-mediated activation of the *bfl-1* promoter (Figure 3.22 panel B). As a functional control for TRAF2 $\Delta$ (6-86), the effect of TRAF2 $\Delta$ (6-86) expression on LMP-1-mediated activation of NF- $\kappa$ B was investigated in a similar experimental strategy but with transfection of the 3 x enh  $\kappa$ B-luc construct instead of the *bfl-1* luciferase construct. In this experiment, transfection of 5.0  $\mu$ g of pSG5TRAF2 $\Delta$ (6-86) resulted in a 40% decrease while transfection of 10.0  $\mu$ g of pSG5TRAF2 $\Delta$ (6-86) caused a 65% decrease in LMP-1 mediated activation of NF- $\kappa$ B (Figure 3.22 panel C). Western blot analysis revealed that the inhibitory effect of

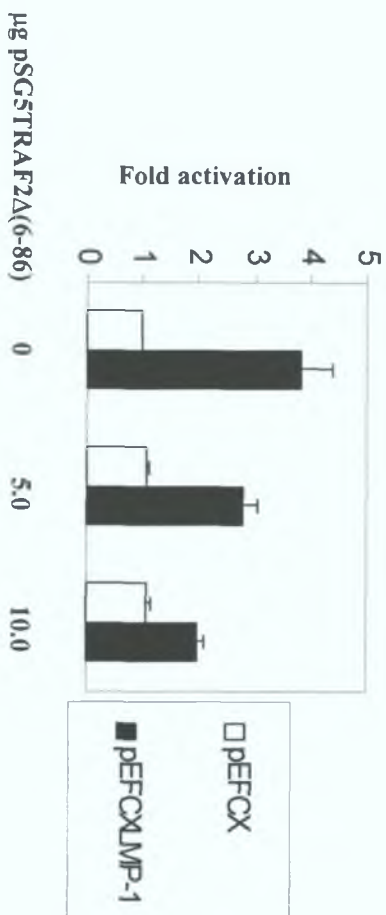
TRAF2 $\Delta$ (6-86) on LMP-1-mediated activation of the *bfl-1* promoter is not due to inhibition of LMP-1 expression (Figure 3 22 panel A) These results therefore implicate the involvement of TRAF2 in LMP-1-mediated upregulation of *bfl-1* promoter activity

The observations with TRAF2 $\Delta$ (6-86) were supported by experiments in which A20 was overexpressed in DG75 cells in the presence of LMP-1 A20 is an anti-apoptotic RING finger protein that interacts with TRAFs and efficiently inhibits NF- $\kappa$ B and JNK activation by LMP-1 by associating with LMP1-TRAF and LMP1-TRADD complexes and displacing the TRAF and TRADD molecules (Eliopoulos *et al* , 1997, 1999, Fries *et al* , 1999) To examine whether A20 expression could inhibit LMP-1-mediated activation of NF- $\kappa$ B activation in DG75 cells, 2.5  $\mu$ g of pEFCXLMP-1 or pEFCX was cotransfected with various amounts of a vector that expresses A20 (0, 2.5, 5.0 or 10.0  $\mu$ g of pSG5-A20) in the presence of the NF- $\kappa$ B-driven luciferase reporter As shown in Figure 3 23 (panel B), A20 expression potently inhibited NF- $\kappa$ B activation by LMP-1 in DG75 cells, with transfection of 5.0  $\mu$ g and 10.0  $\mu$ g of pSG5-A20 resulting in approximately 80% and 94% inhibition of *bfl-1* promoter activation by LMP-1 A similar degree of inhibition of LMP-1-mediated activation of NF- $\kappa$ B was observed when the effect of A20 expression on LMP-1-induced luciferase synthesis from the 3 x enh $\kappa$ B-luciferase reporter was investigated (Figure 3 23 panel C) A20 expression did not alter basal *bfl-1* promoter activity or NF- $\kappa$ B activation suggesting that A20 only affects LMP-1-induced signaling A20 expression did not interfere with LMP-1 expression from pEFCXLMP-1 as assessed by Western blot analysis of lysates prepared from the transfected cell populations (Figure 3 23 panel A) Taken together, the results obtained in the experiments based on the usage of the dominant negative TRAF2 mutant, TRAF2 $\Delta$ (6-86), and the TRAF2 interacting protein, A20, suggest a likely role for TRAF2 in the positive effect of LMP-1 on *bfl-1* promoter activity

μg pSG5TRAF2Δ(6-86)      0      5.0      10.0

C.

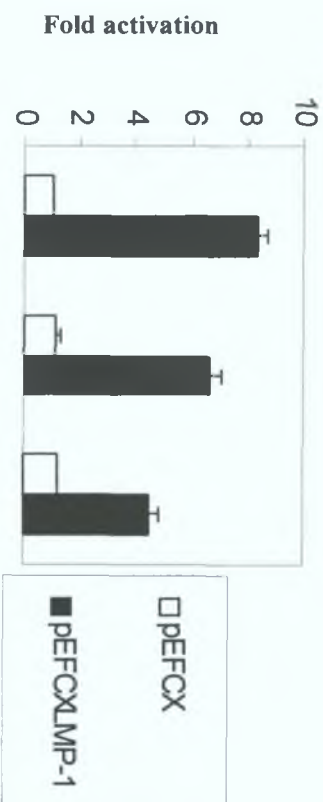
3 x enh KB-luc



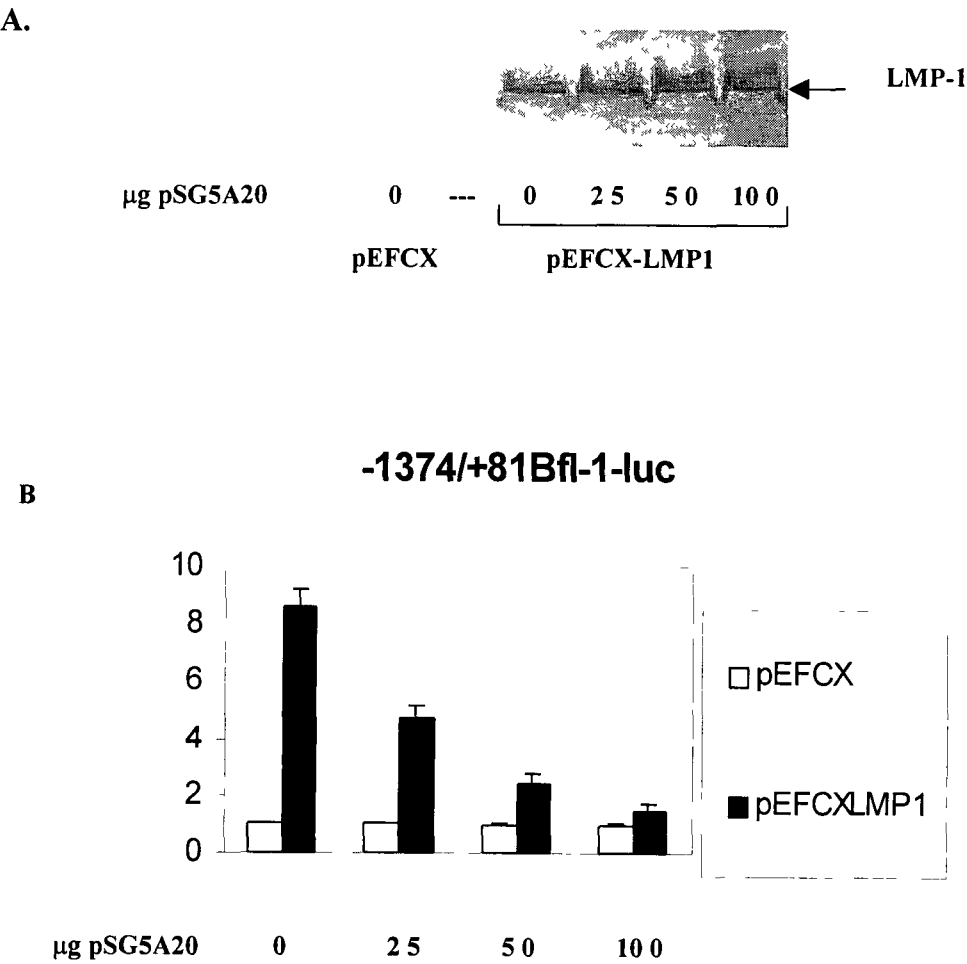


B.

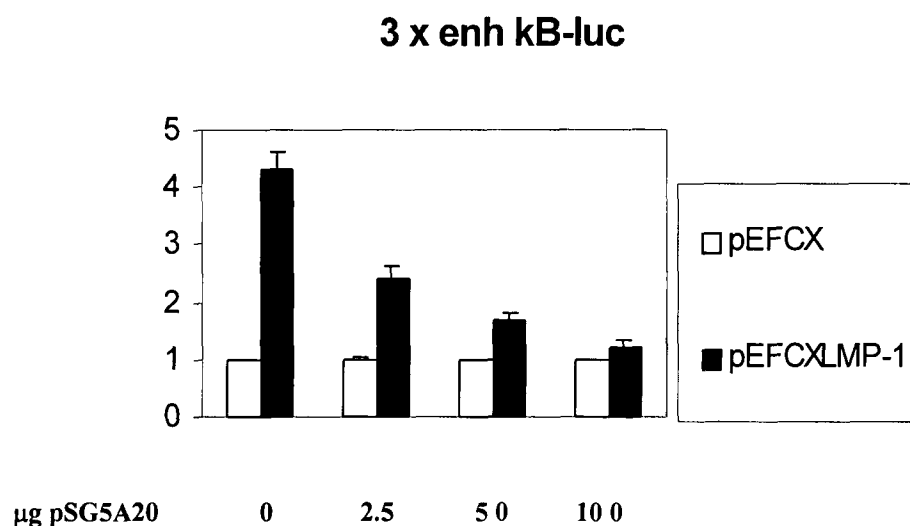
-1374/+81 Bfl-1-luc



**Figure 3 22 Involvement of TRAF2 in LMP-1-mediated activation of the *bfl-1* promoter in DG75 cells** DG75 cells were cotransfected with 2.5 µg of either pEFCX-LMP1 or empty vector pEFCX and 2.5 µg of either p-1374/+81-Bfl-1-luc (panel B) or 3 x enh κB-luc (panel C) together with various amounts (0, 5.0 or 10.0 µg) of a plasmid expressing dominant-negative TRAF2 mutant [pSG5-TRAF2Δ(6-86)]. Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (A) and luciferase activity produced from either the *bfl-1* promoter – luc construct (B) or the NF- κB –luc construct (C). Normalised luciferase activities were expressed as fold activation over control [empty-vector in the absence of expression of TRAF2Δ(6-86)] as before



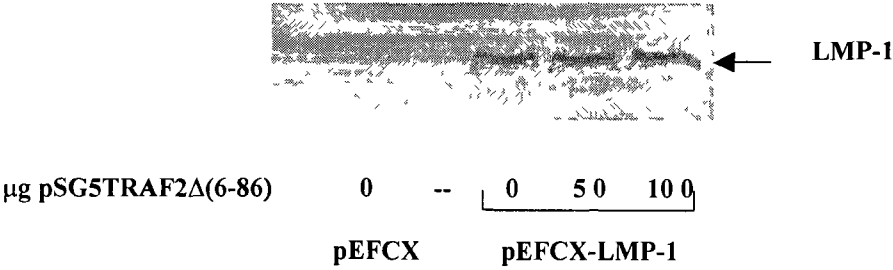
C.



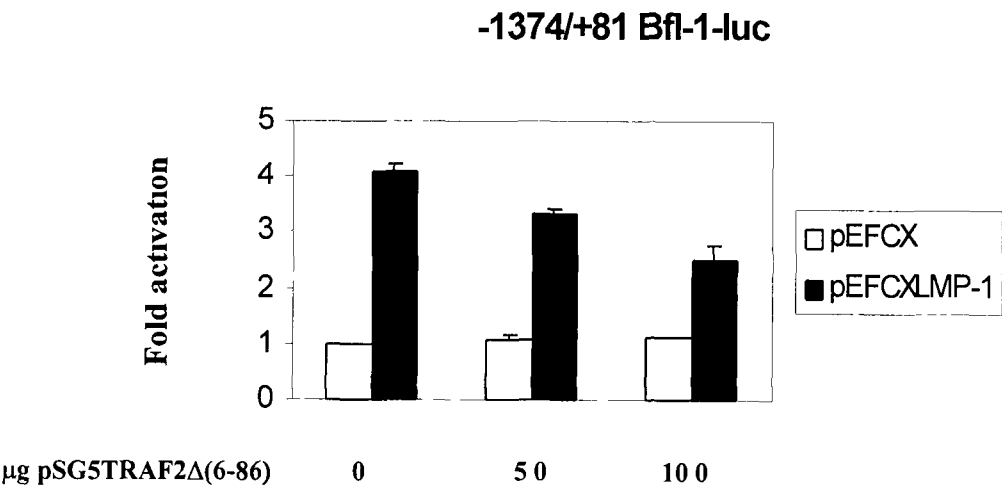
**Figure 3 23 Overexpression of A20 blocks LMP-1-mediated activation of the *bfl-1* promoter in DG75 cells** DG75 cells were cotransfected with 2.5 μg of pEFCX-LMP1 or empty vector pEFCX and 2.5 μg of either p-1374/+81-Bfl-1-luc (panel B) or 3 x enh κB-luc (panel C) together with various amounts (0, 2.5, 5.0 or 10.0 μg) of a plasmid expressing A20 (pSG5-A20). Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (A) and luciferase activity produced from either the *bfl-1* promoter – luc construct (B) or the NF-κB – luc construct (C). Normalised luciferase activities were expressed as fold activation over control (empty vector in the absence of A20 expression=1).

In Jurkat cells, expression of dominant-negative TRAF2 mutant, TRAF2Δ(6-86) (Figure 3 24), or A20 (Figure 3 25) also inhibited LMP-1-mediated activation of NF-κB and the *bfl-1* promoter. As observed in DG75 cells, expression of TRAF2Δ(6-86) caused a partial inhibition [approximately 50% with transfection of 10.0 μg of pSG5 TRAF2Δ(6-86)] of LMP-1-mediated activation of NF-κB and the *bfl-1* promoter and expression of A20 almost completely inhibited these LMP-1-mediated effects in Jurkat cells. Western blot analysis revealed that expression of TRAF2Δ(6-86) or A20 did not alter LMP-1 expression relative to controls.

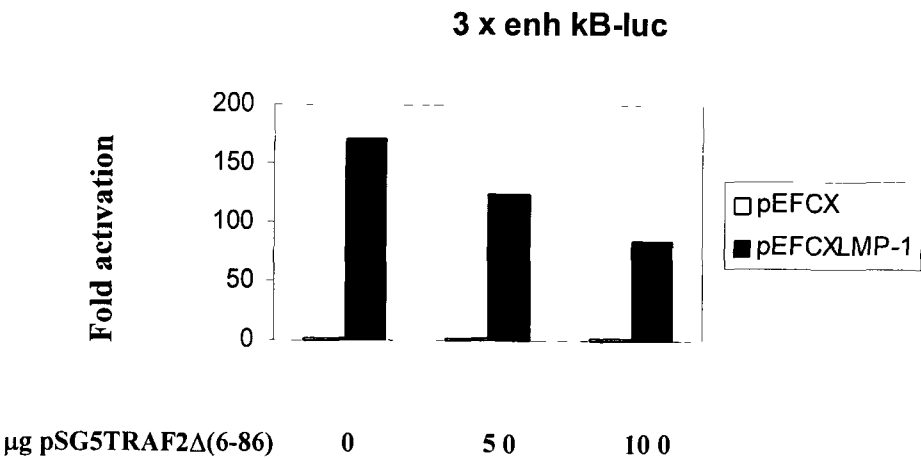
A



B

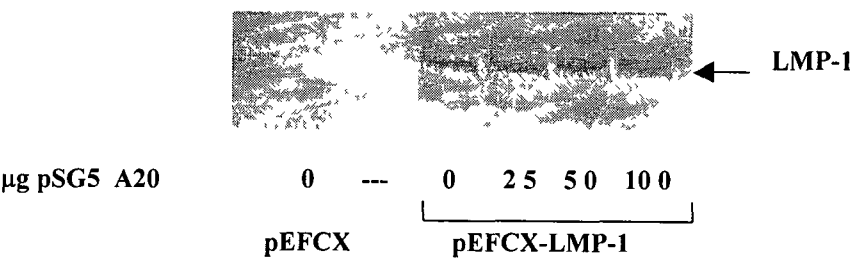


C

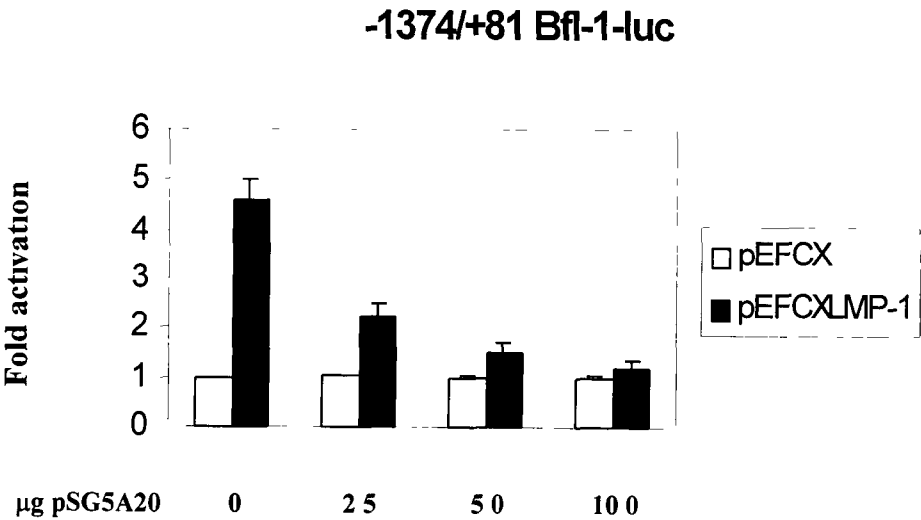


**Figure 3 24 Involvement of TRAF2 in LMP-1-mediated activation of the *bfl-1* promoter in Jurkat T cells** Jurkat cells were cotransfected with 2.5 µg of pEFCX-LMP1 or empty vector pEFCX and 2.5 µg of either p-1374/+81-Bfl-1-luc (panel B) or 3 x enh κB-luc (panel C) together with various amounts (0, 5.0 or 10.0 µg) of a plasmid expressing dominant-negative TRAF2 mutant [pSG5-TRAF2Δ(6-86)]. Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (A) and luciferase activity produced from either the *bfl-1* promoter – luc construct (B) or the NF- κB –luc construct (C). The experiment in panel C was only performed once. Normalised luciferase activities were expressed as fold activation over control [empty-vector in the absence of expression of TRAF2Δ(6-86) = 1].

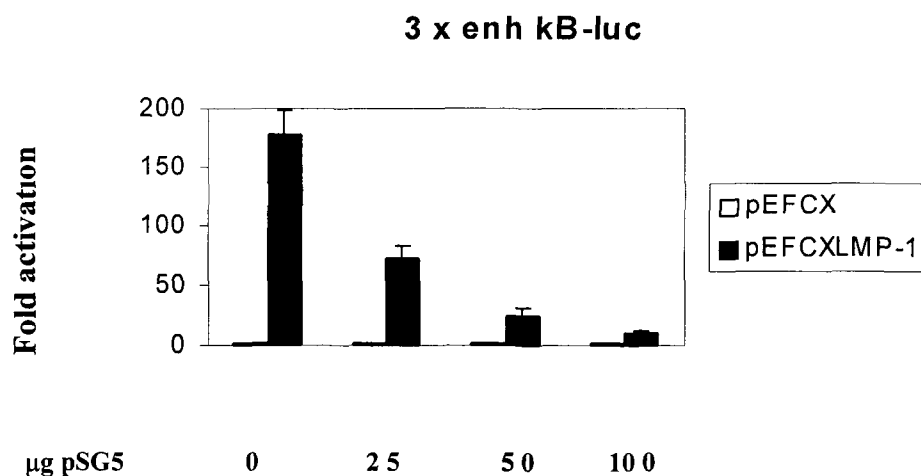
A



B



C.



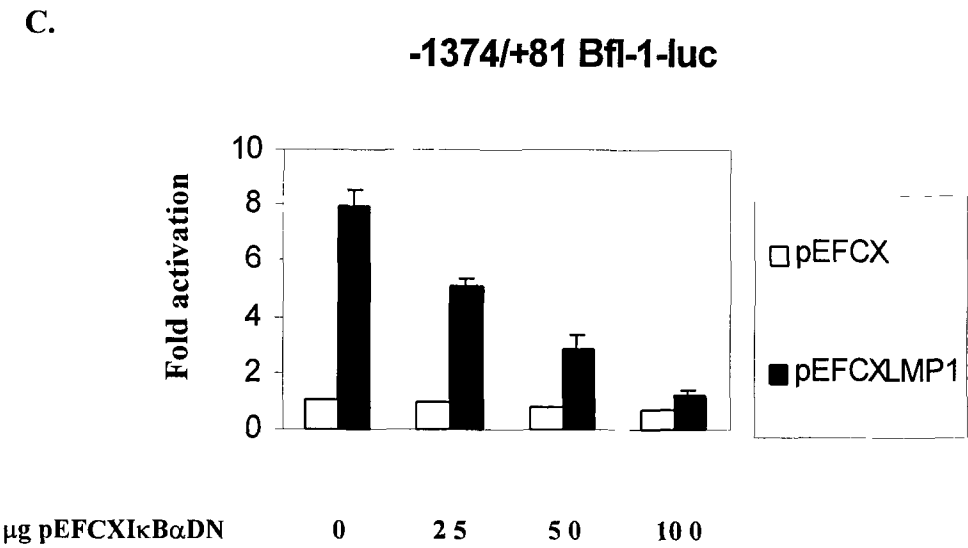
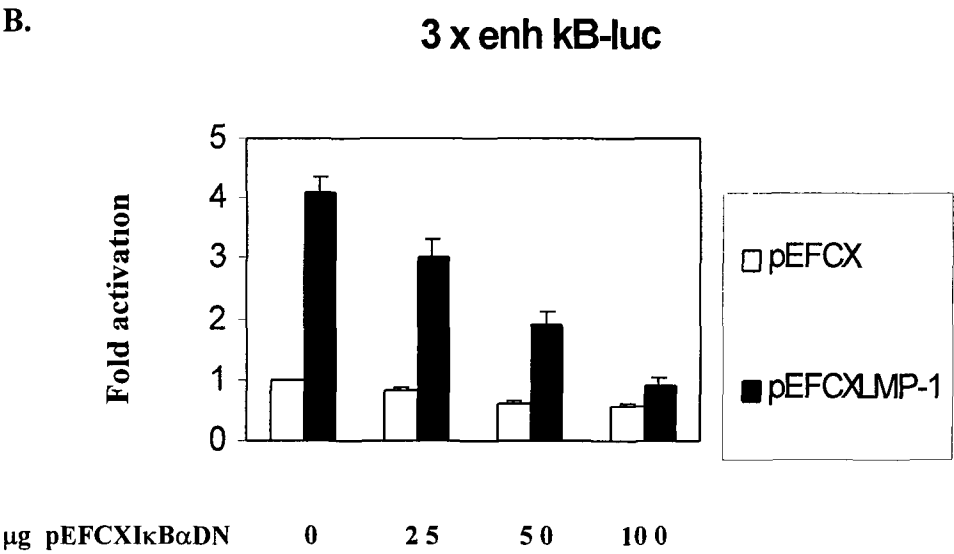
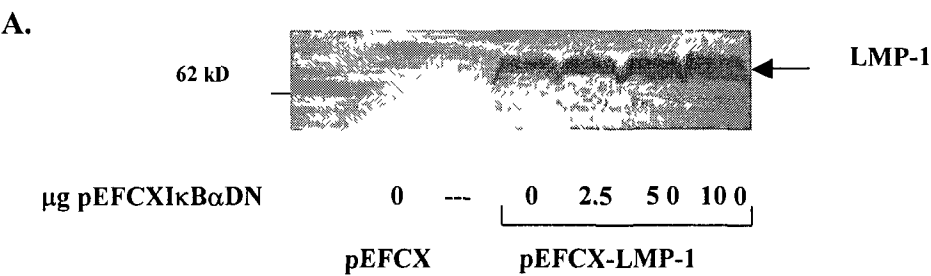
**Figure 3 25 Overexpression of A20 blocks LMP-1-mediated activation of the *bfl-1* promoter in Jurkat T cells** Jurkat cells were cotransfected with 2.5 µg of pEFCX-LMP1 or empty vector pEFCX and 2.5 µg of either p-1374/+81-Bfl-1-luc (panel B) or 3 x enh κB-luc (panel C) together with various amounts (0, 2.5, 5.0 or 10.0 µg) of a plasmid expressing A20 (pSG5-A20). Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (A) and luciferase activity produced from either the *bfl-1* promoter – luc construct (B) or the NF-κB –luc construct (C). Normalised luciferase activities were expressed as fold activation over control (empty-vector in the absence of A20 expression =1)

### 3.2.7.5 Activation of *bfl-1* promoter activity by LMP-1 is mediated by the transcription factor NF- $\kappa$ B

It is now well established that LMP-1 can activate the transcription factor NF- $\kappa$ B in several cellular contexts (including B and T lymphocytes and epithelial cells) and that many of the LMP-1-induced changes in gene expression such as *A20* and *ICAM-1* appear to be regulated by this transcription factor (Hammarskjold and Simurda, 1992; Laherty *et al.*, 1992; Huen *et al.*, 1995; Liljeholm *et al.*, 1998; Eliopoulos *et al.*, 1999; Mehl *et al.*, 2001). Members of the NF- $\kappa$ B family, which include p50 (NF $\kappa$ B1), p52 (NF $\kappa$ B2), p65 (RelA), c-rel and RelB, can form both homodimers and heterodimers (Baeuerle and Baltimore, 1996; Baldwin, 1996). The prototype NF- $\kappa$ B is the NF- $\kappa$ B/Rel heterodimer p50/p65. These dimers are normally held in the cytoplasm as inactive complexes with an inhibitory component, I $\kappa$ B. Activation appears to involve phosphorylation and subsequent release and degradation of the I $\kappa$ B component, thus permitting translocation of the active heterodimers to the nucleus, where they bind to specific DNA sequences in the promoters of the genes they activate. There are a number of different types of I $\kappa$ B, and evidence suggests that LMP-1 activates NF- $\kappa$ B through phosphorylation of I $\kappa$ B $\alpha$  on serine residues at positions 32 and 36 (Herrero *et al.*, 1995).

As observed in section 3.2.7.1, the extent of activation of NF- $\kappa$ B by LMP-1 parallels its effect on *bfl-1* promoter activity in DG75 cells. In addition, TRAF-2 which has been shown to be required for activation of NF- $\kappa$ B by LMP-1, is also required for LMP-1 mediated induction of *bfl-1* promoter activity (section 3.2.7.4). These observations together with the now well-established fact that several of the LMP-1-induced changes in gene expression are NF- $\kappa$ B-mediated suggested that the regulation of *bfl-1* promoter activity by LMP-1 could also be mediated by this transcription factor. To directly test this hypothesis, DG75 cells were cotransfected with either an NF- $\kappa$ B-dependent luciferase reporter construct (3 x enh  $\kappa$ B-luc), or the p-1374/+81 Bfl-1 luc reporter and the pEFCX-LMP-1 plasmid together with a plasmid encoding a dominant negative

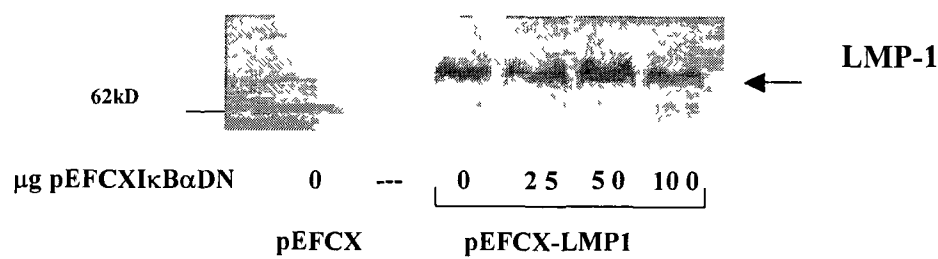
mutant form of I $\kappa$ B $\alpha$  (pEFCX-I $\kappa$ B $\alpha$ DN) I $\kappa$ B $\alpha$ DN is a double mutant with the phosphorylatable serines at positions 32 and 36 mutated to alanines such that it can no longer be phosphorylated and is consequently not proteolysed upon NF- $\kappa$ B stimulation (Brown *et al* , 1995) This mutant efficiently retains NF- $\kappa$ B in the cytoplasm, unable to translocate to the nucleus and induce transcription (Liljeholm *et al* , 1998) Expression of I $\kappa$ B $\alpha$ DN efficiently inhibited LMP-1-induced NF- $\kappa$ B activity in DG75 cells as assessed by measuring luciferase activity after cotransfection of 3 x enh  $\kappa$ B-luc (2.5  $\mu$ g) and pEFCX-LMP-1 (2.5  $\mu$ g) plasmids together with pEFCX-I $\kappa$ B $\alpha$ DN (2.5 to 10.0  $\mu$ g) (Figure 3.26B) The effect of I $\kappa$ B $\alpha$ DN on NF- $\kappa$ B activity was dose-dependent, with 10.0  $\mu$ g of pEFCX-I $\kappa$ B $\alpha$ DN resulting in complete inhibition of LMP-1-induced NF- $\kappa$ B activation Transfection of pEFCX-I $\kappa$ B $\alpha$ DN also inhibited the basal NF- $\kappa$ B activity in DG75 cells with cotransfection of 10.0  $\mu$ g of pEFCX-I $\kappa$ B $\alpha$ DN resulting in an approximately 60% decrease in basal NF- $\kappa$ B activity In a similar experimental strategy but with cotransfection of the p-1374/+81 Bfl-1 luc reporter instead of 3 x enh  $\kappa$ B-luc, expression of I $\kappa$ B $\alpha$ DN also inhibited LMP-1-induced upregulation of *bfl-1* promoter activity in a dose-dependent manner (Figure 3.26C) In this experiment, 10.0  $\mu$ g of pEFCX-I $\kappa$ B $\alpha$ DN, inhibited LMP-1-mediated activation of the *bfl-1* promoter to near basal (vector control) levels This amount (10  $\mu$ g) of pEFCX-I $\kappa$ B $\alpha$ DN, is the same as that which completely inhibited LMP-1-induced NF- $\kappa$ B activity The inhibitory action of I $\kappa$ B $\alpha$ DN on these LMP-1-induced effects on promoter activity is not the result of inhibition of LMP-1 expression from pEFCX-LMP-1 as revealed by Western blot analysis for LMP-1 expression (Figure 3.26A) These results strongly suggest that the activation of the *bfl-1* promoter induced by LMP-1 is NF- $\kappa$ B-dependent Expression of I $\kappa$ B $\alpha$ DN also inhibited basal *bfl-1* promoter activity (transfection of 10.0  $\mu$ g of pEFCX-I $\kappa$ B $\alpha$ DN caused an approximately 30% decrease in basal *bfl-1* promoter activity), thereby suggesting that the basal activity of the *bfl-1* promoter in DG75 cells may be, at least in part, NF- $\kappa$ B-dependent



**Figure 3 26 Inhibition of LMP-1-mediated activation of *bfl-1* promoter activity by overexpression of a dominant negative mutant of IκBα (IκBαDN) in DG75 cells** DG75 cells were cotransfected with 2.5 μg of pEFCX-LMP1 or empty vector pEFCX and 2.5 μg of either 3 x enh κB-luc (panel B) or p-1374/+81-Bfl-1 -luc (panel C) together with various amounts (0, 2.5, 5.0 or 10.0 μg) of a plasmid expressing IκBαDN. Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (A) and luciferase activity produced from either the NF-κB -luc construct (B) or the *bfl-1* promoter - luc construct (C) was measured. Normalised luciferase activities were expressed as fold-activation over control (empty-vector in the absence of expression of IκBαDN =1)

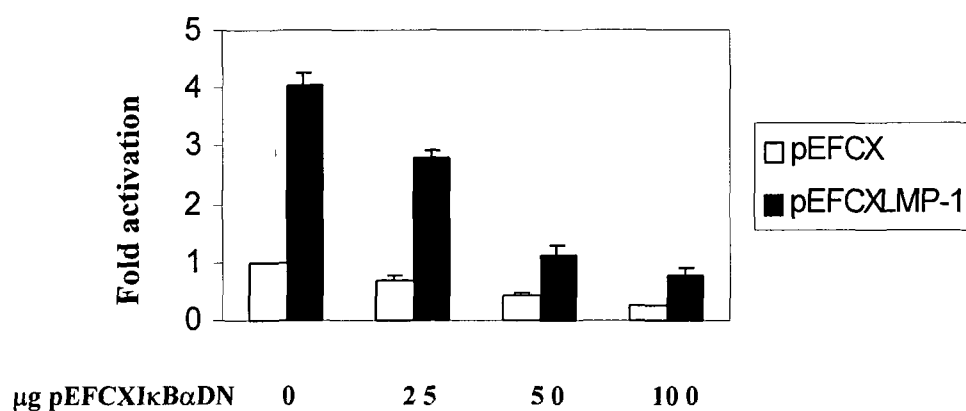
The effect of LMP-1 expression on *bfl-1* promoter activity was also investigated in the context of another EBV-negative BL cell line, BL41, in order to rule out the possibility that the effect is specific to DG75 cells. Indeed, transient expression of LMP-1 in BL41 cells enhanced luciferase synthesis from the NF-κB-luc construct, 3 x enh-κB-luc, approximately 4-fold (Figure 3 27B) and stimulated *bfl-1* promoter activity from the p-1374/+81Bfl-1-luc construct approximately 5.4-fold (Figure 3 27C). As observed with DG75 cells, expression of the dominant negative IκBα mutant, IκBαDN, also inhibited the effect of LMP-1 on NF-κB activation and *bfl-1* promoter activity in BL41 cells in a dose-dependent manner, however much lower amounts (5 μg) of pEFCX- IκBαDN were required to achieve complete inhibition of these effects on reporter activity in BL41 cells (Figure 3 27)

A.



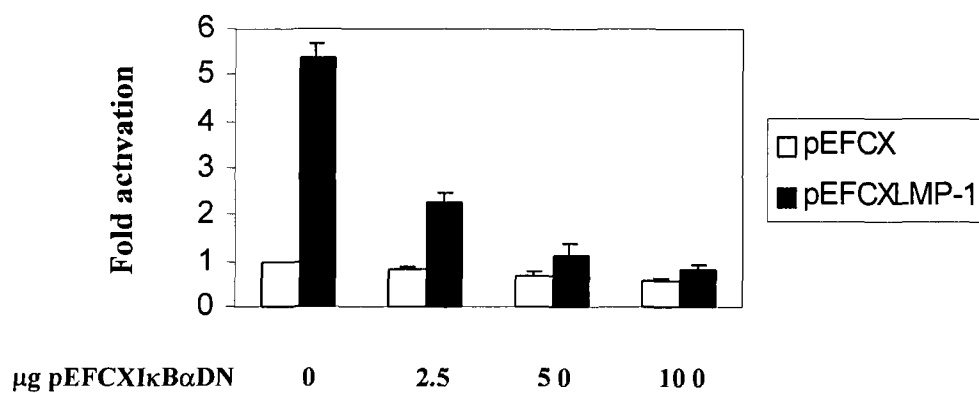
B.

### 3 x enh kB luc



C.

### -1374/+81 WTBfl-1 luc

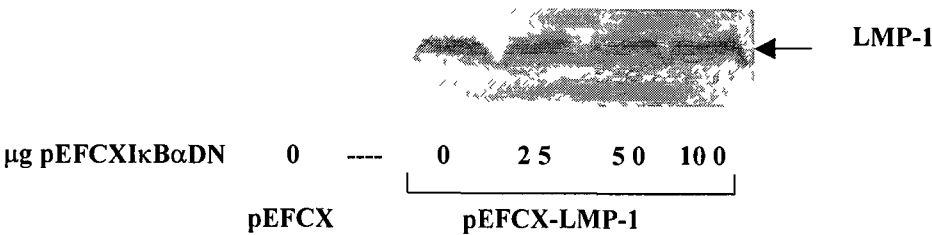


**Figure 3 27 Inhibition of LMP-1-mediated activation of *bfl-1* promoter activity by overexpression of a dominant negative mutant of  $\text{I}\kappa\text{B}\alpha$  ( $\text{I}\kappa\text{B}\alpha\text{DN}$ ) in BL41 cells** BL41 cells were cotransfected with 2.5  $\mu\text{g}$  of pEFCX-LMP1 or empty vector pEFCX and 2.5  $\mu\text{g}$  of either 3 x enh  $\kappa\text{B}$ -luc (panel B) or p-1374/+81-Bfl-1 -luc (panel C) or together with various amounts (0, 2.5, 5.0 or 10.0  $\mu\text{g}$ ) of a plasmid expressing  $\text{I}\kappa\text{B}\alpha\text{DN}$ . Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot

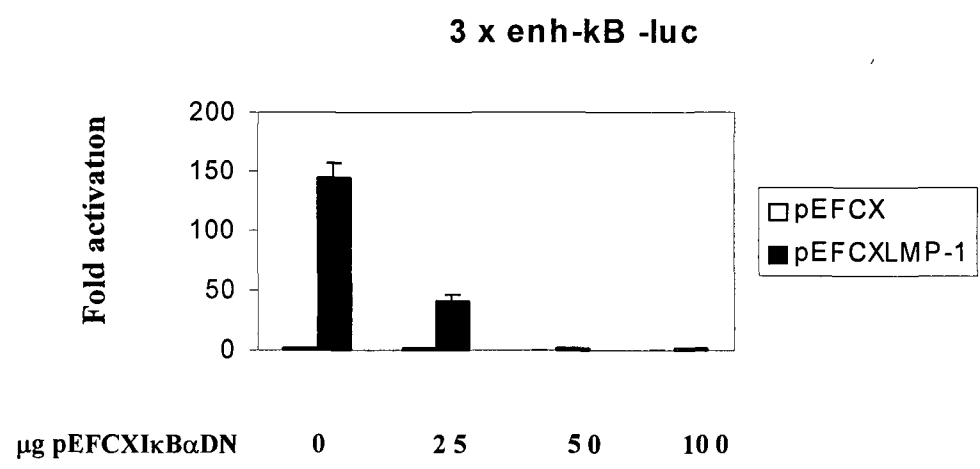
analysis (A) and luciferase activity produced from either the NF- $\kappa$ B-luc construct (B) or the *bfl-1* promoter-luc construct (C) was measured. Normalised luciferase activities were expressed as fold-activation over control (empty-vector in the absence of expression of I $\kappa$ B $\alpha$ DN=1).

A role for NF- $\kappa$ B in mediating LMP-1-induced activation of *bfl-1* promoter activity was also observed in Jurkat T cells. Transient expression of LMP-1 in Jurkat cells activated the NF- $\kappa$ B-luc construct, 3 x enh- $\kappa$ B-luc, approximately 145-fold (Figure 3 28B) and stimulated *bfl-1* promoter activity from the p-1374/+81Bfl-1-luc construct approximately 4.2-fold (Figure 3 28C). Expression of the dominant negative I $\kappa$ B $\alpha$  mutant, I $\kappa$ B $\alpha$ DN, inhibited the effect of LMP-1 on NF- $\kappa$ B activation and *bfl-1* promoter activity in Jurkat cells in a dose-dependent manner, however, unlike DG75 cells much lower amounts (5.0  $\mu$ g) of pEFCX-I $\kappa$ B $\alpha$ DN were required to achieve complete inhibition of the effects on these reporter constructs in Jurkat cells (Figure 3 28 B and C). The basal activity of the *bfl-1* promoter was only marginally inhibited in Jurkat cells in that only a 20% inhibition of basal *bfl-1* promoter activity was detected on transfection of 5.0  $\mu$ g of pEFCX-I $\kappa$ B $\alpha$ DN and the extent of inhibition was not increased on transfection of a higher amount (10.0  $\mu$ g) of pEFCX-I $\kappa$ B $\alpha$ DN even though a dose-dependent decrease in NF- $\kappa$ B activity was detected in a similar experiment but with transfection of the NF- $\kappa$ B-luc reporter. These results suggest that the basal activity of the *bfl-1* promoter in Jurkat cells may not be dependent on NF- $\kappa$ B activity to the same extent as in B cell lines.

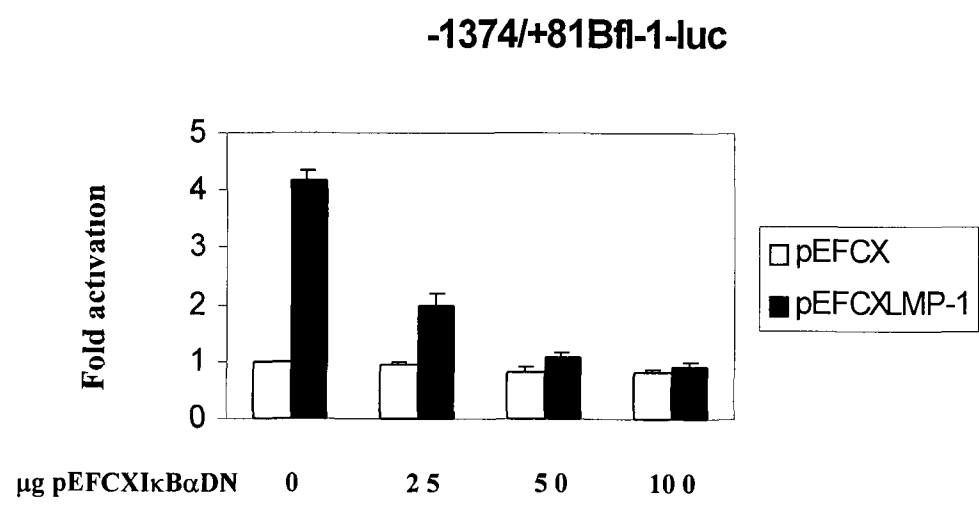
A.



B.



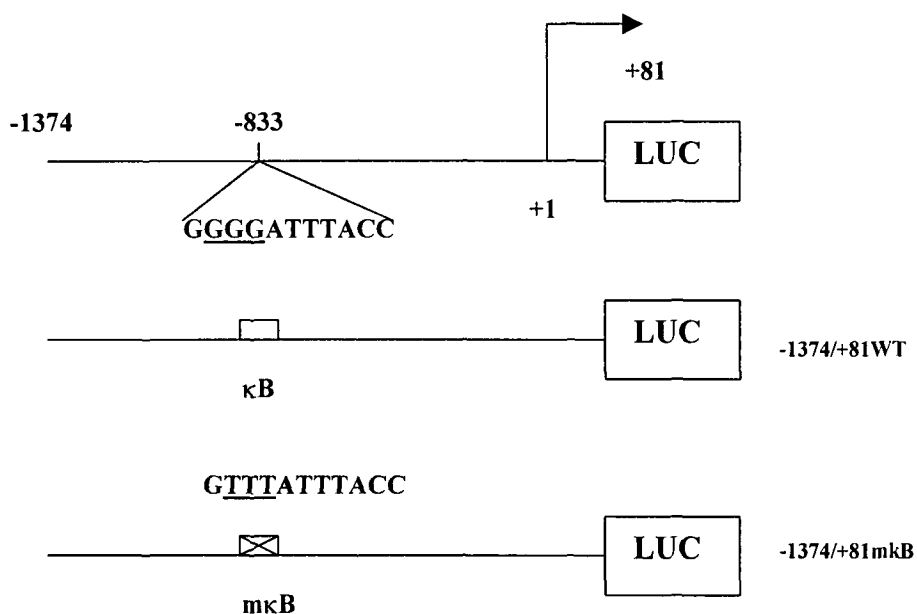
C.



**Figure 3 28 Inhibition of LMP-1-mediated activation of *bfl-1* promoter activity by overexpression of a dominant negative mutant of I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ DN) in Jurkat cells** Jurkat cells were cotransfected with 2.5  $\mu$ g of pEFCX-LMP1 or empty vector pEFCX and 2.5  $\mu$ g of either 3 x enh  $\kappa$ B-luc (panel B) or p-1374/+81-Bfl-1-luc (panel C) together with various amounts (0, 2.5, 5.0 or 10.0  $\mu$ g) of a plasmid expressing I $\kappa$ B $\alpha$ DN. Cells were harvested 24 h post-transfection and analysed for LMP-1 expression by Western blot analysis (A) and luciferase activity produced from either the NF- $\kappa$ B-luc construct (B) or the *bfl-1* promoter-luc construct (C) was measured. Normalised luciferase activities were expressed as fold activation over control (empty-vector in the absence of expression of I $\kappa$ B $\alpha$ DN=1).

### **3.2.7.6 LMP1-induced activation of the *bfl-1* promoter does not involve an NF- $\kappa$ B site at position -833 (-833 to -823)**

LMP-1 upregulates *bfl-1* expression, in part, by increasing the activity of the -1374/+81 region of the gene and this effect is mediated by at least one transcription factor, NF- $\kappa$ B. An NF- $\kappa$ B binding site (GGGGATTACC) identified at position -833 (-833 to -823) relative to the transcription start site of *bfl-1* has previously been shown to be essential for c-rel-mediated activation of the *bfl-1* promoter in the HeLa cell line by mutation analysis (Zong *et al*, 1999). To investigate if this NF- $\kappa$ B site may also be important for the LMP-1-mediated activation of *bfl-1*, a *bfl-1* promoter-luciferase construct, p-1374/+81 m $\kappa$ B Bfl-1-luc, containing a GGG $\rightarrow$ TTT mutation in the NF- $\kappa$ B motif at -833 in the context of the -1374/+81 promoter region (Zong *et al*, 1999) was used in cotransfection studies with the LMP-1-expression plasmid pEFCX-LMP-1. The p-1374/+81m $\kappa$ B-Bfl-1-luc construct was derived from the corresponding CAT-based vector (Zong *et al*, 1999) using the same strategy used for the derivation of the wild type -1374/+81 *bfl-1* promoter containing luciferase reporter construct. DNA sequencing was used to confirm the presence of the mutation in the construct. A schematic representation of the -1374/+81 region of the *bfl-1* promoter showing the position of the NF- $\kappa$ B site at -833 is shown in Figure 3 29.

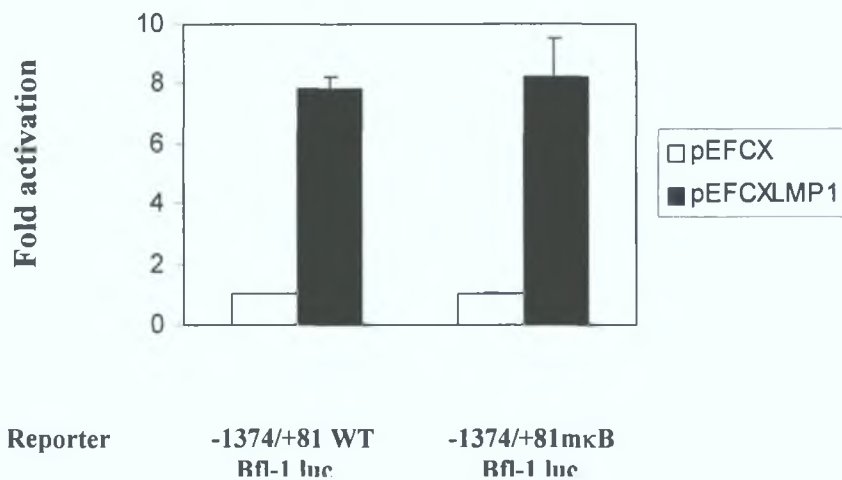


**Figure 3 29** Schematic representation of *Luc* reporter gene constructs driven by the -1374/+81 region of the *bfl-1* gene or a derivative of this region with a mutation in the NF-κB site (GGGGATTACC → GTTTATTACC) at nucleotide position -833 (relative to transcription start site) The sequence of the NF-κB site at position -833 in -1374/+81 WT Bfl-1-luc is indicated and the nucleotides in this site that were mutated in -1374/+81 mκB Bfl-1-luc are underlined

Expression of LMP-1 activated the p-1374/+81WT Bfl-1-luc construct to a similar extent as its NF-κB (-833)-mutated derivative p-1374/+81mκB Bfl-1-luc in transient transfection assays of DG75 and Jurkat cells (Figure 3 30A and B) These results suggest that unlike c-rel, the NF-κB site at position -833 is not essential for the activation of the *bfl-1* promoter by LMP-1 in these B and T lymphocyte cell lines Also, the mutation at position -833 did not significantly affect basal *bfl-1* promoter activity in either cell line

A.

DG75



B.

Jurkat

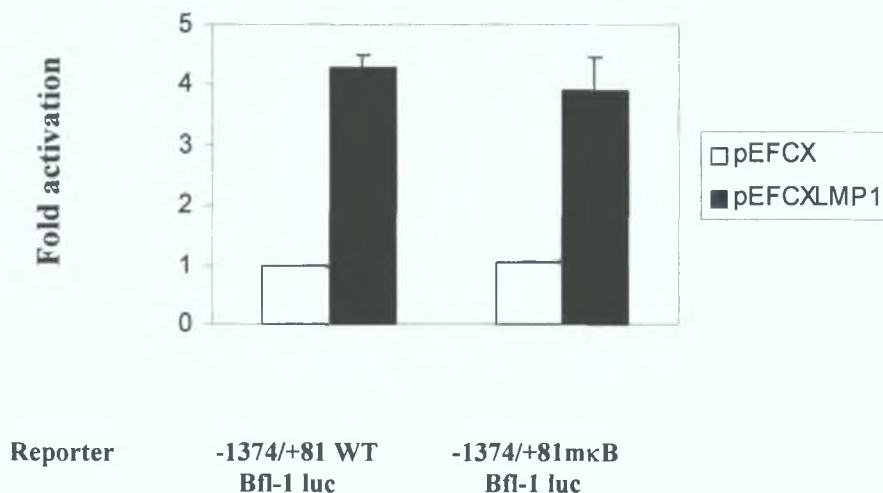


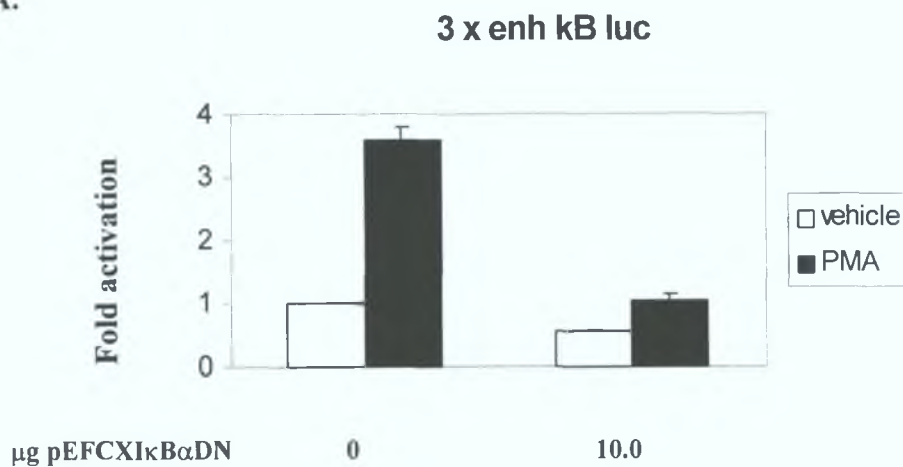
Figure 3.30 LMP-1 mediated activation of the *bfl-1* promoter in B and T lymphocyte cell lines does not require the NF- $\kappa$ B site at position -833. DG75 (panel A), and Jurkat (panel B) cells were cotransfected with 2.5  $\mu$ g of pEFCX-LMP1 or empty vector pEFCX and 2.5  $\mu$ g of either p-

1374/+81WTBfl-1-luc or its NF- $\kappa$ B (-833)-mutated derivative p-1374/+81m $\kappa$ B Bfl-1-luc Cells were harvested 24 h post-transfection and assayed for luciferase activity Normalised luciferase activities were expressed as fold activation over control (transfection of empty-vector and the wild-type bfl-1 promoter-luc construct =1)

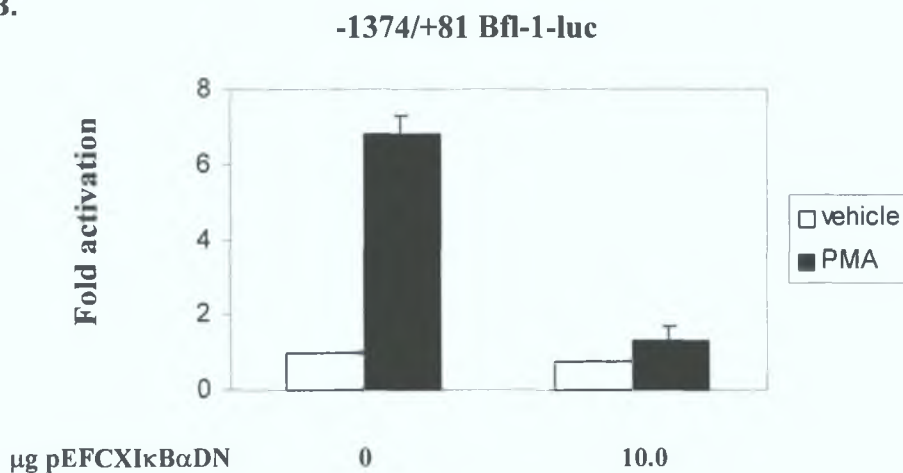
### 3.2.7.7 Activation of the *bfl-1* promoter by PMA is NF- $\kappa$ B-dependent

Treatment of a variety of cell lines including those of B cell origin with the chemical agent phorbol-12-myristate 13-acetate (PMA) has been shown to upregulate *bfl-1* mRNA levels (Moreb and Schweder, 1997) PMA is also a well known activator of the transcription factor NF- $\kappa$ B In order to examine if PMA also activated NF- $\kappa$ B in DG75 cells, luciferase activity from cells transfected with the NF- $\kappa$ B-dependent luciferase reporter, 3 x enh  $\kappa$ B luc, was measured in the presence or absence of PMA treatment In this experiment, treatment with PMA at the same concentration of  $10^{-7}$ M as used by Moreb and Schweder (1997) activated NF- $\kappa$ B approximately 3.5-fold (Figure 3.31A) The effect of PMA treatment on *bfl-1* promoter activity in DG75 cells was also examined using a similar experimental strategy but with transfection of the p-1374/+81 Bfl-1-luc construct In this experiment, PMA treatment resulted in a 6.8-fold increase in *bfl-1* promoter activity (Figure 3.31B) Furthermore, the effect of PMA on the activity of the *bfl-1* promoter was demonstrated to be NF- $\kappa$ B-dependent since transfection of an expression vector for the dominant negative mutant of I $\kappa$ B $\alpha$  (pEFCX-I $\kappa$ B $\alpha$ DN) significantly inhibited the induction of luciferase activity from the cotransfected *bfl-1*-luciferase reporter construct by PMA in these cells (Figure 3.31B)

A.

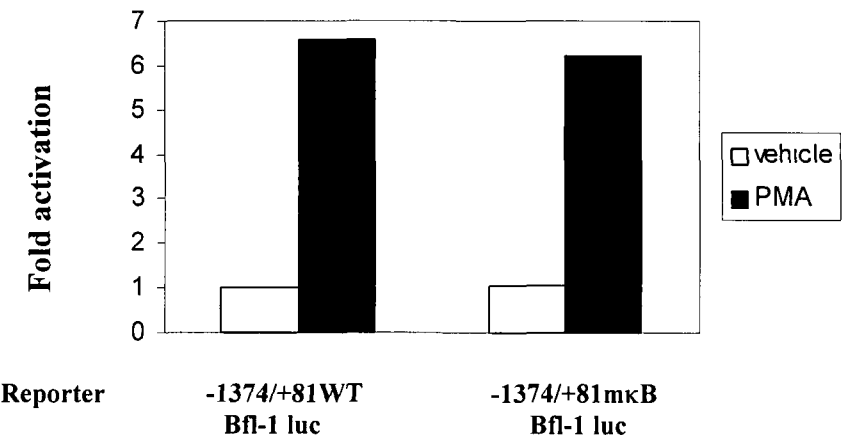


B.



**Figure 3.31 PMA activates *bfl-1* promoter activity in an NF-κB-dependent manner in DG75 cells.** **A)** DG75 cells transfected with 2.5 µg of the NF-κB-dependent luciferase vector, 3 x enh κB-luc in the presence or absence of 10.0 µg pEFCX-IκBαDN were treated with either 10<sup>-7</sup> M PMA or vehicle control (0.062% ethanol) 18h posttransfection. After treatment for 24 h, cells were harvested and analysed for luciferase activity. Normalised luciferase values are expressed as fold-increase over control (= the value obtained with p-1374/+81 Bfl-1-luc-transfected cells treated with vehicle control was arbitrarily assigned a value of 1). **B)** DG75 cells transfected with 2.5 µg of the -1374/+81Bfl-1 luc vector in the presence or absence of 10.0 µg of pEFCX-IκBαDN were treated with either 10<sup>-7</sup> M PMA or vehicle control (0.062% ethanol) 18h posttransfection. Cells were harvested 24 h post-treatment and analysed for luciferase activity as in panel A.

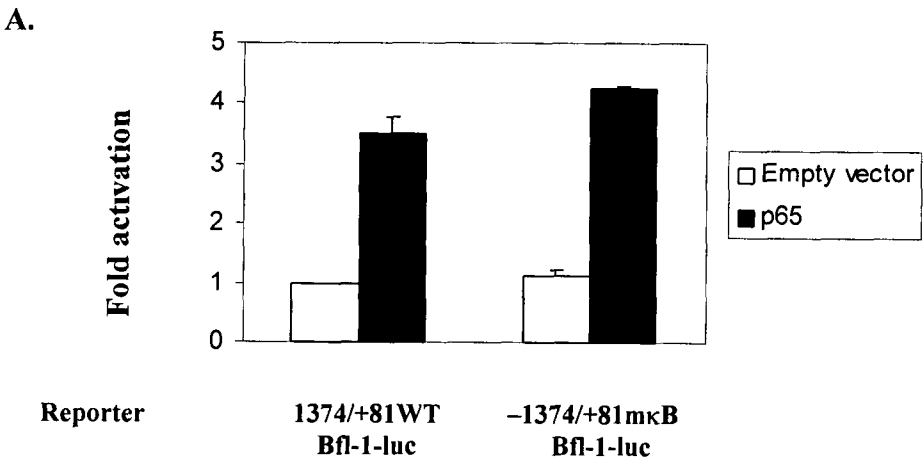
PMA treatment activated the wild-type and NF- $\kappa$ B-mutated Bfl-1 promoter-luciferase construct to approximately the same extent indicating that the NF- $\kappa$ B site at -833 is also not essential for the activation of the *bfl-1* promoter by PMA (Figure 3 32)

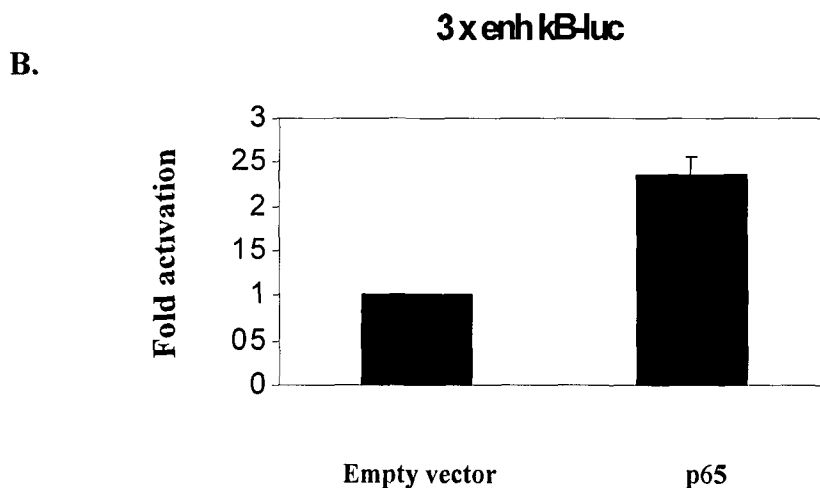


**Figure 3 32 PMA- mediated activation of the *bfl-1* promoter in DG75 cells does not require the NF- $\kappa$ B site at position -833 of the promoter** DG75 cells transfected with 2.5  $\mu$ g of either p-1374/+81WTBfl-1-luc or its NF- $\kappa$ B (-833)-mutated derivative p-1374/+81mκB Bfl-1-luc were treated with either  $10^{-7}$  M PMA or vehicle control (0.062% ethanol ) 18h posttransfection. After treatment for 24 h, cells were harvested and analysed for luciferase activity as before. Normalised luciferase activities were expressed as fold activation over control (transfection of empty-vector and p-1374/+81 WT Bfl-1luc =1). This experiment was performed once.

**3.2.7.8 The *bfl-1* promoter is responsive to the NF-κB subunit protein p65**

To directly demonstrate the responsiveness of the *bfl-1* promoter to NF-κB, the effect of overexpression of the NF-κB subunit p65 (Rel A) on *bfl-1* promoter activity was assessed by cotransfection of an expression vector for p65 together with the *bfl-1* promoter-luciferase construct, p-1374/+81 WT*Bfl-1-luc*, in DG75 cells. It can be seen from Figure 3 33A that expression of p65 results in a 3.5-fold activation of *bfl-1* promoter activity from the cotransfected p-1374/+81 *Bfl-1-luc* construct. p65 expression did not affect luciferase activity from the basal/ promoterless reporter vector (result not shown). Inactivation of the NF-κB site at position -833 of the *bfl-1* promoter did not alter the ability of p65 to activate transcription from the -1374/+81 region of the *bfl-1* promoter, indicating that as with LMP-1-expression and PMA treatment, the NF-κB site at position -833 is also not essential for the p65-mediated activation of the *bfl-1* promoter (Figure 3 33A). As a control for the functional activity of p65, the effect of p65 expression on the activity of a cotransfected NF-κB-dependent luciferase reporter construct was determined. p65 expression resulted in an approximately 2.4-fold increase in luciferase activity from the 3 x enh κB-luc reporter construct (Figure 3 33B).





**Figure 3 33 Overexpression of the NF-κB subunit p65 (Rel A) activates bfl-1 promoter activity in DG75 cells but this effect is not mediated by the NF-κB site at position –833 of the promoter A)** DG75 cells were cotransfected with 5 0 μg of the expression vector for p65 (pSG5-p65) or empty vector (pSG5) together with 2 5 μg of either the *bfl-1* promoter-luciferase reporter construct p-1374/+81 WT*Bfl-1*-luc or the NF-κB-mutated version p-1374/+81 mκB *Bfl-1*-luc Cells were harvested 24 h post-transfection and assayed for luciferase activity as before Normalised luciferase values are expressed as fold-increase over control (transfection of empty vector with wild-type *bfl-1* promoter-luc construct =1) **B)** DG75 cells were cotransfected with 5 0 μg of the expression vector for p65 (pSG5-p65) or empty vector (pSG5) together with 2 5 μg of the NF-κB-dependent luciferase reporter, 3 x enh-κB-luc, and luciferase activity assayed in cells harvested 24 h post-transfection

### 3 2.7.9 Deletion analysis of the *bfl-1* promoter

To identify the regions of the *bfl-1* promoter that mediate induction by LMP-1, a series of *bfl-1* promoter-luciferase constructs containing progressive deletions from the 5' end was used in transient transfection assays Promoter-luciferase constructs containing DNA sequence from the –1240/+81, –367/+81 or –129/+81 regions of the *bfl-1* gene fused to the luciferase gene were derived from corresponding CAT-based constructs using the same strategy as for the derivation of the p–1374/+81 *Bfl-1*-luc construct (Figure 3 34, the –1240/+81 *Bfl-1*luc, –367/+81 *Bfl-1*luc and –129/+81 *Bfl-1*luc reporter constructs

were made by P Pegman in the laboratory) A schematic representation of the luciferase reporter gene constructs driven by various regions of the *bfl-1* promoter is shown in Figure 3 34 Agarose gel electrophoresis confirmed the quality of the *bfl-1*-promoter-luciferase constructs (Figure 3 35)

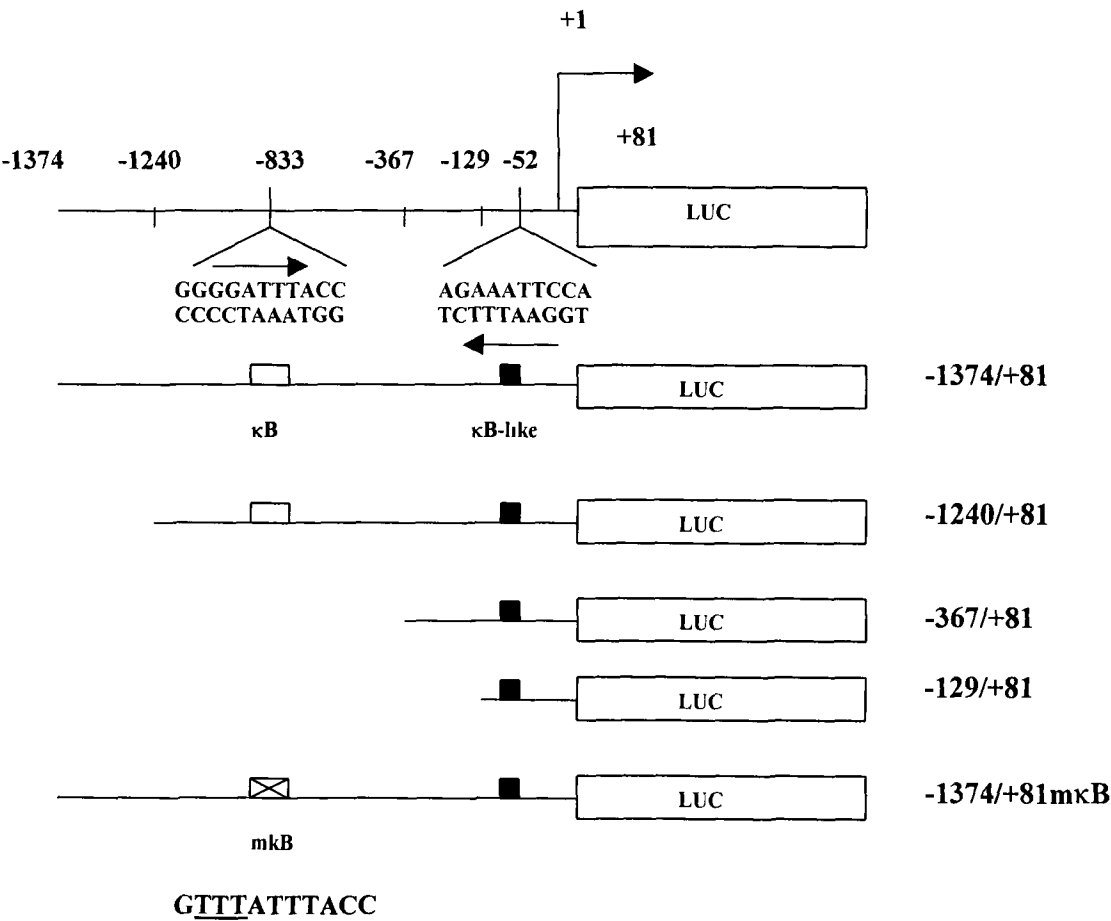
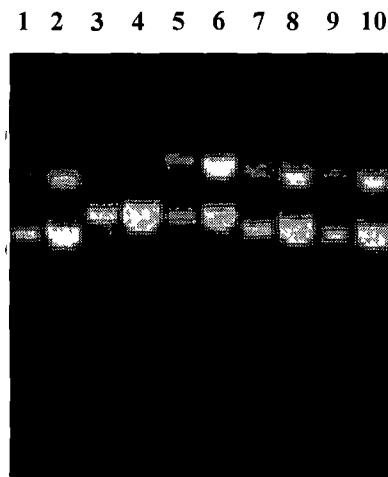


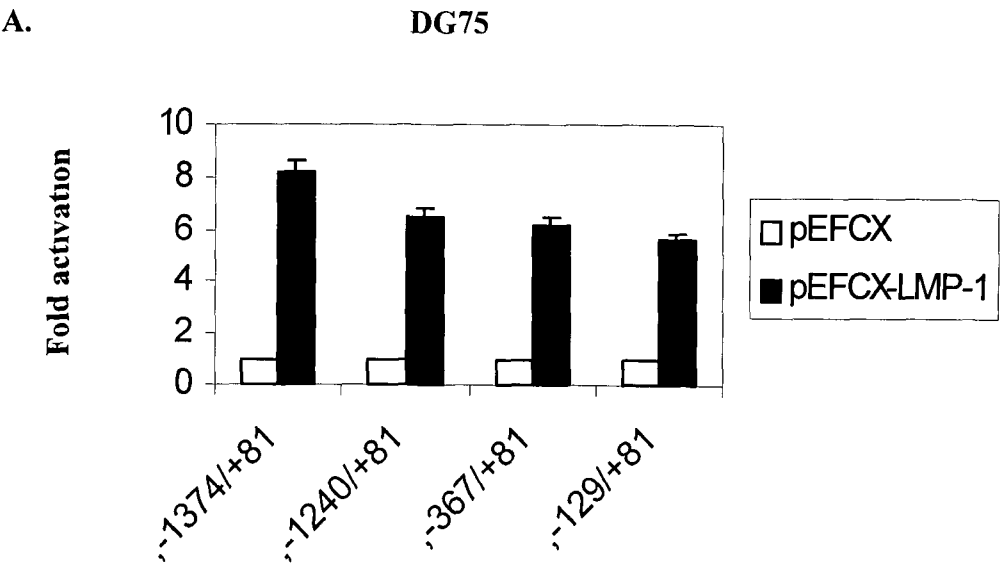
Figure 3 34 Schematic representation of *bfl-1*-promoter-luciferase reporter constructs containing NF-κB (position -833, -1374/+81 mκB) and 5' deletion mutants (-1240/+81, -367/+81, -129/+81) of the -1374/+81 region of *bfl-1* The sequences of the NF-κB site at position -833 and an 'NF-κB-like' site at position -52 are indicated The arrows near the sequence of these sites correspond to the orientation of the binding sites The TATA box is located at position -69 (not indicated in the figure)



**Figure 3 35 Agarose gel electrophoresis of *bfl-1* promoter-luciferase constructs** One (lanes 1,3, 5, 7, 9) or 2 5 (lanes 2, 4, 6, 8, 10)  $\mu\text{g}$  of the promoterless/basic (lanes 1, 2) or *bfl-1*-promoter-luciferase constructs encompassing -1374/+81 (lanes 3, 4 ), -1240/+81 (lanes 5, 6) , -367/+81 (lanes 7, 8) or -129/+81(lanes 9, 10) regions of *bfl-1* were electrophoresed in a 1 % agarose- 1 x TAE gel for 1 h at 100 V

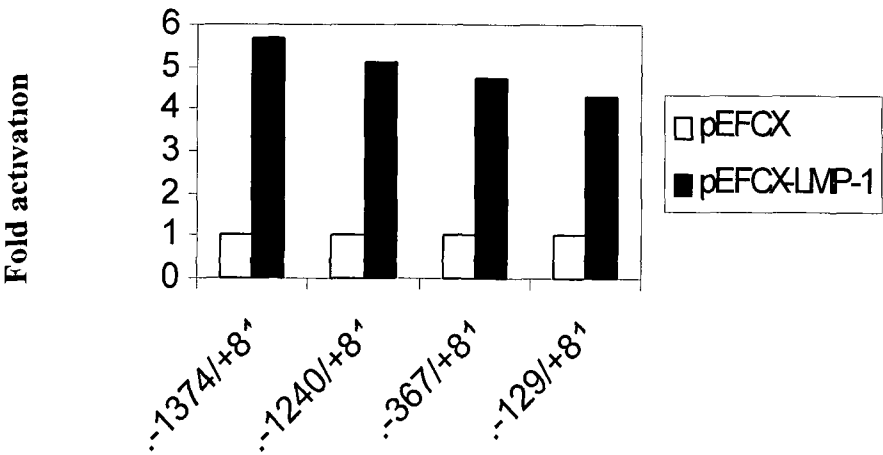
The effect of expression of LMP-1 on the activity of various regions of the *bfl-1* promoter was investigated by cotransfection of the LMP-1 expression plasmid, pEFCX-LMP-1, or the empty vector, pEFCX, together with the individual *bfl-1* promoter-luciferase reporter constructs containing the -1374/+81 region of *bfl-1* or 5' progressive deletions thereof into DG75, BL41 and Jurkat cells and assaying for luciferase activity 24 h post-transfection. As shown in Figure 3 36A, LMP-1 expression in DG75 cells activated transcription from the -1374/+81 region of *bfl-1* approximately 8.2 -fold over control. Surprisingly, LMP-1 expression was still able to induce considerable activation of the promoter fragments bearing progressive 5'deletions, with a 6.5-, 6.2- and 5.6-fold activation measured with the -1240/+81, -367/+81 and -129/+81 *bfl-1* promoter-luciferase constructs, respectively, in DG75 cells. Thus, in DG75 cells, the -129/+81 region of *bfl-1* makes a considerable contribution to the LMP-1-induced activation of *bfl-1* promoter activity, retaining 64% of the LMP-1 -induced activity of the -1374/+81 region of this gene. A similar trend in the extent of activation of the *bfl-1* promoter-deletion luciferase reporter constructs by LMP-1 was also found when the transfections

were performed in BL41 cells, with the -129/+81 region retaining 69% of the LMP-1 -induced activity of the -1374/+81 region of *bfl-1* (Figure 3 36B) A different trend was observed when the same experiments were performed in Jurkat T cells In this cell line, LMP-1 expression enhanced luciferase activity from the -1374/+81 and -1240/+81 promoter constructs by 4.5 and 5.1 -fold respectively However, activity from the -367/+81 and -129/+81 promoter constructs was enhanced 2.2 and 1.8-fold respectively by LMP-1-expression in Jurkat cells (Figure 3 36C) The -367/+81 and -129/+81 regions retained approximately 34% and 23% of the LMP-1-induced activity of the -1374/+81 region of *bfl-1* respectively, which is significantly lower than that observed in similar transfections of DG75 and BL41 cell lines Thus, in the B cell lines DG75 and BL41, the -129/+81 region of *bfl-1* contains elements whose activation by LMP-1 account for the majority of LMP-1-induced activation of the promoter However, in Jurkat cells, the region of the *bfl-1* promoter between -1240 and -367 accounts for the majority of the LMP-1-induced transcriptional activation of this gene It can be concluded from these results that LMP-1 may exert cell type-specific differences in the elements of the *bfl-1* promoter targeted for activation



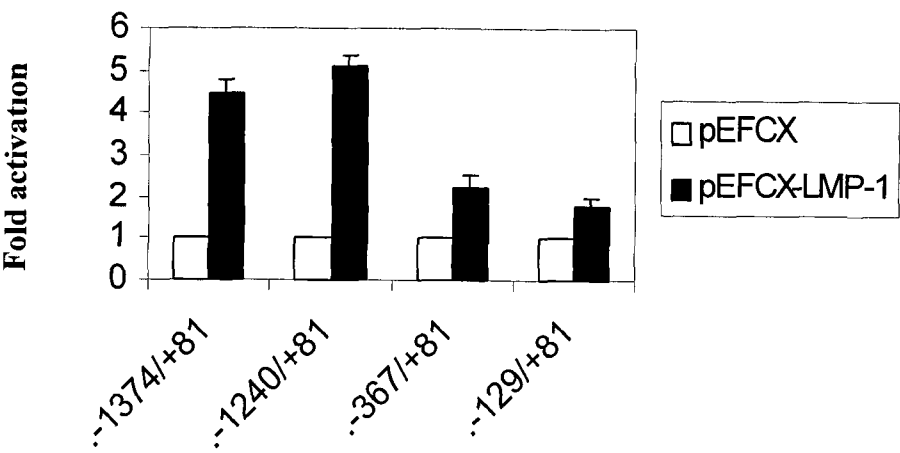
B.

BL41



C.

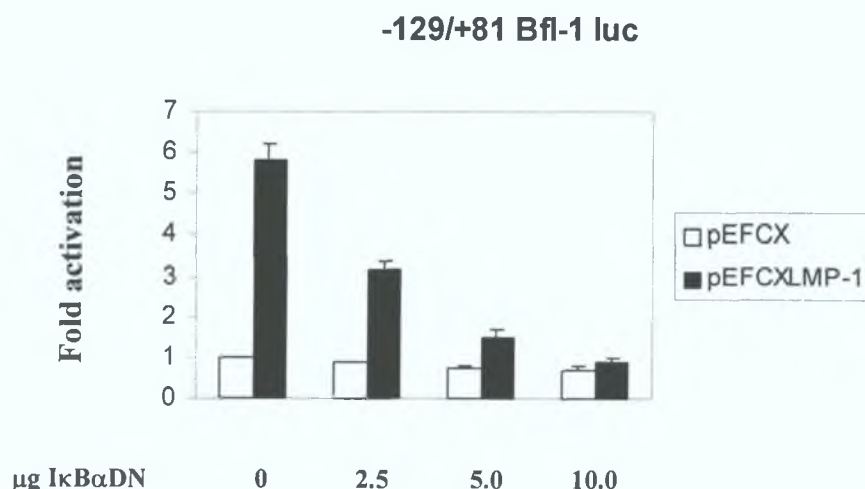
Jurkat



**Figure 3.36 Identification of the regions of the *bfl-1* promoter targeted by LMP1 for activation.** DG75 (panel A), BL41 (panel B) and Jurkat T cells (panel C) were transfected with 2.5µg of either the LMP-1 expression plasmid, pEFCX-LMP-1 or empty vector, pEFCX, together with 2.5µg of the individual *bfl*-promoter-luciferase reporter constructs, -1374/+81 Bfl-1luc, -1240/+81 Bfl-1luc, -367/+81 Bfl-1luc or -129/+81Bfl-1luc. Cells were harvested 24 h post-transfection and assayed for luciferase activity as before. Normalised luciferase activities were expressed as fold-activation over control (empty vector =1). The experiment in panel B was performed once.

### 3.2.7.10 LMP-1-mediated activation of the -129/+81 region of *bfl-1* in DG75 cells is NF-κB-dependent

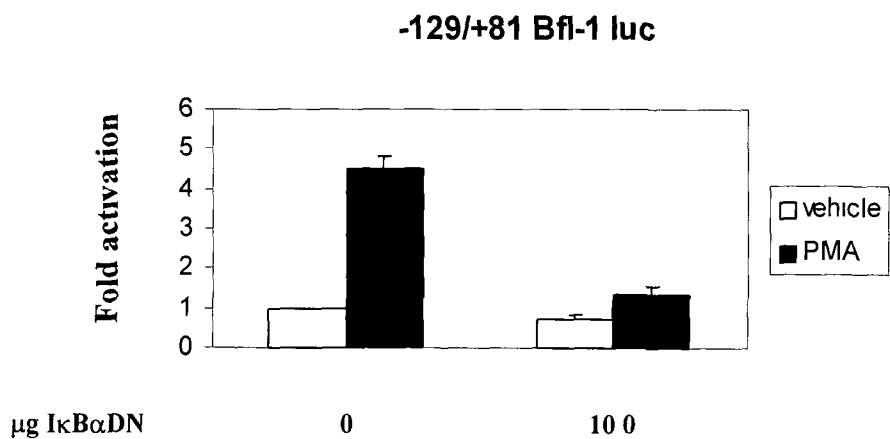
To investigate if the activation of the -129/+81 region of the *bfl-1* gene by LMP-1 in DG75 cells is also NF-κB dependent, the LMP-1-expression plasmid pEFCX-LMP-1 was cotransfected with the *bfl-1*-promoter luciferase reporter p-129/+81Bfl-1-luc in the presence of increasing amounts (0, 2.5, 5.0 or 10.0 µg) of a plasmid expressing the dominant negative mutant IκBαDN. As observed with the -1374/+81 promoter region, expression of IκBαDN inhibited LMP-1-mediated activation of the -129/+81 *bfl-1* promoter region from p-129/+81Bfl-1-luc in a dose-dependent fashion with transfection of 10.0 µg of pEFCX-IκBαDN resulting in complete inhibition (Figure 3.37).



**Figure 3.37 LMP-1 mediated activation of the -129/+81 region of the *bfl-1* promoter is inhibited by expression of a dominant negative mutant of IκBα (IκBαDN) in DG75 cells.** DG75 cells were cotransfected with 2.5 µg of the LMP-1-expression vector, pEFCX-LMP-1 or empty vector pEFCX and 2.5

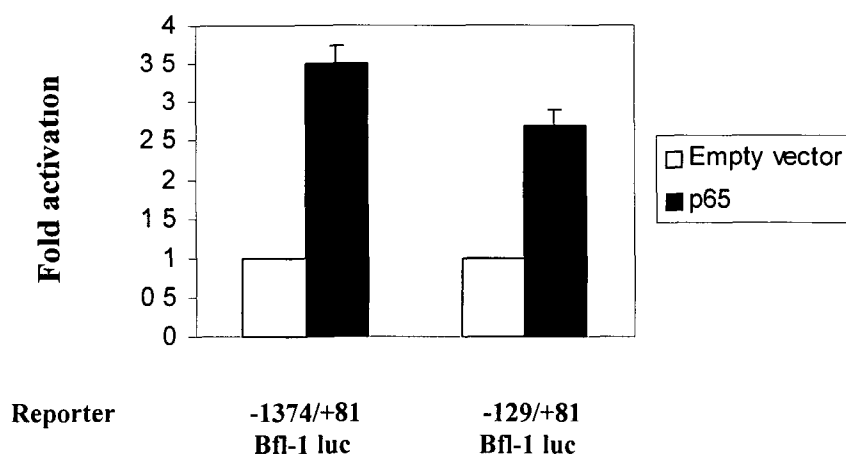
µg of p-129/+81-Bfl-1-luc together with various amounts (0, 2.5, 5.0 or 10.0 µg) of a plasmid expressing IκBαDN. Cells were harvested 24h post-transfection and analysed for luciferase activity. Normalised luciferase activities were expressed as fold activation over control (empty vector in the absence of expression of IκBαDN = 1).

The activity of the -129/+81 region of *bfl-1* could also be stimulated by PMA and as observed with the -1374/+81 region, this effect was NF-κB-dependent (Figure 3.38). PMA stimulated activity from the -129/+81 region by 4.5-fold which represents approximately 60% of the PMA-mediated activation of the -1374/+81 region of *bfl-1* (by comparison to Figure 3.32). Thus, the -129/+81 region is the minimal region of *bfl-1* that is also responsible for PMA activation of the *bfl-1* promoter in DG75 cells.



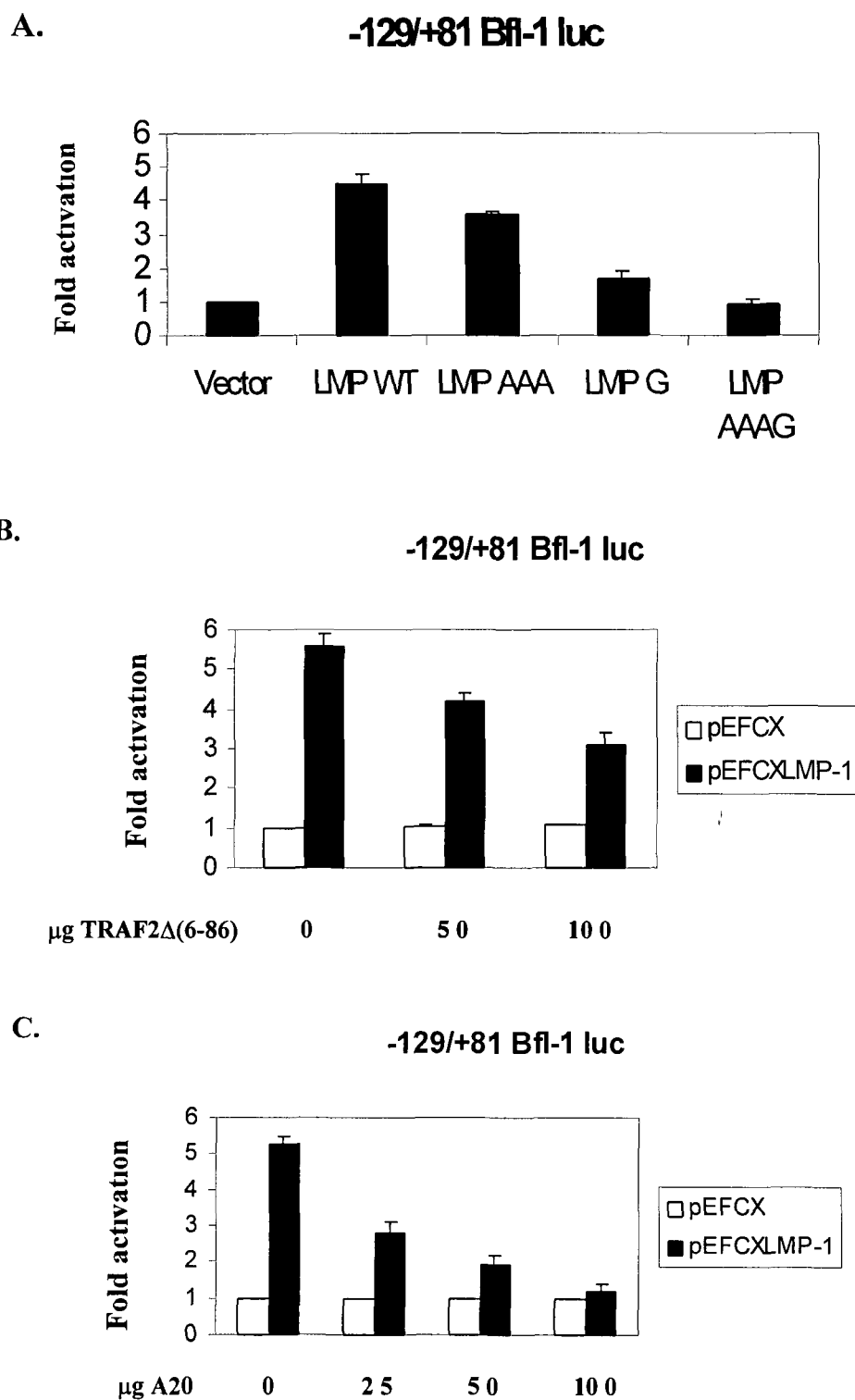
**Figure 3.38 PMA activates the -129/+81 region of the *bfl-1* promoter in an NF-κB-dependent manner in DG75 cells.** DG75 cells transfected with 2.5µg of the -129/+81 Bfl-1luc vector in the presence or absence of 10.0 µg of pEFCX-IκBαDN were treated with either 10<sup>-7</sup> M PMA or vehicle control (0.062% ethanol) 18 h post-transfection. After treatment for 24 h, cells were harvested and analysed for luciferase activity as before. Normalised luciferase values are expressed as fold-increase over control (-129/+81 Bfl-1 luc in the absence of expression of IκBαDN treated with vehicle = 1).

Overexpression of the NF-κB subunit p65 enhanced expression from the -129/+81 promoter luciferase construct approximately 2.7-fold which is lower than that obtained when the -1374/+81 promoter luciferase construct was used (3.5-fold) (Figure 3.39). The results suggest that the -129/+81 region of the *bfl-1* promoter is still significantly responsive to NF-κB.



**Figure 3 39 Overexpression of p65 activates expression from the –129/+81 region of the *bfl-1* promoter in DG75 cells** DG75 cells were transfected with 5 0 µg of an expression vector for p65 (pSG5-p65) or the empty vector (pSG5) together with 2 5 µg the *bfl-1* promoter construct p-1374/+81 Bfl-1-luc or p-129/+81 Bfl-1-luc Cells were harvested 24 h post-transfection and assayed for luciferase activity as before Normalised luciferase values are expressed as fold activation over control (empty vector) Control values were arbitrarily assigned a value of 1

To identify the signaling domains of LMP-1 responsible for activating the –129/+81 region of *bfl-1*, DG75 cells were cotransfected with the –129/+81 *bfl-1* promoter-luciferase construct together with expression vectors for either wild-type (LMP WT) or LMP-1 molecules mutated in either CTAR1 or CTAR2 or both domains It can be seen from Figure 3 40A, that as observed with the –1374/+81 Bfl-1-luc reporter construct, both the CTAR1 and CTAR2 domains contribute to activation of the –129/+81 region of *bfl-1* with the CTAR2 domain exerting a predominant effect A role for TRAF2 in mediating the effect of LMP-1 on the –129/+81 region of the *bfl-1* promoter is again suggested by the observation that overexpression of a dominant negative TRAF2 mutant, TRAF2Δ(6-86), or the TRAF2-interacting protein, A20, significantly inhibited the stimulatory effect of LMP-1 on the activity of the –129/+81 region of the *bfl-1* (Figure 3 40 B and C)

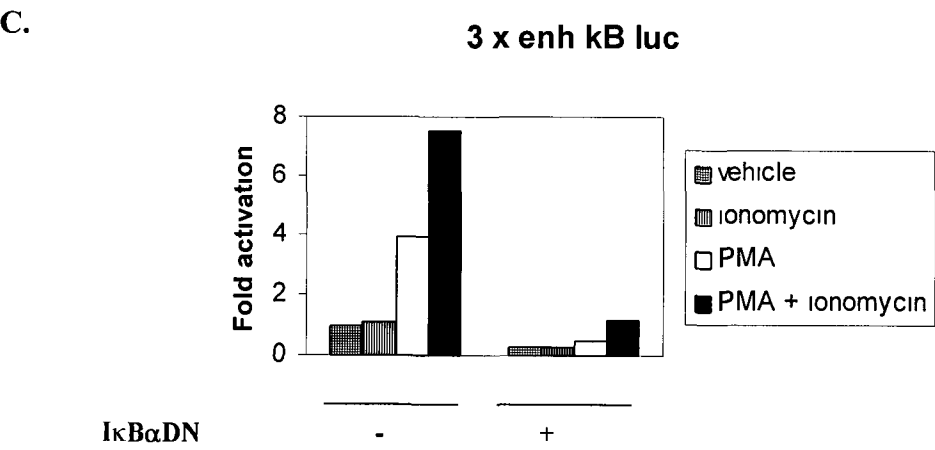
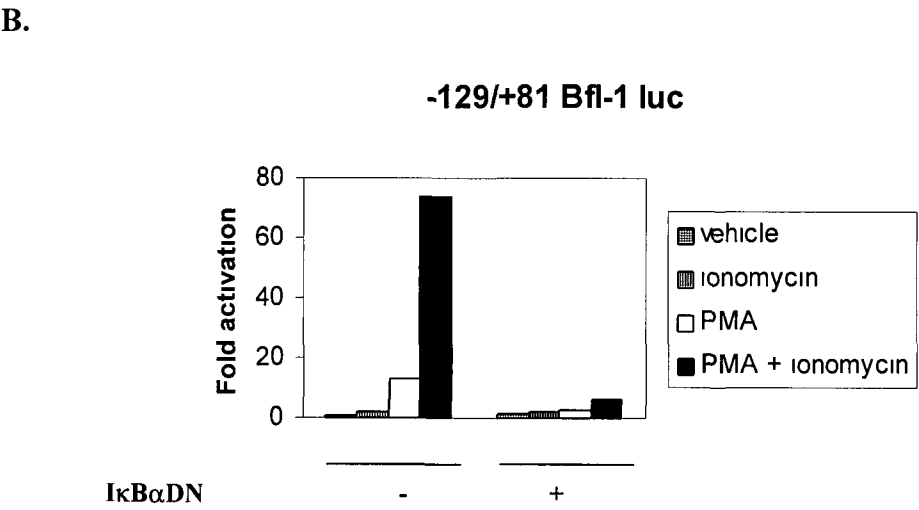
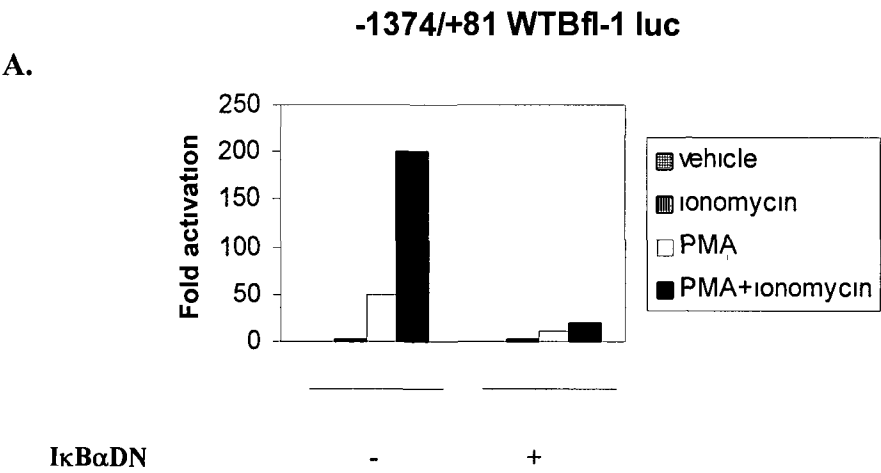


**Figure 3** 40 LMP-1 mediated activation of the -129/+81 region of *bfl-1* requires both the CTAR1 and CTAR2 domains of LMP-1 and involves a TRAF-2 dependent mechanism in DG75 cells A) Identification of the signaling domains of LMP-1 that mediate stimulation of activity of the -129/+81 region of *bfl-1* DG75 cells were cotransfected with 2.5 μg of either pSG5 ('empty vector'), pSG5-

LMP1WT (expressing wild-type LMP1), pSG5-LMP AAA (expressing LMP1 with a non-functional CTAR1 domain), pSG5-LMP1 G (expressing LMP1 with a non-functional CTAR2 domain) or pSG5-LMP1 AAAG (expressing LMP1 that is non-functional in both the CTAR1 and CTAR2 domains) together with 2.5 µg of p-129/+81 Bfl-1-luc. **B)** Inhibition of LMP1-mediated activation of the -129/+81 region of bfl-1 by the dominant negative mutant of TRAF2, TRAF2Δ(6-86). DG75 cells were cotransfected with 2.5 µg of pEFCX-LMP1 or pEFCX and 2.5 µg of -129/+81 Bfl-1-luc in the absence or presence of a plasmid expressing TRAF2Δ(6-86) (5.0 or 10.0 µg). **C)** Inhibition of LMP-1-mediated activation of the -129/+81 region of *bfl-1* by expression of the TRAF2-interacting protein, A20, in DG75 cells. DG75 cells were cotransfected with 2.5 µg of pEFCX-LMP1 or pEFCX and 2.5 µg of p-129/+81 Bfl-1-luc in the absence or presence of a plasmid expressing A20 (2.5, 5.0 or 10.0 µg). Cells were harvested 24 h post-transfection and luciferase activity measured as before.

### 3.2.7.11 Treatment of Jurkat T cells with PMA plus ionomycin transactivates the *bfl-1* promoter

Treatment of Jurkat T cells with PMA plus ionomycin has been previously shown to upregulate steady-state *bfl-1* mRNA levels in an NF- $\kappa$ B-dependent manner (Zong *et al* , 1999) However, analysis of *bfl-1* promoter activity was not included in this study To investigate if the effect of PMA plus ionomycin treatment on *bfl-1* mRNA is due to, at least in part, an effect on *bfl-1* promoter activity, Jurkat cells were transfected with the –1374/+81 *bfl-1*-promoter-luciferase or –129/+81 *bfl-1* promoter-luciferase reporter construct and treated with PMA plus ionomycin 18 h post transfection for 24 h after which luciferase activity was measured It can be seen from Figure 3 41 that PMA/ionomycin treatment transactivated the –1374/+81 promoter region approximately 200-fold and the –129/+81 region to a lesser extent of approximately 74-fold (equal to approximately 37% of activation of the –1374/+81 region) PMA-treatment alone led to a 50-fold transactivation of the –1374/+81 region and a 12 8 -fold transactivation of the –129/+81 region By comparison, ionomycin treatment on its own resulted in only a less than 3-fold transactivation of either promoter region These results suggest that PMA and ionomycin synergise in activating the *bfl-1* promoter in Jurkat T cells Furthermore, the effect of the treatment of Jurkat cells with PMA and ionomycin on *bfl-1* promoter activity is NF- $\kappa$ B-dependent since expression of a dominant negative mutant of I $\kappa$ B $\alpha$  abrogated this effect The observation that the –129/+81 region of the *bfl-1* promoter accounts for only 35% of the extent of transactivation of the –1374/+81 region by PMA + ionomycin is similar to that obtained when LMP-1 was used as the effector in transfections of Jurkat cells Thus, the differential effect of LMP-1 expression or PMA/ionomycin treatment on the activity of different regions of the *bfl-1* promoter may be cell-type dependent



**Figure 3 41 Treatment of Jurkat T cells with PMA and ionomycin activates the *bfl-1* promoter** Jurkat T cells were transfected with either the -1374/+81 Bfl-1 luc (A) or -129/+81 bfl-1 luc (B) reporter constructs or the 3 x enh κB luc (C) reporter in the presence or absence of 10 0 μg of pEFCXIκBαDN and treated at 18 h post-transfection with either vehicle control (0 062% ethanol, 0 05% DMSO) or 1 μM ionomycin or 10<sup>-7</sup> M PMA or a combination of 1 μM ionomycin and 10<sup>-7</sup> M PMA After 24 h treatment with the various agents, cells were harvested and analysed for luciferase activity as before Normalised luciferase values were expressed as fold activation relative to control (vehicle treatment in the absence of expression of IκBαDN=1) This experiment was performed once

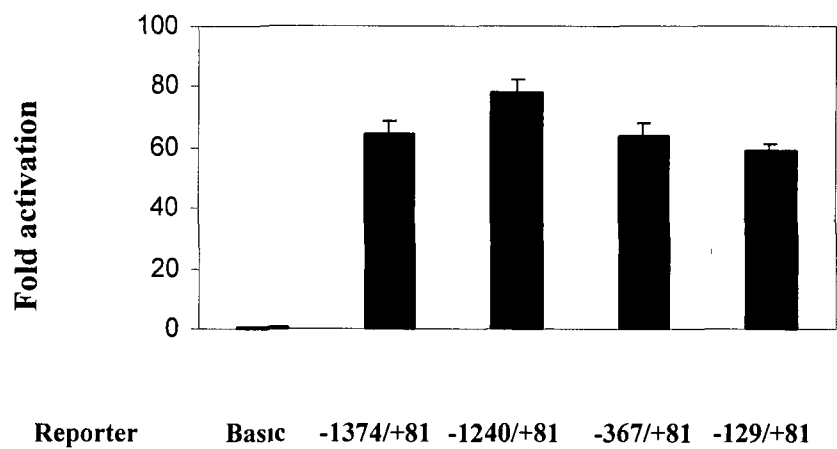
### 3.2.8 LMP-1 regulates *bfl-1* promoter activity in lymphoblastoid cell lines

LMP-1 can induce several of the changes in gene expression that are observed during EBV infection of primary B-lymphocytes. Studies addressing the role of LMP-1 during EBV transformation of B-lymphocytes have been based on the usage of EBV recombinants that are mutated in different functional domains of LMP-1. These studies have revealed that EBV recombinants that express LMP-1 molecules containing only aminoacids 1-231 (i.e. lacking CTAR2) can drive initial proliferation of infected B lymphocytes but are functionally impaired in causing LCL outgrowth (Izumi *et al* , 1997). Further studies using EBV recombinants expressing LMP-1 molecules with point mutations in CTAR2 confirmed that this domain of LMP-1 is critical in enabling efficient, long-term LCL outgrowth (Izumi and Kieff, 1997). In LCLs, LMP-1 is constitutively associated with TRAF molecules and TRADD (Devergne *et al* , 1996, Izumi *et al* , 1999).

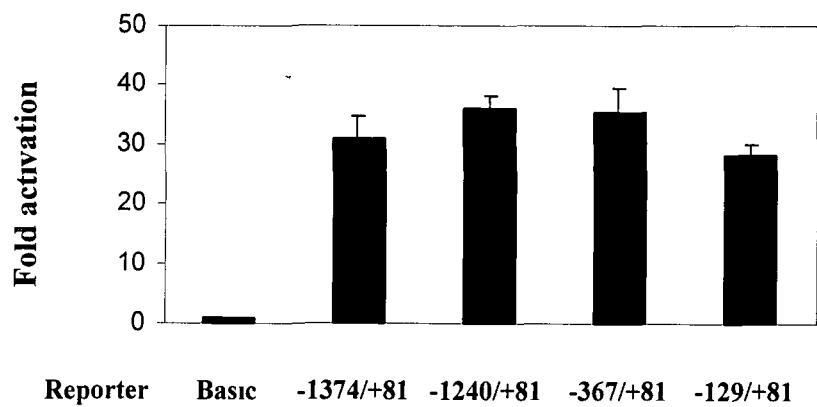
LCLs express substantial levels of *bfl-1* mRNA (Figures 3.2 and 3.5). To examine the extent of activation of the *bfl-1* promoter in LCLs, the *bfl-1*-promoter-luciferase reporter constructs were transfected into IARC 290B and IB4 and luciferase activity measured 24h post-electroporation. The IARC 290B and IB4 LCLs were found to be more efficient to transfect amongst a range of LCLs (including IARC171 and OKULCL) examined and were hence chosen as target cell lines for the promoter studies. Relative to the activity obtained with the basic (promoterless) luciferase reporter construct, an approximately 64-fold activation of the -1374/+81 region of the *bfl-1* promoter was obtained in IARC 290B cells (Figure 3.42A). Progressive 5' deletions of the -1374/+81 region of the *bfl-1* promoter to position -129 (Figure 3.34) did not significantly alter the extent of activation. Thus, relative to the promoterless luciferase construct, expression from the -1240/+81, -367/+81 and -129/+81 promoter luciferase constructs was enhanced approximately 78-fold, 64-fold and 59-fold respectively (Figure 3.42A). Although a lower extent of activation was observed in IB4 cells than in IARC 290B cells a similar trend in activation of different regions of the *bfl-1* promoter was observed in IB4 cells (Figure 3.42B). Transfections in IB4 cells revealed that relative to the activity obtained with the basic

reporter construct, a 31-, 36-, 35- and 28- fold increase in luciferase expression was obtained from the -1374/+81, -1240/+81, -367/+81 and -129/+81 promoter luciferase constructs respectively. These results suggest that in both IARC 290B and IB4, the -129/+81 region is sufficient to drive optimal transactivation of the *bfl-1* gene. Further mapping studies were not carried out on the promoter in this study.

**A. IARC 290B**



**B. IB4**



**Figure 3 42 Activity of *bfl-1* promoter-luciferase constructs in the LCLs IARC290B (A) and IB4 (B)**

Ten million cells were electroporated (300 V, 960  $\mu$ F) with 2.5  $\mu$ g of the promoterless-luciferase reporter or the *bfl-1* promoter-luciferase reporter constructs containing *bfl-1* promoter regions -1374/+81, -1240/+81, -367/+81, -129/+81 and then incubated under normal growth conditions for 24h before harvesting for analysis of luciferase activity. Normalised luciferase values were expressed as fold-activation relative to control (basal/promoterless luciferase vector).

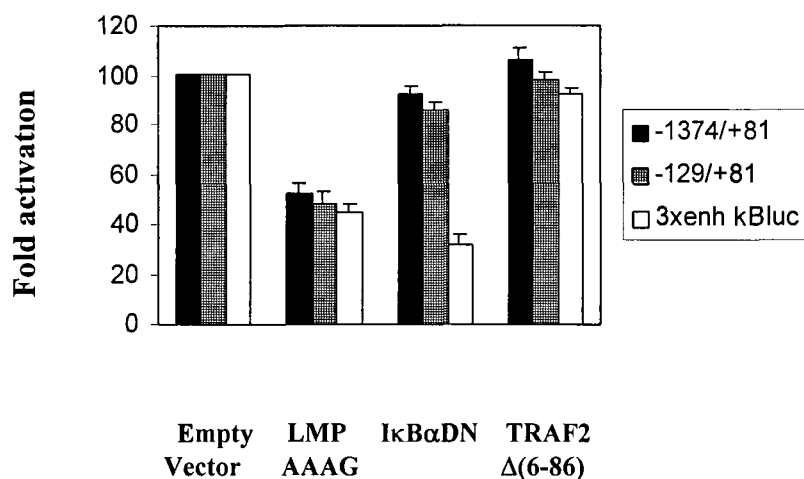
Several lines of evidence indicate that the high level of activation of NF- $\kappa$ B and AP-1 observed in LCLs is maintained by LMP-1 (Kilger *et al*, 1998, Spender *et al*, 1999). Also, association of LMP-1 with the TRAF molecules TRAF-1, 2, 3 and 5 and also with TRADD and RIP has been demonstrated in LCLs (Devergne *et al*, 1998, Izumi *et al*, 1999). Recently, the growth and survival of LCLs has been shown to be NF- $\kappa$ B-dependent and inhibition of NF- $\kappa$ B activity in the LCL IB4 was shown to result in downregulation of *bfl-1* expression but not of *bcl-2* or *bcl-x/L* (Cahir-McFarland *et al*, 2000). Based on these observations it was reasonable to hypothesise that the high levels of *bfl-1* mRNA in LCLs may be maintained by LMP-1.

In a preliminary experiment it was found that expression of LMP-AAAG (Figure 3 21) or a dominant negative I $\kappa$ B $\alpha$  (Figure 3 26) mutant could efficiently inhibit wild-type LMP-1-mediated activation of NF- $\kappa$ B and the *bfl-1* promoter in transient transfection assays in DG75 cells. It was therefore of interest to examine the efficiency of these dominant negative mutants in inhibiting *bfl-1* promoter activity and NF- $\kappa$ B activation when present as constitutive signals such as exists in LCLs. To this end, the LCLs IARC290B and IB4 were chosen as target cell lines. It was found that expression of the dominant negative LMP1 mutant, LMP-AAAG, reduced NF- $\kappa$ B activity by approximately 55% in IARC 290B (Figure 3 43A) and by 60% in IB4 (Figure 3 43B) suggesting that LMP-1 contributes significantly to the level of NF- $\kappa$ B activation in these cells. Importantly, expression of the mutant LMP AAAG also resulted in a parallel reduction of *bfl-1* promoter activity from the -129/+81 *bfl-1* promoter-luciferase construct in these cell lines, resulting in an approximately 52% and 64% inhibition in IARC290B (Figure 3 43A) and IB4 (Figure 3 43B) respectively. These results suggest an important role for

LMP-1 in maintaining *bfl-1* transcriptional activity in LCLs. In order to investigate if NF- $\kappa$ B activation is responsible for the high level of activation of *bfl-1* promoter activity in IARC290B and IB4, the effect of expression of the dominant negative I $\kappa$ B $\alpha$  mutant, I $\kappa$ B $\alpha$ DN, on *bfl-1* promoter activity was investigated. Surprisingly, although expression of I $\kappa$ B $\alpha$ DN reduced *bfl-1* promoter activity by approximately 66% in IB4 cells, I $\kappa$ B $\alpha$ DN expression resulted in only an 8% reduction of activity from the -1374/+81 region and a 14% reduction from the -129/+81 region of *bfl-1* in IARC 290B cells (Figure 3.43). The inhibitory effects of LMP AAAG and dominant negative I $\kappa$ B $\alpha$  on NF- $\kappa$ B activation and *bfl-1* promoter activity did not seem to be due to a decrease in viability of the transfected cells since their transfection did not significantly affect the activity of  $\beta$ -galactosidase expressed from a cotransfected plasmid. The minor effect on *bfl-1* promoter activity in IARC 290B cells was not likely to be due to a lack of functional expression of I $\kappa$ B $\alpha$ DN since a 68% reduction in activity of an NF- $\kappa$ B-dependent reporter was observed on expression of I $\kappa$ B $\alpha$ DN in these cells. The extent of reduction in NF- $\kappa$ B activity on expression of I $\kappa$ B $\alpha$ DN in IB4 cells is similar to that observed in IARC290B and excludes the possibility that the lack of a major effect on the *bfl-1* promoter was due to an insufficient reduction in NF- $\kappa$ B activity by I $\kappa$ B $\alpha$ DN. Interestingly, expression of the dominant negative TRAF2 mutant, TRAF2 $\Delta$ (6-86), in IARC-290B cells did not significantly affect *bfl-1* promoter activity or NF- $\kappa$ B activity. Based on these results it can be concluded that LMP1 makes a major contribution to NF- $\kappa$ B activity and *bfl-1* promoter activity in the LCLs IARC 290B and IB4 and that CTAR1 and/or CTAR2 are required for this effect. Furthermore, at least in IARC290B, the mechanism by which LMP-1 upregulates *bfl-1* is most likely TRAF-2-independent. However, NF- $\kappa$ B activation may not be the only mechanism responsible for activating the *bfl-1* promoter in different LCLs and other mechanisms such as activation of AP-1 may also play an important role. These observations have implications for the mechanisms used by LMP1 in regulating gene expression in different LCLs and warrants further investigation.

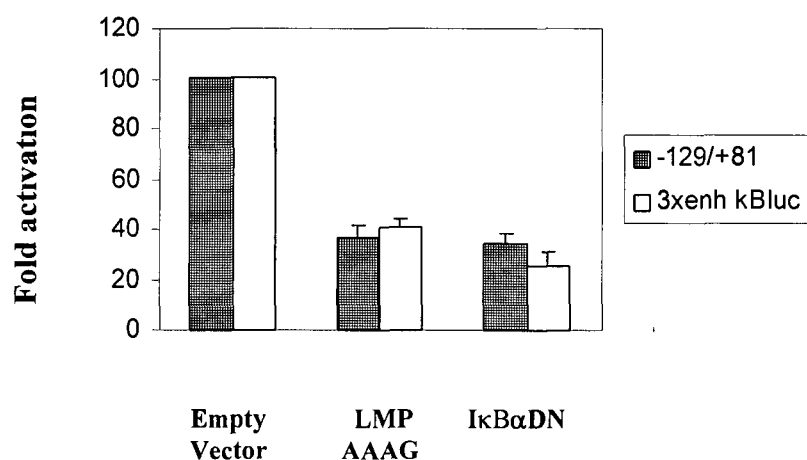
A.

# IARC 290B



B.

# IB4



**Figure 3 43** LMP1 contributes to *bfl-1* promoter activation in the LCLs by both NF-κB-dependent and -independent mechanisms IARC 290B (A) and IB4 (B) were transfected with 10.0 μg of either empty vector (pSG5) or pSG5-based vectors expressing either the dominant negative LMP1 mutant LMP-AAAG, dominant negative IκBα, IκBαDN or dominant negative TRAF2, TRAF2Δ(6-86) (the latter was only transfected into IARC-290B) and 2.5 μg of either the -1374/+81- (only transfected into IARC 290B) or -129/+81- *bfl-1* promoter luciferase constructs or the NF-κB-dependent luciferase reporter constructs. Cells were harvested and analysed for luciferase activity as before. Normalised luciferase activities were expressed relative to values obtained for empty vector that was assigned a value of 100%.

## 3.2.9 Bandshift analysis

### 3.2.9.1 Introduction

The results presented so far indicate that increased transcriptional activity of the *bfl-1* gene partly accounts for the LMP-1-mediated upregulation of the steady-state levels of *bfl-1* mRNA in B cells. Furthermore, deletion analysis of the 5' region of the gene, suggests that the major elements mediating transcriptional activation of the *bfl-1* gene by LMP-1 in this cellular context are located between positions –129 and +81 [positions are relative to the transcriptional start site proposed by Zong *et al*, (1999)] (Figure 3.36). To identify potential regulatory elements within this region that may mediate the effect of LMP1 on the *bfl-1* promoter activity, electrophoretic mobility shift assays (EMSA) were performed.

EMSA is widely used in studying the sequence-specific binding of nuclear proteins such as transcription factors and is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free double stranded DNA fragments or oligonucleotides. The gel shift assay is performed by incubating a purified protein or a complex mixture of proteins (such as nuclear extracts) with a <sup>32</sup>P end-labeled DNA fragment containing the putative protein-binding site, followed by analysis on a non denaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using unlabeled DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

The finding that the transactivation of the –129/+81 region of *bfl-1* by LMP-1 is NF-κB-dependent suggests that there may be NF-κB-binding site(s) located within this region that mediate this effect. A transcription factor search of the –129/+81 region of the *bfl-1* gene using the TESS program (Transcription Element Search Software, website [www.cbil.upenn.edu/tess](http://www.cbil.upenn.edu/tess)) revealed the presence of a number of potential transcription factor binding sites including a NF-κB binding site at position –52 (bp –52 to –43,

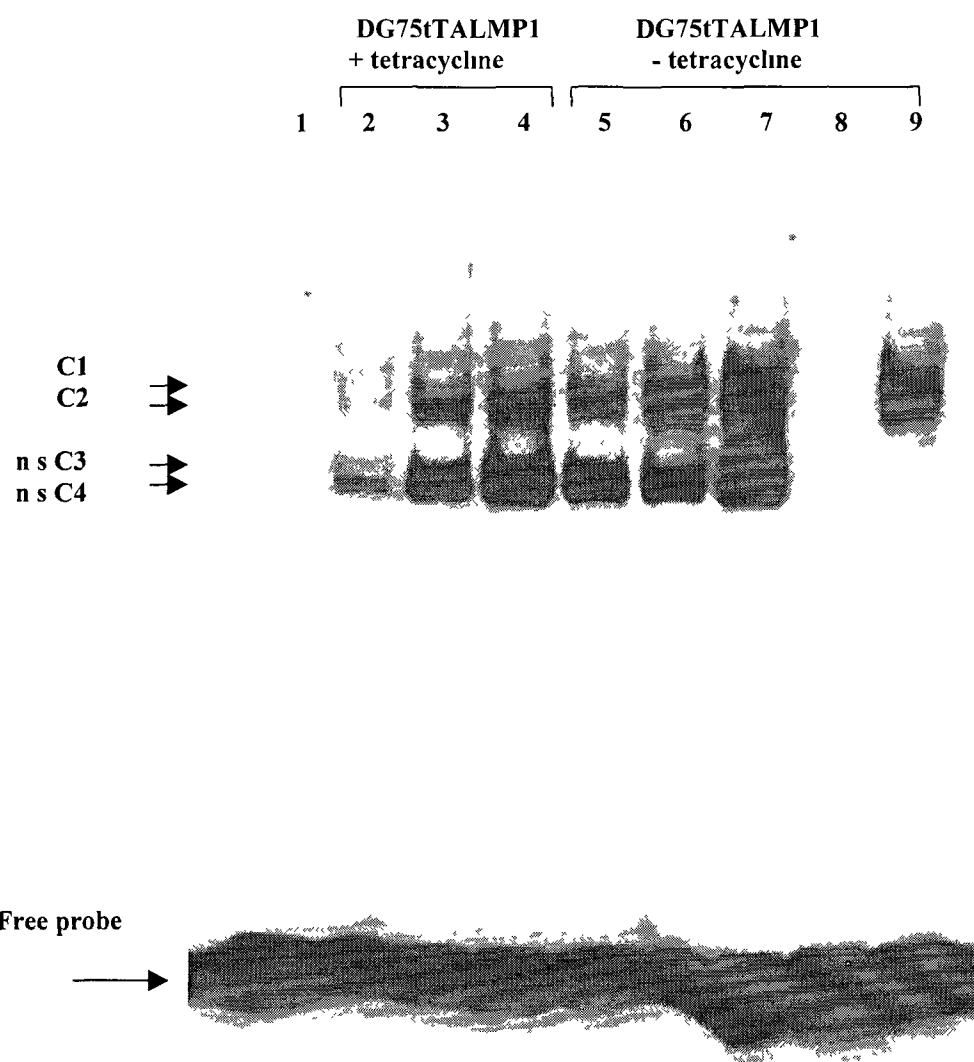


including anti-apoptotic genes such as *bcl-x* and *c-IAP-2* and cytokines such as *IL-8* (Chen *et al* , 1999, Hong *et al* , 2000, Okamoto *et al* , 1994) The potential involvement of the NF- $\kappa$ B-like site at position -52 of *bfl-1* in the LMP-1-induced enhancement of *bfl-1* promoter activity was investigated by electrophoretic mobility shift assays (EMSA) using nuclear extracts prepared from DG75-tTA-LMP1 cells before and after induction of LMP1 expression Since the studies of the kinetics of induction of *bfl-1* mRNA by LMP-1 in these cells indicated that the magnitude of induction was maximal by 48h post-induction (Figure 3 3), nuclear extracts were prepared from the DG75-tTA-LMP1 cells at the earlier time-point of LMP-1 induction of 36h when *bfl-1* mRNA levels are still increasing

### **3.2.9.2 LMP-1 induces activation of p65-containing NF- $\kappa$ B complexes in DG75-tTA-LMP1 cells**

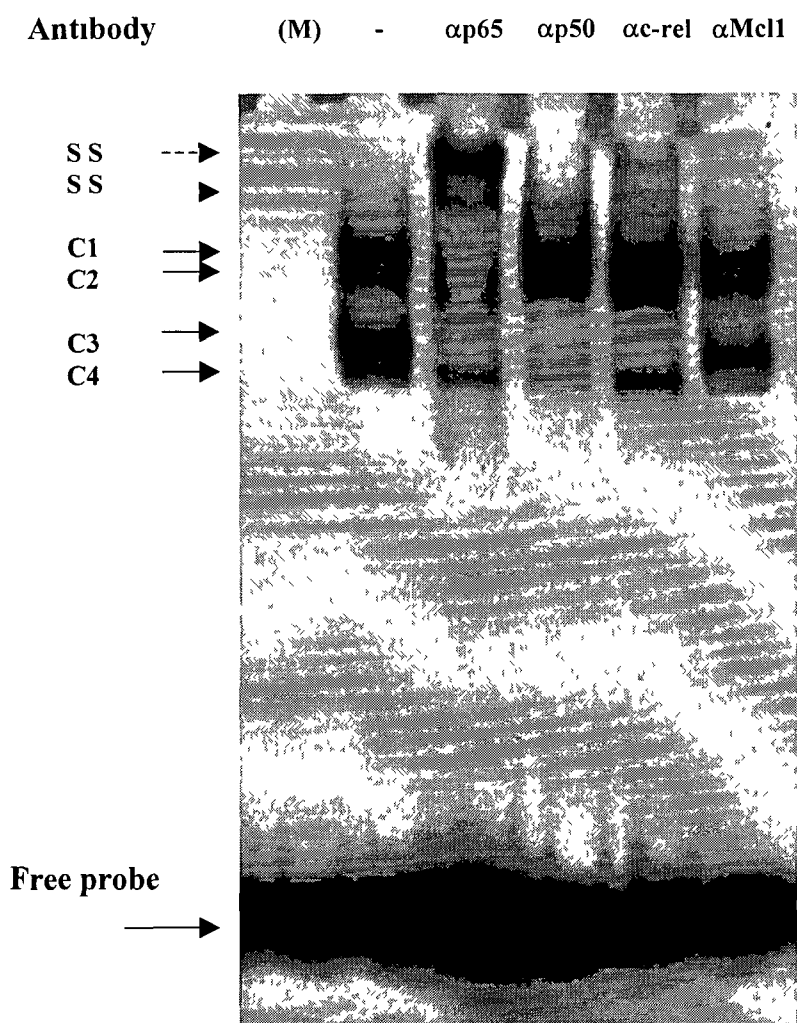
As a preliminary experiment, the effect of LMP-1 induction on NF- $\kappa$ B binding activity in DG75-tTA-LMP1 cells was assessed To this end, EMSA was performed with nuclear extracts prepared from DG75-tTA-LMP1 cells before and 36 h after induction of LMP1 using a 22 bp oligonucleotide containing the NF- $\kappa$ B site (5'-GGGGACTTCC-3') from the Ig $\kappa$  light chain promoter This oligonucleotide will henceforth be referred to as the 'consensus' NF- $\kappa$ B oligonucleotide for the purposes of this thesis The sequence of this NF- $\kappa$ B site is the same as that present in the NF- $\kappa$ B-dependent luciferase reporter construct (3 x enh  $\kappa$ B luc) used for assessing NF- $\kappa$ B activation by LMP-1 in transient transfection assays A titration experiment of the amount of nuclear protein used in the binding reaction with the consensus NF- $\kappa$ B oligonucleotide was first conducted to establish optimal binding conditions It can be seen from figure 3 45 that four protein-DNA complexes (C1, C2, C3 and C4) were detected with extracts from the uninduced DG75-tTA-LMP-1 cells Induction of LMP-1 did not result in the formation of any new complexes and although complexes of similar mobility were detected the intensity of complexes C1, C2 was consistently higher with extracts from the LMP-1 expressing cells compared to the non-LMP1 -expressing counterpart irrespective of the amount of protein used To confirm that these shifted complexes are NF- $\kappa$ B specific, the binding

experiment was repeated but with pre-incubation of the extract with a 100-fold molar excess of either 'cold' (unlabeled) NF- $\kappa$ B consensus oligonucleotide or a 'cold' mutagenic version of this oligonucleotide as competitor before addition of the labeled target oligonucleotide. It can be seen from figure 3.45 that when cold NF- $\kappa$ B oligonucleotide is used as competitor, formation of all four complexes is no longer detectable (i.e. the four complexes are outcompeted). This result on its own would indicate that the four complexes are NF- $\kappa$ B specific. However, when the competition experiment was repeated but with the mutagenic NF- $\kappa$ B oligonucleotide, it was only the formation of complexes 3 and 4 that was inhibited, the intensity of complexes 1 and 2 remained unchanged. Taken together, the results of the two competition experiments indicate that it is only complexes C1 and C2 that are NF- $\kappa$ B-specific, formation of complexes 3 and 4 is more likely to be the result of non-specific binding of nuclear proteins to the labeled oligonucleotide. The ability to detect activated NF- $\kappa$ B complexes in the DG75-tTA-LMP1 cells in the absence of LMP-1-induction is consistent with the high basal level of NF- $\kappa$ B activation reported to be present in this BL cell line (Huen *et al*, 1995, Rowe *et al*, 1994). The increase in the extent of formation of NF- $\kappa$ B-DNA complexes on induction of LMP-1 validates the experimental system and suggests that NF- $\kappa$ B-binding activity is preserved in the nuclear extracts under the conditions used.



**Figure 3 45 EMSA of the Ig $\kappa$  light chain ('consensus') NF- $\kappa$ B oligonucleotide with nuclear extracts from DG75-tTA-LMP-1 cells before and 36 h after LMP-1 induction** A double stranded, end-labeled oligonucleotide spanning the Ig $\kappa$  light chain NF- $\kappa$ B site (consensus) was incubated with 5 (lanes 2, 5), 10 (lanes 3, 6) or 15 (lanes 4, 7)  $\mu$ g of nuclear extracts from uninduced DG75-tTA-LMP1 cells or cells induced to express LMP-1 36 h after removal of tetracycline Competition experiments were performed by pre-incubation of 10  $\mu$ g of extract from the LMP-1-induced cells with a 100-fold molar excess of either unlabeled consensus NF- $\kappa$ B oligonucleotide (lane 8) or NF- $\kappa$ B-mutated oligonucleotide (lane 9) before addition of labeled oligonucleotide to the binding reaction A mock binding reaction containing all components except for nuclear extract was loaded in lane 1 After separating the protein-DNA complexes by nondenaturing polyacrylamide gel (5%) electrophoresis (140 V, 2 h), the shifted bands were visualised using autoradiography Complexes C1 and C2 are NF- $\kappa$ B specific complexes, complexes C3 and C4 are non-specific (n s ) The position of the free probe is indicated

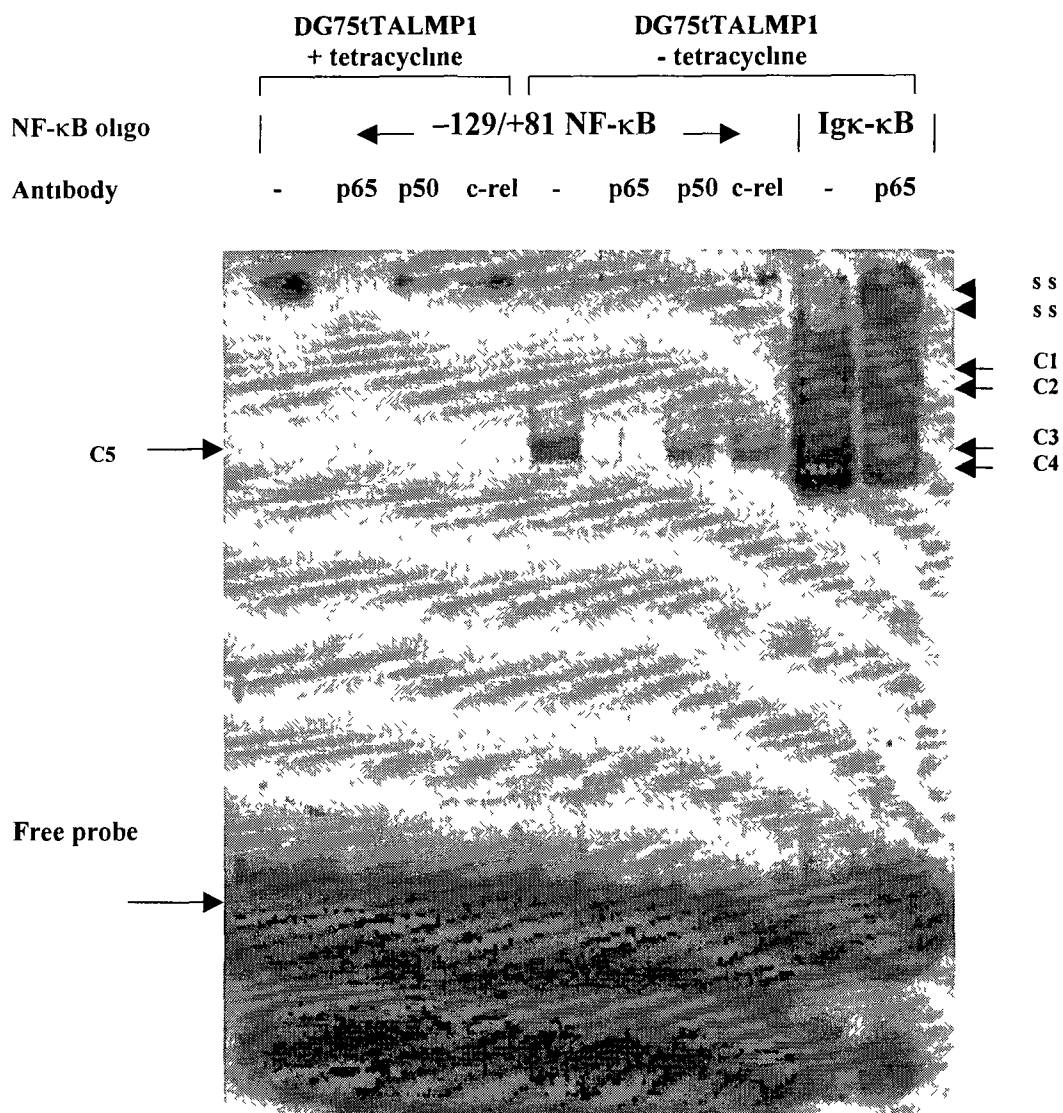
To characterise the nuclear protein complexes bound to the consensus NF- $\kappa$ B oligonucleotide, supershift assays were performed using antibodies against the NF- $\kappa$ B subunits p65, p50 and c-rel and an unrelated anti-Mcl-1 antibody. In supershift assays, preincubation of the nuclear extract with an antibody to a protein suspected to be involved in complex formation with the labeled oligonucleotide either diminishes complex formation or further retards the mobility of the complex, depending on whether the antibody recognises an epitope in the DNA binding region of the protein or not. Supershift assays with the consensus NF- $\kappa$ B oligonucleotide were performed by preincubation of extracts from the LMP-1 expressing DG75-tTA-LMP-1 cells with antibodies to p65, p50, c-rel and Mcl-1 followed by addition of the labeled consensus NF- $\kappa$ B oligonucleotide. It can be seen from figure 3.46 that when the anti-p65 antibodies were used in the supershift assays both of the specific complexes, namely C1 and C2 were further retarded in mobility. However, antibodies to p50 and c-rel did not significantly diminish nor supershift either of the specific complexes. Antibodies to Mcl-1 used as a negative control did not supershift the specific complexes as expected indicating the specificity of the reaction of the p65 antibodies. Thus, it can be concluded that each of C1 and C2 are NF- $\kappa$ B- specific complexes containing p65 as at least one NF- $\kappa$ B subunit and that the extent of formation of these complexes is increased upon induction of LMP-1 expression.



**Figure 3 46** Supershift analysis of the NF- $\kappa$ B-DNA complexes formed between nuclear proteins from the DG75-tTA-LMP1 cells after LMP-1 induction and the oligonucleotide containing the Ig $\kappa$  light chain ('consensus') NF- $\kappa$ B site. 10  $\mu$ g of nuclear extract prepared from the DG75-tTA-LMP-1 cells 36 post- LMP-1 induction was left untreated (-) or preincubated with antibodies to either p65, p50, c-rel or Mcl-1 (control) for 30 min on ice before incubation with end-labeled consensus NF- $\kappa$ B oligonucleotide. A mock binding reaction (M) was also set up as before. Protein-DNA complexes were analysed by non-denaturing PAGE and the shifted bands visualised by autoradiography. The  $\kappa$ B-specific complexes C1 and C2 and the non-specific complexes (n.s.) C3 and C4 are indicated. The supershifted complexes are indicated as ss.

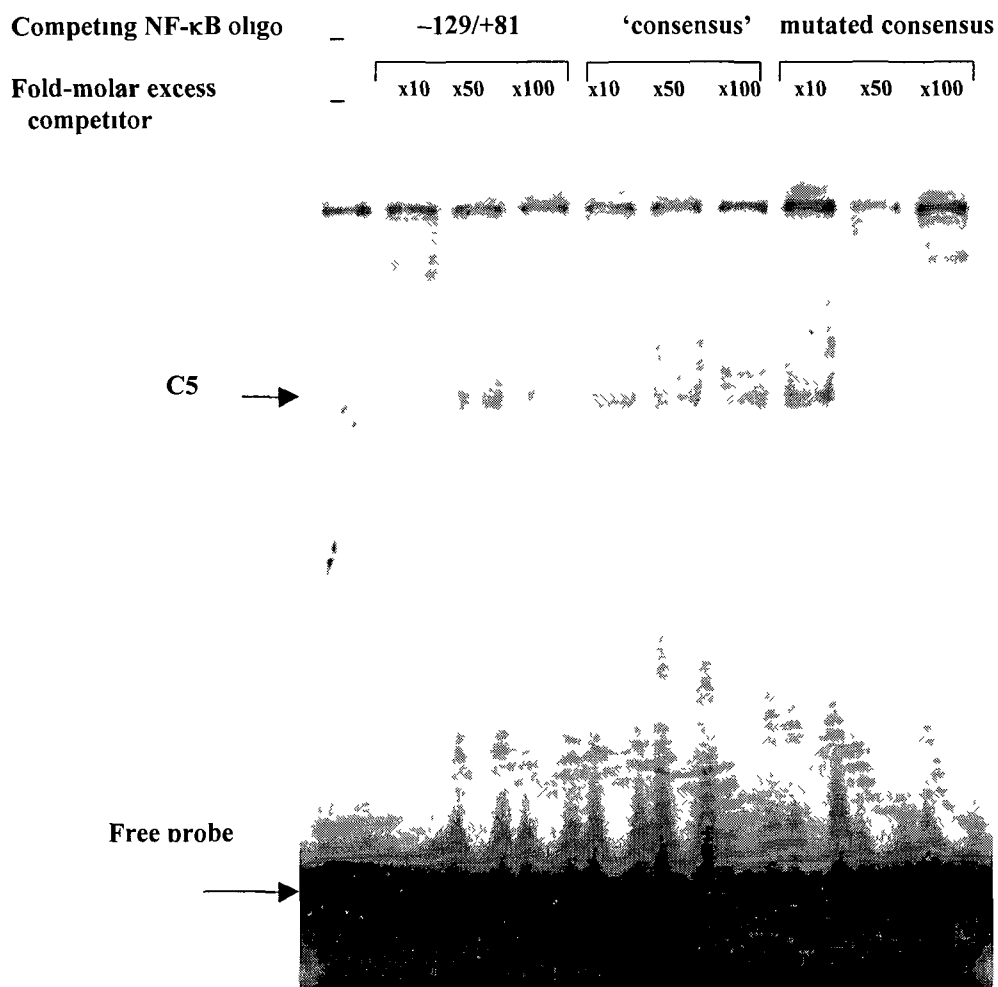
### **3.2.9.3 Lack of demonstrable binding of a specific LMP-1-activated NF- $\kappa$ B complex to the NF- $\kappa$ B-like site at –52 to –43 of the *bfl-1* promoter region in DG75-tTA-LMP1 cells**

To determine whether nuclear proteins could bind to the NF- $\kappa$ B-like site within the –129/+81 region of the *bfl-1* promoter, EMSA was performed using a 14 bp oligonucleotide derived from the *bfl-1* promoter sequence that includes the  $\kappa$ B-like site at position –52 and the same nuclear extracts used to demonstrate LMP-1-mediated enhancement of NF- $\kappa$ B-DNA complex formation in DG75-tTA-LMP-1 cells. The usage of an oligonucleotide of this length was justified since 14 bp oligonucleotides have been used successfully in EMSA to demonstrate involvement of particular NF- $\kappa$ B-like sites in LMP1, TNF- $\alpha$  and CD40 -mediated activation of the *c-IAP-2* promoter (Hong *et al*, 2000). As can be seen in figure 3 47, EMSA revealed that expression of LMP-1 in DG75-tTA-LMP-1 cells resulted in the induction of a single nuclear protein complex with the 14 bp oligonucleotide encompassing the NF- $\kappa$ B-like site in the –129/+81 region of *bfl-1*. Supershift assays indicated that while antibodies to p65 almost completely inhibited formation of this complex, antibodies to p50 or c-rel did not (Figure 3 47).



**Figure 3 47 EMSA and supershift analysis with a 14bp oligonucleotide spanning the NF-κB-like site present at position -52 of the *bfl-1* promoter for nuclear protein NF-κB binding activity** 10 0 μg of nuclear extracts prepared from uninduced DG75-tTA-LMP1 cells or from cells 36 h post-induction of LMP-1 were left untreated or precubated with antibodies to either p65, p50 or c-rel for 30 min on ice before addition of a double stranded end-labeled oligonucleotide spanning the NF-κB-like site present at position -52 of the *bfl-1* promoter As a control, 10 μg of nuclear extract from the LMP-1-expressing DG75-tTA-LMP1 cells was subjected to supershift analysis with antibodies to p65 and the labeled consensus NF-κB oligonucleotide (Igκ κB site) The protein-DNA complexes were analysed as before C5= nuclear protein complex formed with the oligonucleotide containing the NF-κB site at position -52 of *bfl-1* Other annotations as in Figure 3 46

The antibody to p65 is directed to an epitope located near the carboxy-terminal region of the protein (Chemicon International Inc). This region is not involved in DNA binding but rather has transactivating functions. It would therefore be expected that in supershift assays with this p65 antibody, p65-containing complexes will be further retarded in mobility rather than diminished. It was therefore curious that the anti-p65 antibody inhibited complex formation in supershift assays with the oligonucleotide spanning the NF- $\kappa$ B site at position -52. Secondly, this complex (C5) was of similar mobility to the non-specific complex C3 that was formed when the consensus NF- $\kappa$ B oligonucleotide was used in the binding reaction (Figure 3 47), suggesting that this complex may indeed be non-specific. To investigate whether the shifted complex formed with the oligonucleotide spanning the -129/+81 NF- $\kappa$ B site is specific, the binding experiments were repeated using nuclear extracts from the LMP-1 expressing DG75-tTA-LMP-1 cells and either cold oligonucleotide spanning this  $\kappa$ B site or cold consensus NF- $\kappa$ B oligonucleotide or cold mutated consensus NF- $\kappa$ B oligonucleotide in competition with labeled oligonucleotide containing the  $\kappa$ B site at -52 of *bfl-1*. As shown in Figure 3 48, neither the oligonucleotide spanning the  $\kappa$ B site at position -52 of *bfl-1* nor the consensus NF- $\kappa$ B-containing oligonucleotide or the corresponding mutagenic consensus oligonucleotide could compete for binding to labeled oligonucleotide containing the -129/+81  $\kappa$ B site even when 100-fold molar excess of cold oligonucleotide was used. Thus, the complex C5 formed with the oligonucleotide containing the  $\kappa$ B site at position -52 of the *bfl-1* promoter is most likely non-specific. It may be concluded from these experiments that it was not possible to demonstrate binding of NF- $\kappa$ B proteins to the 14 bp oligonucleotide spanning the NF- $\kappa$ B-hke site at position -52 of *bfl-1* on induction of LMP-1 in the DG75tTALMP-1 cell line.

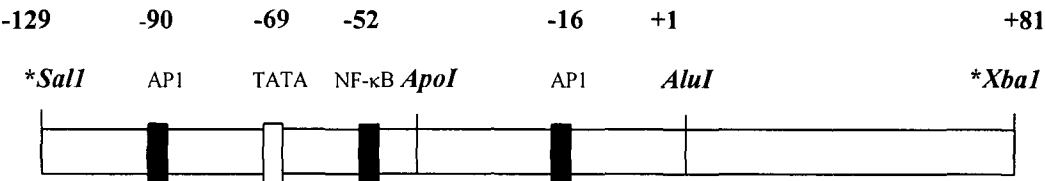


**Figure 3 48** Competition studies of binding of nuclear proteins from LMP-1 expressing DG75-tTA-LMP-1 cells to the labeled oligonucleotide containing the NF- $\kappa$ B-like site at position -52 of the *bfl-1* promoter 10 0  $\mu$ g of nuclear extract from LMP-1-expressing DG75-tTA-LMP1 cells was untreated (-) or preincubated with either 10-fold, 50-fold or 100-fold- molar excess of unlabeled oligonucleotide containing either the NF- $\kappa$ B site at position -52 of *bfl-1* or the consensus (Ig $\kappa$  light chain) NF- $\kappa$ B oligonucleotide or the mutated consensus NF- $\kappa$ B oligonucleotide before addition of the labeled oligonucleotide containing the  $\kappa$ B site embedded within the -129/+81 region of *bfl-1* The protein-DNA complexes were analysed as before

#### **3 2.9.4 Lack of demonstrable binding of NF- $\kappa$ B to the -129/+81 region of *bfl-1* in DG75-tTA-LMP1 cells irrespective of expression of LMP1**

A second approach towards identifying potential elements within the -129/+81 region of *bfl-1* that may be involved in the upregulation of activity of this promoter region by LMP-1, involved using different DNA fragments spanning this region as 'probes' in EMSA with the same nuclear extracts from DG75-tTA-LMP1 cells as used in the previous studies. A partial restriction map of the -129/+81 region of *bfl-1* and the relative positions of the promoter-specific restriction fragments used as probes in EMSA is shown in figure 3 49A. The end labeled DNA fragments encompassing various portions of the -129/+81 region of *bfl-1* were size-fractionated in a non-denaturing polyacrylamide gel and then excised from the gel and purified as described in section 2 7 4 3. The autoradiogram of the gel after electrophoresis of the fragments is also shown in figure 3 49B. Four different restriction fragments were used in EMSA: the *Sal1-Xba1* (210bp, which contains the entire sequence of the -129/+81 region), *Sal1-Apo1* (91bp), *Sal1-Alu1* (140bp) and *Apo1-Xba1* (130 bp).

A.



SalI – ApoI



91bp

SalI – AluI



140bp

SalI – XbaI

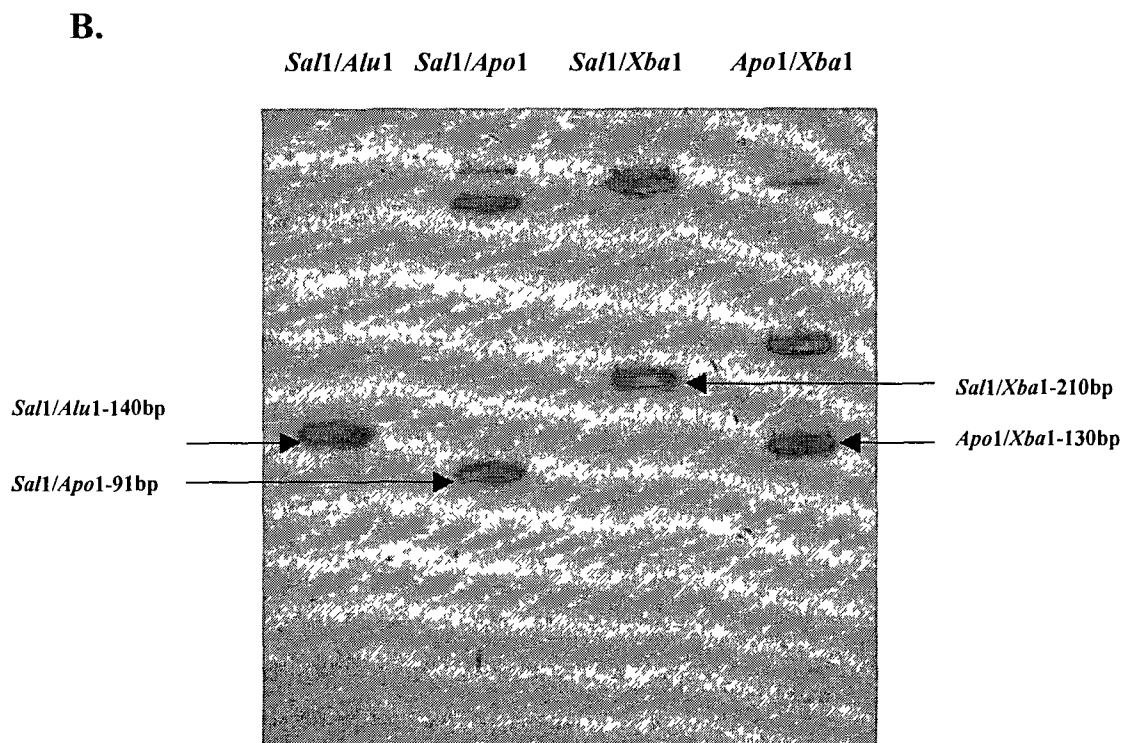


210bp

ApoI-XbaI



130bp



**Figure 3 49 Preparation of end-labeled restriction fragments encompassing the -129/+81 region of the *bfl-1* gene or different portions thereof for use as probes in EMSA** A) Partial restriction map of the -129/+81 region of *bfl-1* showing the restriction sites delineating the different fragments used as probes in EMSA The *bfl-1*-specific restriction fragments generated from *Sal1/Alu1*, *Sal1/Apo1*, *Sal1/Xba1* and *Apo1/Xba1* double digests of p-129/Bfl-1 CAT plasmid to be used as probes in EMSA are schematically shown as black boxes The relative positions of the transcription factor sites AP-1 (-90 and -16), the NF- $\kappa$ B-like site (-52), the TATA box (-69), and the transcription start site (+1) are also indicated The restriction sites on the map are given in italics and the positions of the transcription factor sites are marked on the map as black boxes B) Autoradiogram of the gel after size-fractionation of the end-labeled fragments Size fractionation was carried out as described in section 2 7 4 3 The different restriction digests used to generate the probes are indicated above each lane and the position of the labeled fragments of interest that were excised are indicated by an arrow Undesired but labeled fragments represent sequences from the plasmid backbone and do not include *bfl-1* specific sequences

The end-labeled *Sal1-Alu1*, *Sal1-Apo1*, *Sal1-Xba1* and *Apo1-Xba1* fragments were used in EMSA with nuclear extracts from DG75-tTA-LMP-1 cells prepared before and after induction of LMP-1 using similar conditions as for the NF- $\kappa$ B oligonucleotides except

that the electrophoresis of the complexes was carried out at 200 V for approximately 2.5 h. Under these electrophoresis conditions, specific binding of nuclear proteins to the consensus NF- $\kappa$ B oligonucleotide was preserved (result not shown). As would be expected when DNA fragments of this length are used in EMSA, several protein-DNA complexes were detected with each of the four different probes (Figure 3.50 A and B). However, there did not appear to be any significant difference in the pattern of complexes between the extracts prepared before and after LMP-1 induction in DG75-tTA-LMP1 cells except perhaps for an increase in intensity of two slow mobility complexes (complexes C6 and C7) with extracts from cells expressing LMP-1 when the *SalI*-*AluI* fragment was used (Figure 3.50) and a decrease in intensity of one (complex C8) and a complete absence of another (complex C9) slower mobility complex when the *ApoI*-*XbaI* fragment was used as the target in one experiment (Figure 3.50). The *SalI*-*XbaI* fragment contains the DNA sequence present in the *SalI*-*AluI* and *ApoI*-*XbaI* fragments and similar changes in the extent of complex formation was not observed with this fragment thereby questioning the validity of the results with these fragments.

A.

Probe

*Sal1/Alu1*

*Sal1/Apo1*

*Sal1/Xba1*

*Apo1/Xba1*

DG75tTALMP1

Tetracycline =t

+t

-t

+t

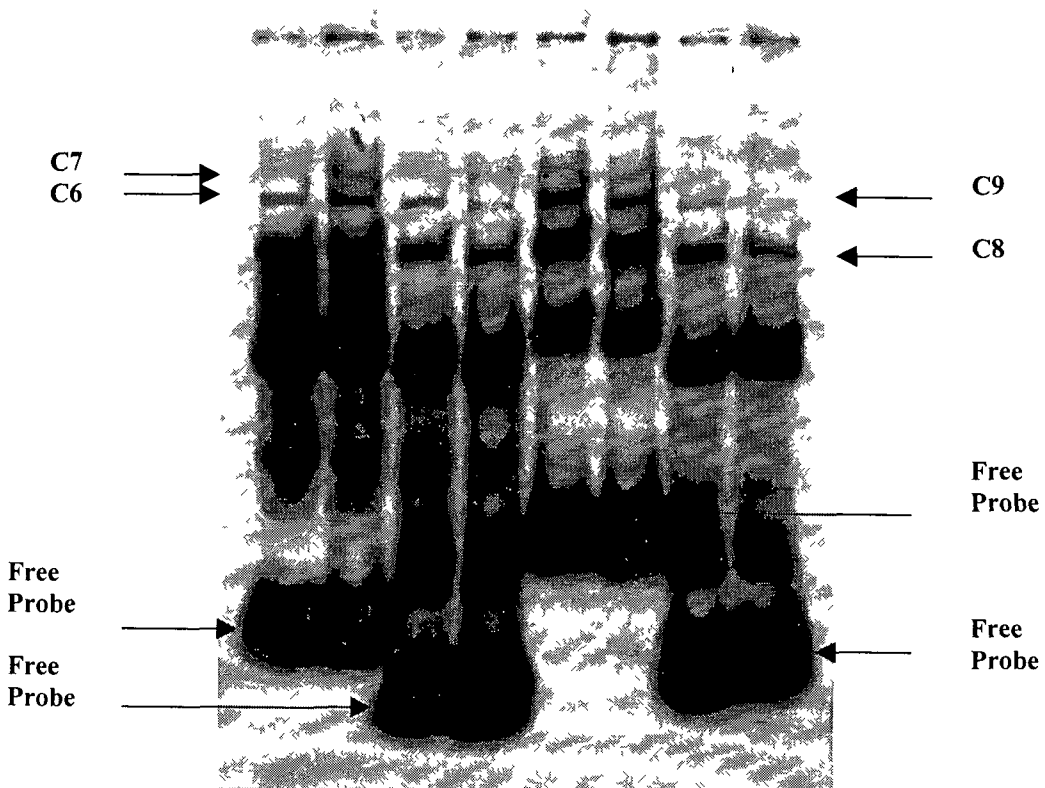
-t

+t

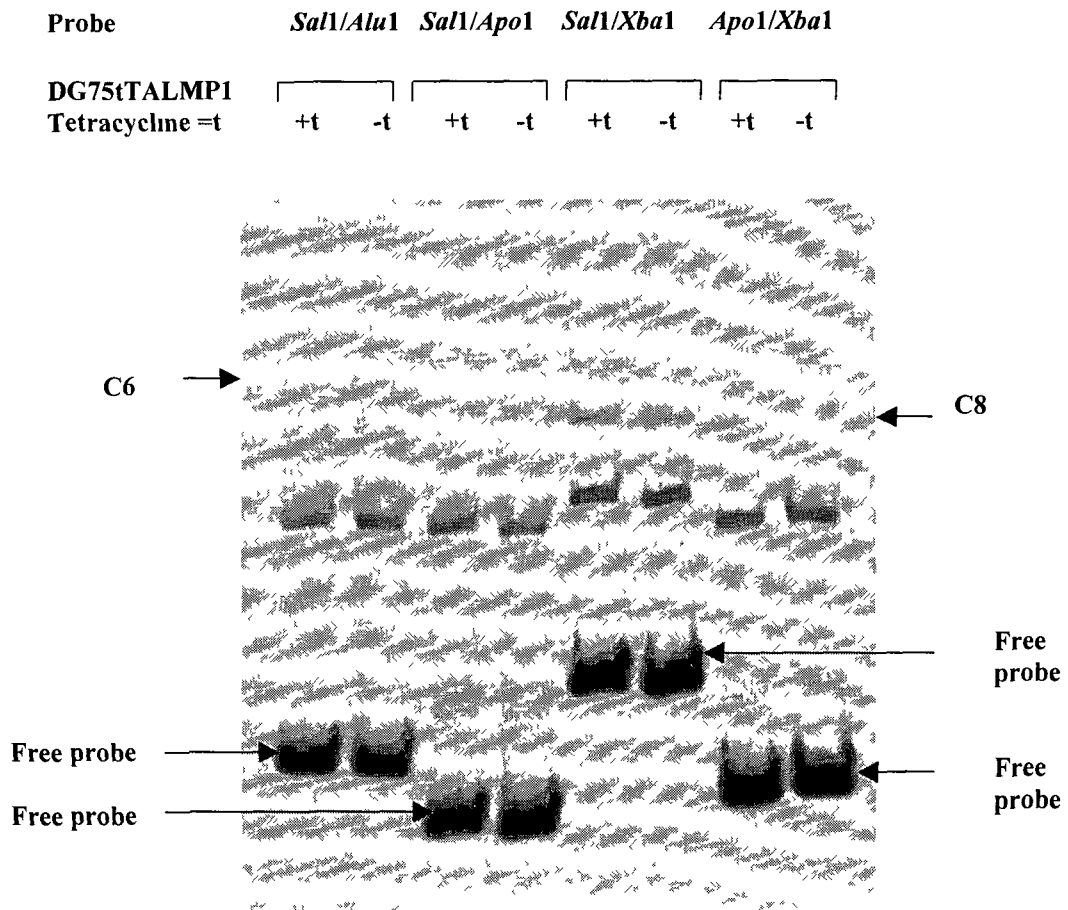
-t

+t

-t



B.



**Figure 3 50** EMSA of DNA fragments spanning different parts of the  $-129/+81$  region of *bfl-1* with nuclear extracts from DG75tTA-LMP-1 cells before and 36 h after LMP-1 induction Double stranded end-labeled restriction fragments (*Sal1/Alu1*, *Sal1/Apo1*, *Sal1/Xba1* and *Apo1/Xba1*) spanning different lengths of the  $-129/+81$  region of *bfl-1* were each incubated with 10 0  $\mu$ g of nuclear extract from uninduced (+ tetracycline) DG75-tTA-LMP1 cells or cells induced to express LMP1 36 h after growth in the absence of tetracycline removal The protein-DNA complexes were analysed by electrophoresis in a 4% non-denaturing polyacrylamide gel at 200 V for 2 5 h followed by autoradiography The restriction fragments used as probes are indicated above the respective lanes The position of complexes C6 and C7 formed with the *Sal1/Alu1* fragment and complexes C8 and C9 formed with the *Apo1/Xba1* fragment are indicated by arrows Panel B is a shorter exposure of panel A

The lack of either an increase in the extent of formation of any of the complexes or formation of new complexes with the probe encompassing the  $-129/+81$  region of *bfl-1*

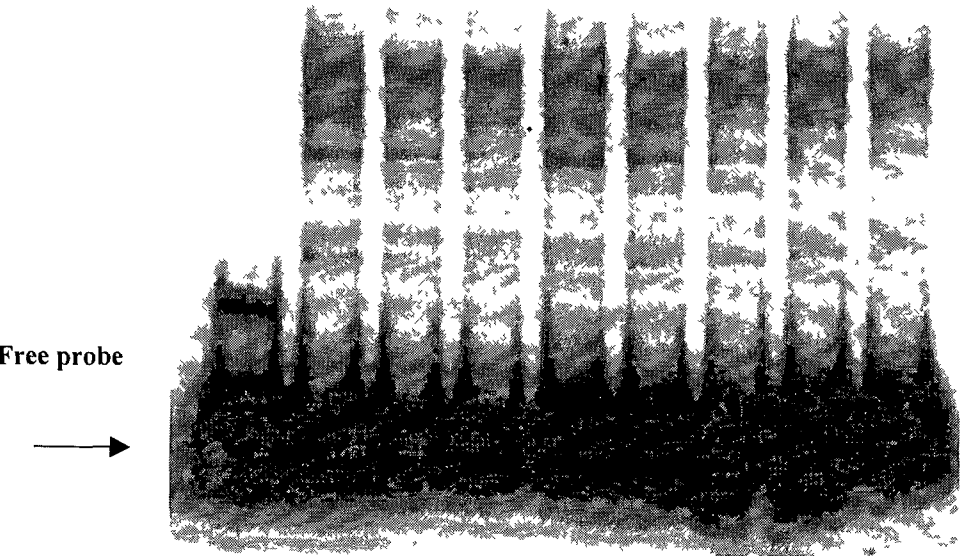
on induction of LMP-1 does not necessarily exclude the involvement of NF- $\kappa$ B. Recent reports suggest that in addition to the more commonly known mode of activating transcription of NF- $\kappa$ B-dependent genes by stimulus-induced occupation of NF- $\kappa$ B sites in the promoter region of the genes, some genes may already have occupied NF- $\kappa$ B sites and the stimulus-induced activation of these genes involves an increase in the transactivation potential of the already bound NF- $\kappa$ B. For instance, IL-1 which is also a member of the TNFR superfamily, has been shown to increase the transactivation potential of p65 by enhancing its level of phosphorylation and thus increases the level of transcription of several of its targets genes (Madrid *et al.*, 2000; Sizemore *et al.*, 1999). To investigate if such a mechanism may be involved in the LMP-1-mediated transactivation of the -129/+81 region of *bfl-1*, it was necessary to investigate if the nuclear protein complexes formed with the restriction fragment encompassing this region, namely, the *Sal1/Xba1* fragment, contained any of the NF- $\kappa$ B subunits. To this end, supershift analysis were conducted by preincubating nuclear extracts from LMP-1-expressing DG75-tTA-LMP-1 cells with antibodies to either p65, p50 or c-rel before incubation with the labeled *Sal1/Xba1* fragment. Competition studies were also conducted simultaneously using a 100-fold molar excess of either wild-type or mutated consensus NF- $\kappa$ B oligonucleotide. The molar amount of the *Sal1/Xba1* fragment could not be accurately determined and was estimated without taking into account any losses that may have occurred during its preparation as a probe; hence, the amount of competitor added in relation to the amount of the *Sal1/Xba1* fragment is likely to be in vast excess. Surprisingly, in this experiment (Figure 3.51) the pattern and relative intensity of the complexes formed with the *Sal1/Xba1* fragment was different to what was observed in the initial experiment (Figure 3.51A). Several of the complexes of slow mobility observed in the initial experiment were not detected and several complexes of faster mobility were observed in this experiment. Nevertheless, with the pattern of complexes detected in this experiment, there was neither any obvious supershift or specific competition observed. Similar inconsistencies were also observed when the *Sal1-Alu1* fragment was used as the probe in a similar experimental strategy as with the *Sal1/Xba1* fragment (Figure 3.51B). Owing to the inconsistencies observed in the pattern of nuclear protein complexes, it was not possible to draw any firm conclusions from these

experiments These inconsistencies are likely to be the result of a procedural problem and possible contributing factors will be alluded to in the discussion section (section 4.6) of this thesis

Although NF- $\kappa$ B plays an important role in mediating the activation of the -129/+81 region of *bfl-1* by LMP-1, it was not possible to demonstrate binding of NF- $\kappa$ B to this region Although a procedural defect cannot be excluded, it is possible that NF- $\kappa$ B may be playing an indirect role in this effect as is thought to be the case with the NF- $\kappa$ B-mediated process of upregulation of CD40 and LFA-3 by LMP-1 (Devergne *et al* , 1998)

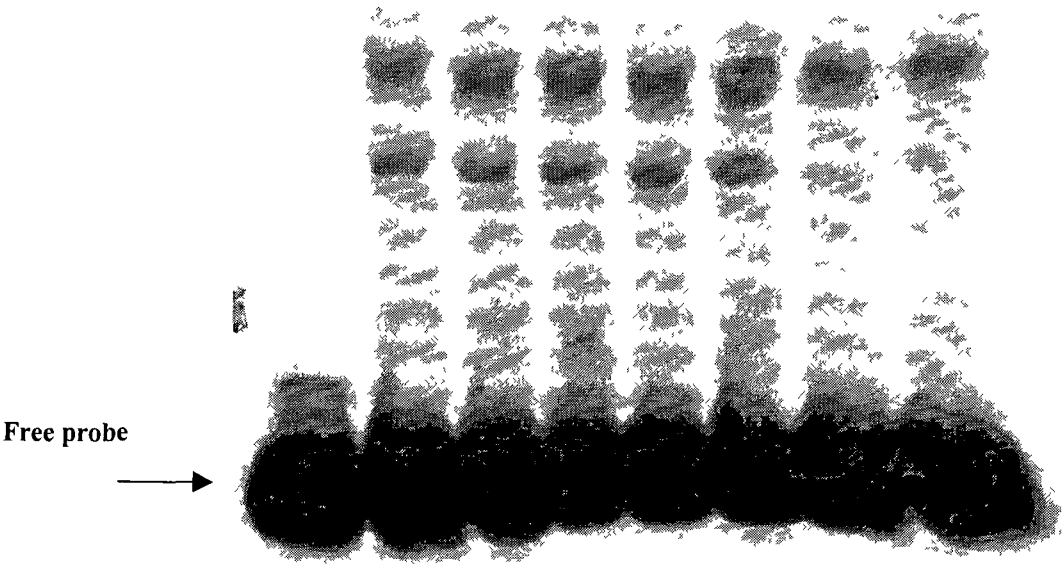
A.

DG75tTALMP1 (tetracycline=t)	-	+t	-t	-t	-t	-t	-t	-t	-t
Antibody/ Competitor $\kappa$ B oligonucleotide	-	-	-	$\alpha$ p65	$\alpha$ p50	$\alpha$ c-rel	$\alpha$ Mcl-1	wt CON	mutCON
	*	*	*	*	*	*	*	*	*



B.

DG75tTALMP1 (tetracycline=t)	-	+t	-t	-t	-t	-t	-t	-t
Antibody/ Competitor κB oligonucleotide	-	-	-	αp65	αp50	αc-rel	wt CON	mutCON



**Figure 3 51** Supershift and competition studies of the binding of nuclear extracts from DG75-tTA-LMP-1 cells to DNA fragments spanning the -129/+81 region of *bfl-1*. 10 0 μg of nuclear extracts from DG75-tTA-LMP-1 cells induced to express LMP-1 36 h after tetracycline removal were preincubated with either the antibodies to the NF-κB subunits p65, p50 or c-rel or control antibody (α-Mcl-1) for supershift assays or an excess of unlabeled wild-type (wt) or mutated (mut) consensus (CON) NF-κB oligonucleotide as competitor before addition of labeled *Sal1/Xba1* (A) or *Sal1/Apo1* (B) fragments as target DNA fragments in the binding reaction. The protein-DNA complexes were size-fractionated as mentioned in Figure 3 50 and visualised by autoradiography.

### 3. 2.10 Activation of CD40 upregulates *bfl-1* promoter activity

#### 3.2.10.1 Introduction

CD40 is a 48 kDa membrane receptor of the tumour necrosis factor receptor (TNFR) family. It is activated by CD154 (CD40L), its membrane-bound cognate ligand. CD40 was initially described as a B-lymphocyte activation antigen, but is now known to be expressed by a variety of other cell types, especially endothelial and epithelial cells. In resting B-cells, signaling through CD40 stimulates cell survival, growth and differentiation (Stamenkovic *et al*, 1989, Banchereau *et al*, 1994, Clark and Ledbetter, 1994, Gordon, 1995). A key interaction of CD40/CD40L in the humoral immune response involves CD40 expressed on B cells with its ligand expressed on helper T-cells resulting in costimulation of the two lymphocytic cell types.

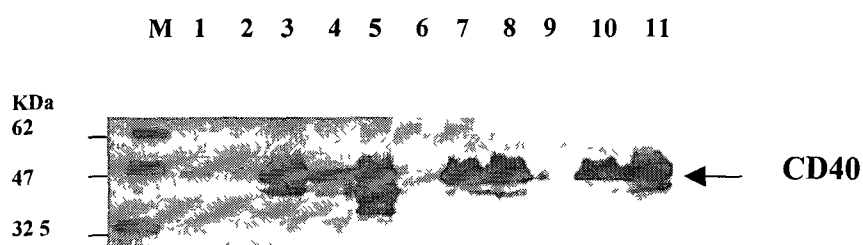
CD40 ligation in B cells induces a number of phenotypic changes similar to those observed upon LMP1 expression, including upregulation of CD23 and CD54 expression, and induction of IL-6 production and homotypic cell adhesion (Busch and Bishop, 1999, Devergne *et al*, 1998, Eliopoulos *et al*, 1997, Laman *et al*, 1996). The similarities between the effects of CD40 activation and LMP-1 signaling is most likely a reflection of the fact that common biochemical pathways are activated by these molecules (Tewari and Dixit, 1996, Baker and Reddy, 1996, Busch and Bishop, 1999). Both CD40 and LMP-1 stimulate activation of the transcription factors NF- $\kappa$ B and AP-1 via recruitment of TRAF proteins. Thus, TRAF2 and TRAF3 associate with the carboxy-terminal domains of both LMP-1 and CD40 through the PXQXT/S domain, whilst TRAF1 interacts directly with LMP-1 but not with CD40 (Hu *et al*, 1994, Cheng *et al*, 1995, Mosialos *et al*, 1995, Pullen *et al*, 1999, Kehry, 1996). TRAF2 has been shown to be an important mediator of NF- $\kappa$ B activation in the case of both molecules (Rothe *et al*, 1995, Kaye *et al*, 1996).

Both LMP-1 and CD40 signaling result in upregulation of the expression of certain anti-apoptotic proteins, such as A20, in B-cells, which may at least partly account for the

survival advantage conferred by CD40 engagement or LMP-1 expression in B cells (Henderson *et al* , 1991, Laherty *et al* , 1992, Sarma *et al* , 1995) Furthermore, CD40 engagement on BL cells has recently been shown to also upregulate expression of *bfl-1* in addition to *bcl-x* (Lee *et al* , 1999) However, this study did not include any investigation of the *bfl-1* promoter It was therefore of interest to investigate if CD40 -mediated upregulation of *bfl-1* mRNA involved promoter-activation and if LMP1 and CD40 can cooperate in upregulating *bfl-1* promoter activity

### **3.2.10.2 Expression of LMP1 correlates with an upregulation of CD40 levels**

It is now well established that one of the effects of EBV infection of primary B-lymphocytes or LMP-1 expression in BL cells is the upregulation of CD40 expression (Gregory, 1995) Thus, examination of the level of CD40 expression in a series of BL lines and LCLs by Western blotting using an anti-CD40 monoclonal antibody, G28 5, revealed that while EBV-negative (DG75, BL41) and EBV-positive cell lines with a latency type I phenotype (Mutu I, OKU BL) exhibit undetectable or low levels of expression of CD40, EBV-positive cell lines with a latency type III phenotype (Mutu III, BL41B5 8) and LCLs (IARC-171, OKU LCL, IARC 290B) exhibit relatively higher levels of expression of this molecule (Figure 3 52 ) The lack of detection of CD40 in some of the EBV-negative BL cell lines and EBV-positive cell lines with a group I phenotype is likely to be due to the lower sensitivity of this assay since expression of CD40, albeit at low levels, has been detected in these cell lines by the more sensitive method of FACS (Fluorescence activated cell sorter) analysis (Henriquez *et al* , 1999) Also, as shown by others, induction of LMP-1 expression in DG75tTA-LMP-1 cells also results in upregulation of expression of CD40 (Floettmann *et al* , 1996) CD40 monomers migrate with a molecular weight of 43-47 kDa , the lower molecular weight proteins detected in some of the cell lines, most likely represent deglycosylated forms of the monomers (Braesch-Andersen *et al* , 1989)



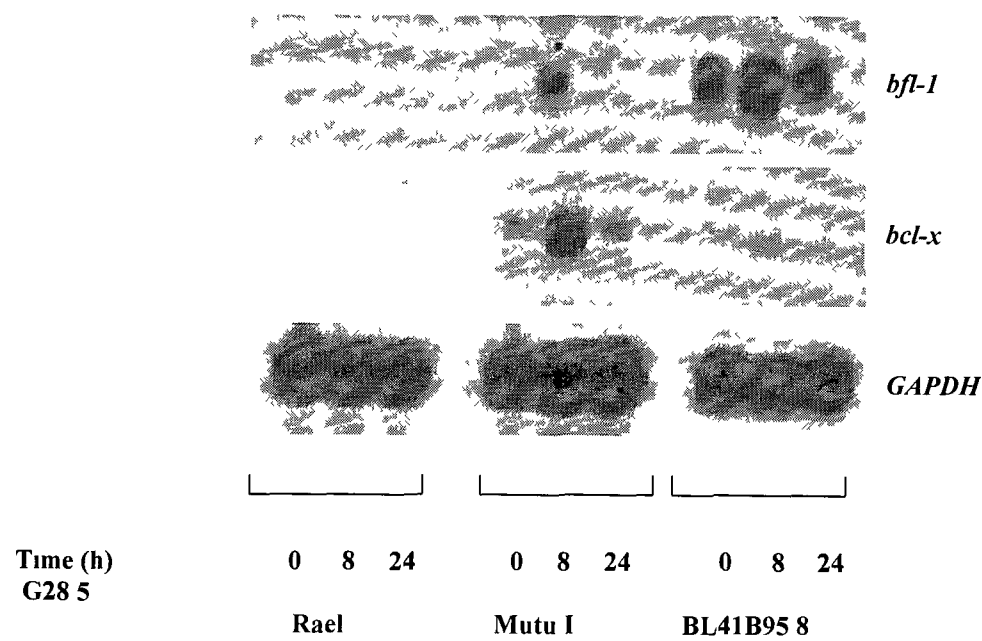
**Figure 3 52 Analysis of expression of CD40 in a series of EBV-negative and -positive BL cell lines, LCLs and DG75tTALMP-1 cells induced to express LMP-1** Western blot analysis of CD40 expression in a series of EBV-negative BL cell lines [ DG75 (lane 1), BL41 (lane6)], EBV-positive BL lines with either a group I phenotype [ Mutu I (lane 4), OKU BL (lane 9)] or a group III phenotype [ Mutu III (lane 5), BL41B95 8 (lane 7)], LCLs [ IARC-171 (lane 8), OKU LCL (lane 10), IARC 290B (lane 11)], uninduced DG75tTALMP-1 cells (lane 2) and DG75 tTALMP-1 cells induced to express LMP-1 48 h after teracycline removal (lane 3) The position of the protein molecular weight markers is indicated on the left-hand side of the photograph and the position of the CD40 protein (48 kDa)is indicated by an arrow

### 3.2.10.3 Ligation of CD40 upregulates expression of *bfl-1* mRNA

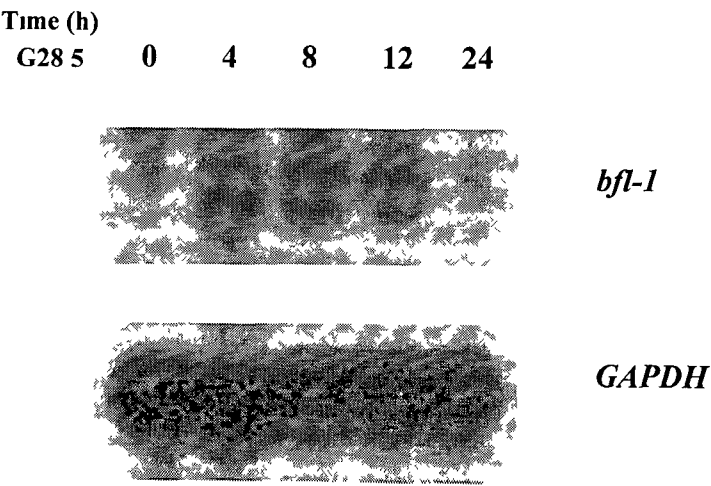
Ligation of CD40 on B-lymphocytes has been shown to elicit a cell survival signal at least part of which involves the upregulation of expression of the anti-apoptotic gene *bclx/L* (Lee *et al* , 1999, Tuscano *et al* , 1996) More recently, activation of CD40 has been shown to also upregulate the expression of *bfl-1* in B cell lines (Lee *et al* , 1999) Indeed, Northern blot analysis revealed that treatment of the BL cell lines Mutu I and BL41-B95 8 with the agonistic anti-CD40 antibodies, G28 5, resulted in an upregulation of expression of *bfl-1* and *bcl-x* (Figure 3 53A) The upregulation of *bfl-1* and *bcl-x* on CD40 ligation was found to be transient with a significant upregulation detected by 8 h followed by a return to basal levels (i e levels in untreated cells) by 24 h post-CD40 ligation The transient nature of upregulation of these mRNAs by CD40 activation has been found by others (Lee *et al* , 1999) However, treatment of the BL cell line Rael with the G28 5 antibodies did not have an effect on *bfl-1* and *bcl-x* mRNA levels (Figure 3 53A) Although Rael expresses CD40 it has been shown by others to be unresponsive to

several CD40-mediated effects on gene expression (Henriquez *et al* , 1999) A more detailed analysis of the kinetics of upregulation of *bfl-1* mRNA by CD40 ligation was attempted in DG75 cells (Figure 3 53B) In this experiment, upregulation of *bfl-1* mRNA levels was detected as early as 4 h post-stimulation and these levels were maintained for at least 12 h and then decreased to near-basal levels by 24 h post-treatment with anti-CD40 antibodies G28 5

A.



B.



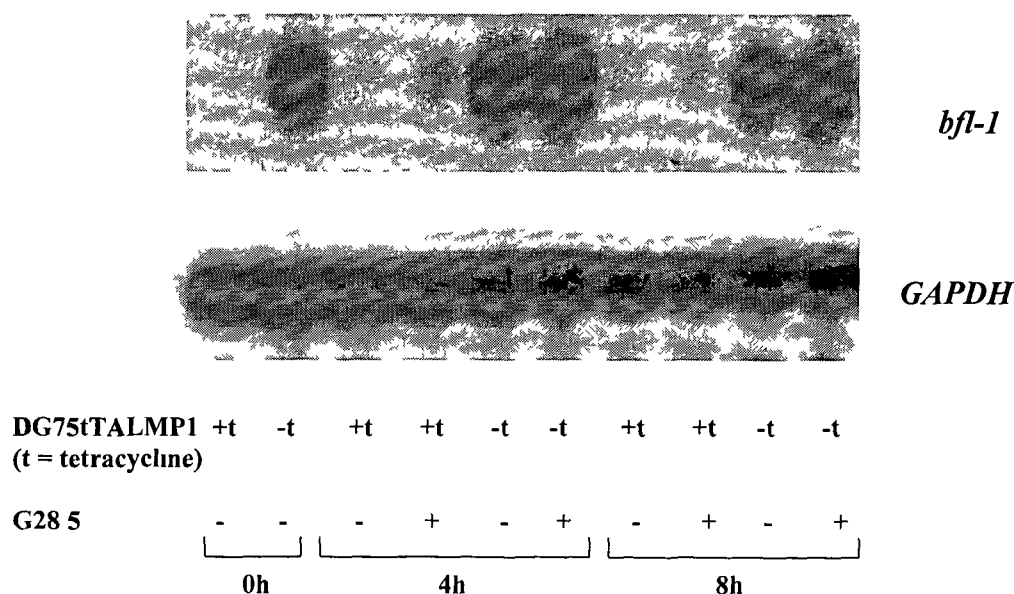
**Figure 3 53 Ligation of CD40 upregulates expression of *bfl-1* and *bcl-x* mRNAs in BL cell lines** Rael, Mutu 1, BL41-B95 8 (A) and DG75 (B) cells were left untreated or treated with the CD40 agonistic antibodies G28 5 either as a 1 4000 dilution of ascites (Rael, Mutu 1, BL41 B95 8) or at a concentration of 1 µg/ml of purified antibody (DG75) Total RNA samples prepared from cells at 0, 8 and 24h (Rael, Mutu 1, BL41-B95 8) or at 0, 4, 8, 12 and 24 h (DG75) post-treatment were subjected to Northern analysis with an antisense *bfl-1* riboprobe (upper panel of Figures A and B) The blots were then sequentially stripped and reprobed with antisense *bcl-x* (middle panel of Figure A) and *GAPDH* (lower panels of Figures A and B) probes

### **3.2.10.4 Investigation of a cooperative effect between CD40 and LMP-1 in upregulating steady-state levels of *bfl-1* mRNA**

Simultaneous provision of signals from LMP-1 and CD40 has been shown to result in an additive or greater than an additive effect of the individual signals on the expression of cell surface proteins such as B7 1 and on IgM secretion (Busch and Bishop, 1999) The effect of the dual signals on the expression of other surface molecules such as ICAM-1, CD23 and LFA-1 was not found to be as dramatic and was not found to be greater than the effect of either signal alone (Busch and Bishop, 1999) The effect of dual signaling from CD40 and LMP-1 on protein expression is dependent on the nature of the signaling pathway(s) involved i e whether there is a dependence on distinct intracellular pathways or a dependence on the availability of intracellular components of a single pathway

To analyse the effect of dual signaling from LMP-1 and CD40 on *bfl-1* mRNA expression, the effect of CD40 ligation on uninduced DG75tTA-LMP-1 cells and on the cells induced to express LMP-1 36 h after tetracycline removal was examined by Northern analysis (Figure 3 54) As expected, induction of LMP-1 in DG75tTA-LMP-1 cells 36 h after tetracycline removal, resulted in a marked upregulation of *bfl-1* mRNA CD40 ligation on the LMP-1 expressing cells resulted in a further increase in *bfl-1* mRNA levels as detected 4 h after CD40 stimulation and these levels were maintained for at least 4h thereafter (Figure 3 54) The minor effect of CD40 ligation on *bfl-1* mRNA levels in the uninduced DG75tTA-LMP-1 cells may be a reflection of the low levels of CD40 expression in these cells (even lower than that in DG75 cells) which are significantly elevated on induction of LMP-1 expression (Figure 3 52, Floettmann *et al* ,

1996) The increase in *bfl-1* mRNA expression on ligation of CD40 in the LMP-1-expressing DG75-tTA-LMP1 cells may therefore be a result of the increased levels of expression of CD40 observed on induction of LMP-1. Thus, LMP-1 and CD40 exert a cooperative effect in upregulating *bfl-1* mRNA levels in DG75-tTA-LMP-1 cells, though the contribution by CD40 may also be a result of the upregulation of CD40 expression by LMP-1.



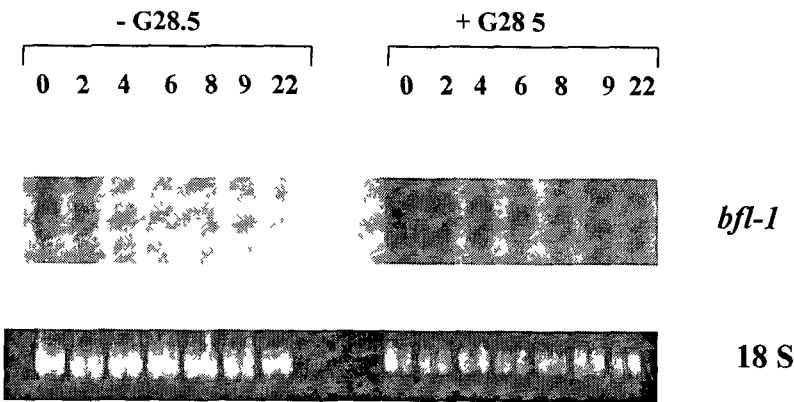
**Figure 3.54** LMP1 and CD40 cooperate in upregulating *bfl-1* mRNA in DG75tTA-LMP-1 cells. Uninduced DG75tTA-LMP-1 cells (+tetracycline) or DG75tTA-LMP-1 cells induced to express LMP-1 36 h after tetracycline removal (- tetracycline) were left untreated or stimulated with 1 µg/ml of the anti-CD40 antibody, G28.5. Total RNA extracted from cells at 0, 4 and 8 h after stimulation with G28.5 was used in Northern analysis with an antisense *bfl-1* riboprobe (upper panel). The blots were sequentially stripped and reprobed with a GAPDH (lower panel) riboprobe. Exposure to X-ray film was for 18 – 24 h.

### 3.2.10.5 The regulation of *bfl-1* expression by CD40 does not occur at the level of mRNA stability.

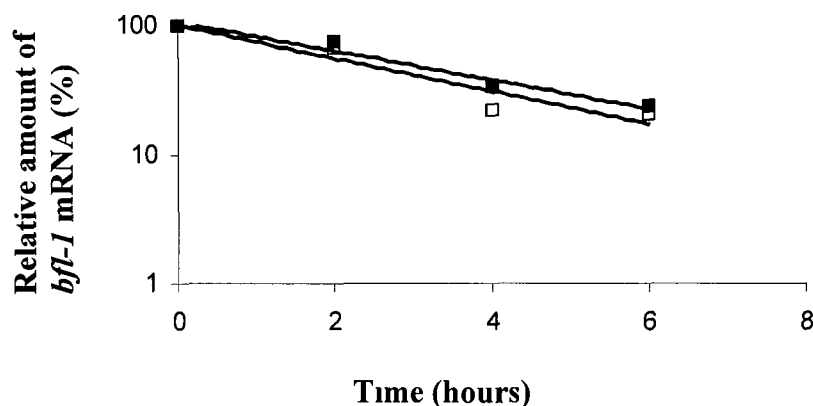
Control of mRNA expression occurs at the level of either mRNA stability or rate of transcription or a combination of the two. To investigate if the CD40-mediated upregulation of *bfl-1* mRNA is due to an increase in mRNA stability, mRNA half-life studies were conducted in the EBV-negative BL cell line BJABtTA. The BJAB cell line

expresses readily detectable levels of *bfl-1* mRNA which are upregulated on stimulation of CD40 by G28.5 monoclonal antibodies (Lee *et al* , 1999). CD40 stimulation of BJABtTA cells was also found to result in upregulation of *bfl-1* mRNA levels (Figure 3.55). The half-life of *bfl-1* mRNA was determined in BJABtTA cells in the presence or absence of CD40 stimulation (5h treatment with G28.5 monoclonal antibodies) as described in section 2.5.5. The half-life of *bfl-1* mRNA in unstimulated cells was found to be approximately 2.4 h. Stimulation with the agonistic anti-CD40 antibodies resulted in only a marginal increase in the stability of *bfl-1* mRNA to 3.0h (Figure 3.55). Thus, unlike the effect of EBV-infection or LMP-1 expression (Figures 3.10 and 3.11), increased mRNA stability does not appear to make a major contribution to the CD40-induced upregulation of *bfl-1* mRNA steady-state levels.

A.



B.



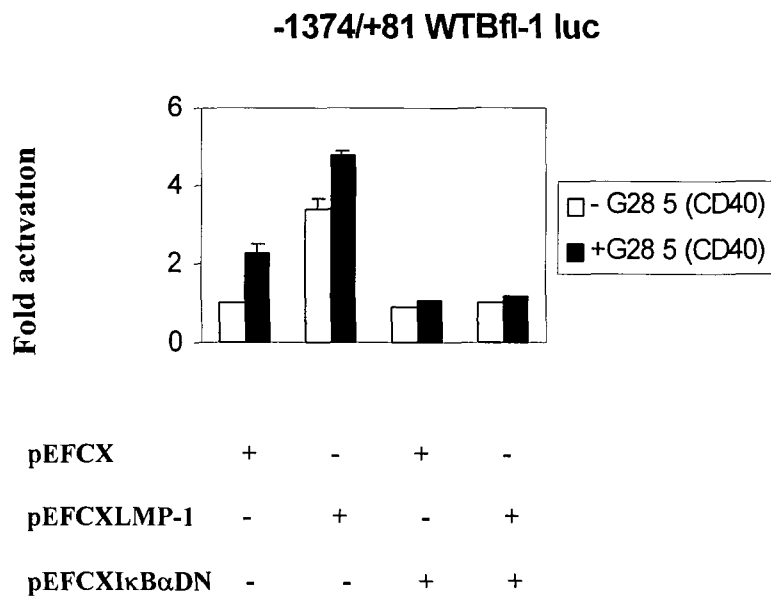
**Figure 3 55 Increased mRNA stability does not make a major contribution to CD40-mediated upregulation of *bfl-1* mRNA levels in BJABtTA cells** Unstimulated BJABtTA cells or cells stimulated for 5 h with 1  $\mu$ g/ml of the anti-CD40 antibody, G28 5, were treated with 5  $\mu$ g/ml actinomycin D to block RNA synthesis. Total RNA was then extracted from cells harvested at 0, 2, 4, 6, 8, 9 and 22 h after treatment with actinomycin D and the rate of decay of *bfl-1* mRNA monitored by Northern analysis. **A)** The upper panel shows the Northern blot analysis of the decay of *bfl-1* mRNA over time (indicated in hours above each lane) after treatment with actinomycin D in unstimulated and CD40-stimulated cells. The lower panel is a photograph of the 18S rRNA band from the same ethidium bromide-stained gel used for blotting. **B)** The graphs show the best-fit semilogarithmic lines generated from the relative amounts of *bfl-1* mRNA as determined from densitometric scanning of the autoradiograms of the Northern blots in panel A plotted against time of exposure to actinomycin D. These values were normalised for loading based on the intensity of the 18S rRNA bands. Owing to the high level of background hybridisation obtained with the *bfl-1* riboprobe in this Northern Blotting experiment, it was only possible to obtain densitometric values for the '*bfl-1* bands' corresponding to the 0, 2, 4 and 6 h timepoints of actinomycin D treatment and the graphs were plotted using the normalised values obtained for only these time points. Relative levels of *bfl-1* mRNA as determined in unstimulated BJABtTA cells (□) and cells stimulated with G28 5 for 5 h (■).

### 3.2.10.6 LMP-1 and CD40 cooperate in upregulating *bfl-1* promoter activity

Since mRNA stability was excluded as a mechanism involved in the CD40-mediated upregulation of *bfl-1* mRNA, it was of interest to investigate the possible involvement of the alternative mechanism of increased promoter activity in this effect. To this end, DG75 cells transfected with the -1374/+81 *bfl-1*-luciferase reporter construct were left

untreated or treated with 1 µg/ml of G28 5 at 36 h post-transfection. After treatment with the anti-CD40 antibody for 12 h, cells were harvested and luciferase activity measured and compared to that of untreated cells. It can be seen from Figure 3 56 that stimulation of CD40 resulted in a 2.2-fold increase in activity of the *bfl-1* promoter suggesting that CD40-induced upregulation of *bfl-1* mRNA levels is mediated by an increase in promoter activity. Expression of LMP-1 from the cotransfected pEFCX-LMP-1 plasmid resulted in a 3.4-fold-increase in *bfl-1* promoter activity from the -1374/+81 *bfl-1*-luciferase reporter and this value was increased further to 4.8-fold on activation of CD40 signaling by G28 5. The extent of activation of the *bfl-1* promoter as a result of the dual signaling from CD40 and LMP-1 is greater than that provided by each of the individual signals but is less than that expected of an additive effect of both signals. These findings suggest that CD40 and LMP-1 signaling pathways leading to upregulation of *bfl-1* promoter activity most likely converge. However, it is possible that part of the effect of CD40 activation on *bfl-1* promoter activity in the LMP-1 transfected cells is due to the increased surface expression of CD40 by LMP-1 in these cells. The lower extent of activation of the -1374/+81 *bfl-1* luc reporter by LMP-1 in these studies compared to the studies reported in section 3 2 7, is likely to be due to the differences in the time of harvesting of the transfected cells for the luciferase assays (Rowe *et al* , 1994, Wang *et al* , 1996).

As observed in section 3 2 7 5 LMP-1-mediated upregulation of *bfl-1* expression is NF-κB-dependent. To investigate if CD40-mediated upregulation of *bfl-1* promoter activity is also NF-κB-dependent, DG75 cells were cotransfected with a plasmid expressing the dominant negative mutant IκBαDN (pEFCX-IκBαDN) and the -1374/+81 *bfl-1*-luciferase reporter construct and then stimulated with the G28 5 antibody. Expression of IκBαDN inhibited the CD40-mediated activation of *bfl-1* promoter activity suggesting that CD40-mediated activation of the *bfl-1* promoter is NF-κB-dependent (Figure 3 56). The dual effect of LMP-1 and CD40 activation on this parameter also appears to be NF-κB-dependent (Figure 3 56). However, in this case an indirect effect on the NF-κB-mediated process of upregulation of CD40 levels by LMP-1 cannot be excluded (Devergne *et al* , 1998).



**Figure 3 56** Expression of a dominant negative mutant of  $\text{I}\kappa\text{B}\alpha$  inhibits the activation of the *bfl-1* promoter by CD40 signaling and by dual signaling from CD40 and LMP-1 DG75 cells were cotransfected with 2.5  $\mu\text{g}$  of the LMP-1 expression plasmid, pEFCX-LMP-1, or empty vector, pEFCX, and 2.5  $\mu\text{g}$  of the *bfl-1* promoter-luciferase construct, p-1374/+81 *Bfl-1*-luc in the absence or presence of 10.0  $\mu\text{g}$  of the  $\text{I}\kappa\text{B}\alpha\text{DN}$  expression plasmid, pEFCX  $\text{I}\kappa\text{B}\alpha\text{DN}$ . At 36h post transfection, the cells were either left untreated or treated with 1  $\mu\text{g}/\text{ml}$  G28.5 for 12 h prior to harvesting for measurement of luciferase activity. Normalised luciferase activities are expressed as fold activation over control (empty vector in the absence of expression of  $\text{I}\kappa\text{B}\alpha$  DN or treatment with G28.5 =1).

### 3.2.11 Mechanism of upregulation of Bcl-2 expression by LMP-1

#### 3.2.11.1 BCL-2

*bcl-2* was discovered by virtue of its association with the t(14,18) reciprocal chromosomal translocation commonly found in follicular lymphoma (reviewed in Bruckheimer *et al* , 1998) The *bcl-2* open reading frame is not disrupted by the t(14,18) translocation and generates an otherwise normal *bcl-2* gene product in inappropriate amounts Bcl-2 was shown to be a unique oncogene in that its deregulation did not result in an increase in cell proliferation, but rather enhancement of cell survival

The assignment of *bcl-2* as a survival gene is based largely on *in vitro* observations Thus, introduction of *bcl-2* into IL-3 dependent cell lines results in their survival after IL-3 withdrawal (Vaux *et al* , 1988, Nunez *et al* , 1990) In the absence of *bcl-2*, withdrawal of IL-3 from dependent cells results in their rapid demise by apoptosis Expression of *bcl-2* *in vivo* also suggests a survival function studies of *bcl-2* –transgenic mice indicate that the gene causes B-cell hyperplasia but not tumorigenesis (Bruckheimer *et al* , 1998) Deregulated Bcl-2 expression is thought to be a primary genetic change that prolongs cell survival in order to permit the acquisition of further pre-malignant changes Indeed, *bcl-2* was shown to complement *c-myc* in the neoplastic process (Korsmeyer, 1995) Germinal center B cells (centrocytes) can be rescued from apoptosis by exposure *in vitro* to immobilised anti-Ig and CD40 monoclonal antibody (Liu *et al* , 1991) However, Bcl-2 expression is induced relatively late during CD40-mediated rescue of germinal center B cells (Holder *et al* , 1993)

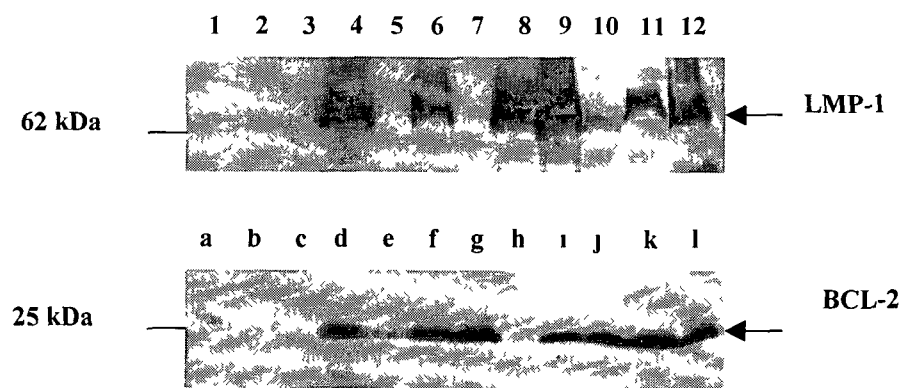
Bcl-2 is a transmembrane protein and localises to the nucleus, rough ER and mitochondria (Hockenberry *et al* , 1991, Korsmeyer, 1995) It is normally expressed in pro- and mature B cells but is downregulated in pre- and immature B-lymphocytes (Bruckheimer *et al* , 1998) This differential expression points to the survival role of Bcl-2 in B lymphocyte development High levels of Bcl-2 are needed to ensure the survival of pro-B cells and mature B cells in order to maintain a population of functional

lymphocytes But low levels of Bcl-2 are necessary for cells that do not express functional surface immunoglobulin or are self-reactive to undergo apoptosis

EBV-positive BL cell lines can be readily triggered into apoptosis unless the full complement of EBV-latent proteins is expressed by the cells (Gregory *et al* , 1991) This protection against apoptosis is accompanied by upregulation of Bcl-2 (Liu *et al* , 1991, Henderson *et al* , 1991) which is also induced following expression of LMP-1 (Henderson *et al* , 1991) Furthermore, ectopic expression of *bcl-2* can provide BL cell lines with a survival advantage Although the induction of *bcl-2* expression by expression of the entire complement of EBV latent genes or *LMP-1* only is well documented (Finke *et al* , 1992, Henderson *et al* , 1991), the mechanism involved in its regulation has not been worked out This section attempts to address the gap in our knowledge of the mechanism involved in the regulation of Bcl-2 expression by LMP1

**3.2.11.2 Correlation between expression of LMP-1 and Bcl-2 in BL cell lines**

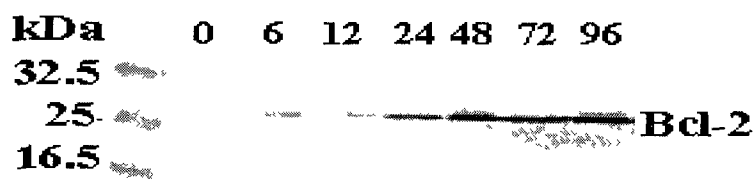
As an initial step towards studying the mechanism of regulation of Bcl-2 expression by LMP-1, the expression of LMP-1 and Bcl-2 in a series of BL cell lines was examined Indeed, as has been reported by others (Henderson *et al* , 1991, Finke *et al* , 1992), Western blot analysis revealed that cell lines that expressed LMP-1 (LCLs and BL lines with a group III phenotype) exhibited higher levels of expression of Bcl-2 protein than non-LMP-1 expressing BL lines (Figure 3 57)



**Figure 3 57 Expression of LMP-1 correlates with upregulated expression of Bcl-2 in BL cell lines** Western blot analysis of LMP-1 expression (upper panel) and Bcl-2 expression (lower panel) in a series of EBV-negative BL cell lines [DG75 (lanes 1, a), BL41 (lanes 7, e)], EBV- positive BL lines with either a group I phenotype [Rael (lanes 2, b), Mutu I (lanes 3, c), OKU BL (lanes 5, h)] or a group III phenotype [ Mutu III (lanes 4, d), BL41-B95 8 (lanes 8, f), Ag876 (lanes 12, l)] and LCLs [ OKU LCL (lanes 6, i), IARC171 (lanes 9, g), X50-7 (lanes 10, j) IARC 290B (lanes 11, k)] The positions of the 62 kDa and 25 kDa molecular weight markers are indicated on the left hand side of the photograph, whilst the positions of the LMP-1 (64 kDa) and Bcl-2 (26 kDa) proteins are indicated by an arrow on the right hand side LMP-1 expression was detected using the CS1-4 antibodies while Bcl-2 was detected using the Bcl-2 100/124 antibodies

### 3.2.11.3Induction of LMP-1 expression results in upregulation of Bcl-2 protein levels

The effect of LMP-1 on Bcl-2 expression was addressed using the DG75tTA-LMP1 cell line in which the expression of LMP1 is under the control of a tetracycline-based system Indeed, induction of LMP-1 on removal of tetracycline from the growth medium resulted in induction of Bcl-2 protein as assessed by Western blot analysis of the same lysates used for analysis of LMP-1 induction in figure 3 3 (Figure 3 58) Upregulation of Bcl-2 expression was detected 24 h after tetracycline removal and the levels rose thereafter for the duration of the experiment with maximal levels detected by 72-96 h post induction of LMP-1

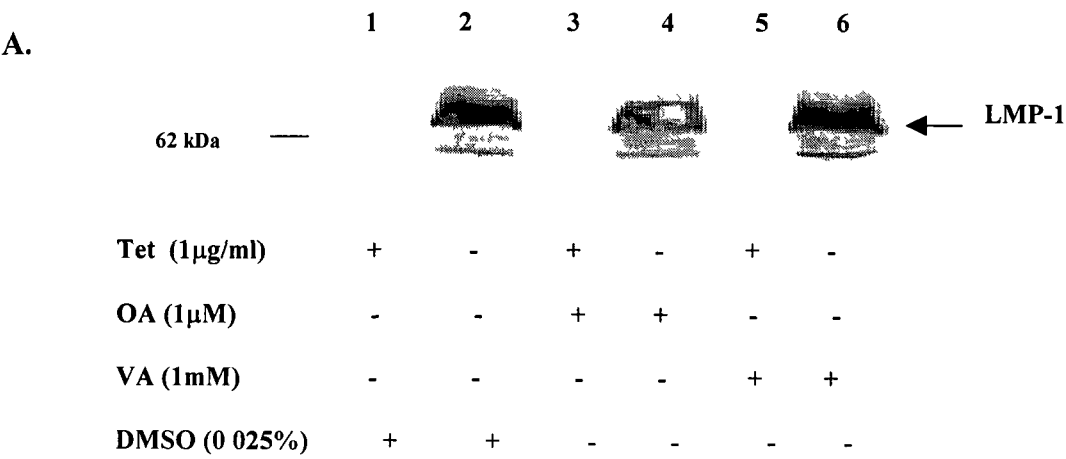


**Figure 3 58 Western blot analysis of Bcl-2 levels in DG75-tTA-LMP-1 cells upon induction of LMP-1** Cells were harvested at various times (indicated in hours above each lane) after removal of tetracycline from the medium, and total cellular protein extracted from 5 x 10<sup>5</sup> cells used in Western blot analysis with anti-bcl-2 antibodies (bcl2100/124) The position of the protein molecular weight markers are indicated on the left hand side of the photograph

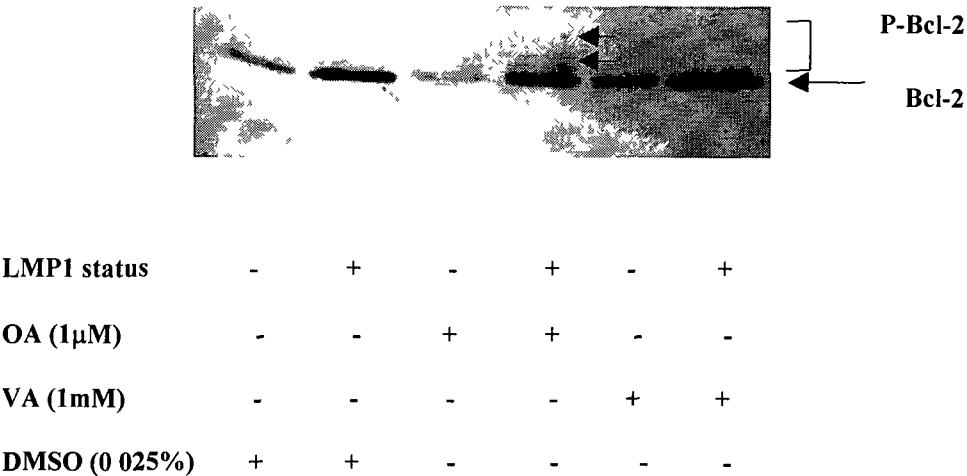
#### 3.2.11.4 Analysis of the phosphorylation status of Bcl-2

Serine-phosphorylation represents a post-translational modification that provides an additional mechanism of control of function of the Bcl-2 protein. Phosphorylation of Bcl-2 has been shown to inactivate its function in lymphoid cells, making them susceptible to apoptosis (Haldar *et al.*, 1995; Srivastava *et al.*, 1998). In these studies treatment of cells with phosphatase inhibitors such as okadaic acid (OA) and calyculin A or chemotherapeutic agents such as taxol induced slower mobility forms of Bcl-2 as revealed by Western blot analysis. However, in an EBV-immortalised lymphoblastoid cell line, GM1500, OA treatment did not result in Bcl-2 phosphorylation or apoptotic death suggesting that the kinase responsible for phosphorylating Bcl-2 is inactive in these cells (Haldar *et al.*, 1995). Since LCLs express LMP-1, it was hypothesised that LMP-1 may be responsible for inactivating this kinase and thus maintain Bcl-2 in an unphosphorylated state. To investigate this possibility, the effect of OA treatment on the phosphorylation status of Bcl-2 protein in DG75 tTALMP-1 cells before and after induction of LMP1 was assessed. As a control for the effect of OA, cells were treated in parallel with the tyrosine phosphatase inhibitor, sodium orthovanadate (VA) for 6h. Surprisingly, Western blot analysis revealed that treatment of DG75 tTA-LMP-1 cells with 1  $\mu$ M OA before or 96 h after LMP-1 induction induced slower mobility forms of Bcl-2 (Figure 3.59B). The presence of the altered forms of Bcl-2 is more obvious in the case of the OA-treated LMP-1 expressing cells perhaps due to the relatively larger amount of Bcl-2 protein induced by LMP-1 in these cells. These results would suggest that LMP-1 does not inactivate the kinase(s) involved in phosphorylating Bcl-2 in this cell line. Trypan blue staining of cells at the time of harvesting did not reveal an effect of OA treatment on cell death. The untreated cells lacked any evidence of slower mobility forms of Bcl-2 either before or after LMP-1 induction. This suggests that Bcl-2 induced in the cells upon expression of LMP-1 is mostly of the non-phosphorylated form. However, it is possible that the lesser phosphorylated forms migrate at apparently the same molecular weight as the unphosphorylated form of Bcl-2 because of insufficient resolution on the gel. If this is indeed the case then the pattern of the phosphorylated forms and hence extent of phosphorylation of Bcl-2 in the LMP-1 expressing cells is not

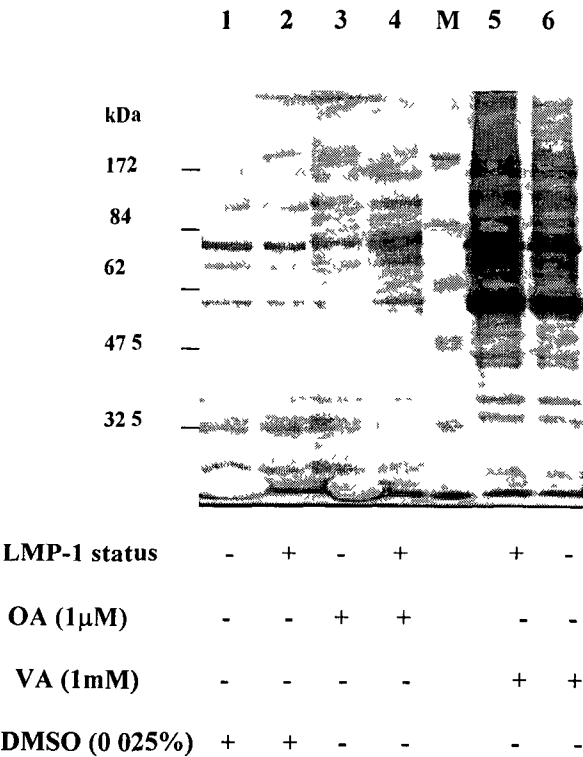
as great as that induced upon OA treatment. Treatment of the cells with the tyrosine phosphatase inhibitor, VA, did not induce any detectable alteration in the mobility of Bcl-2, suggesting that the induction of the slower mobility forms is specific to serine/threonine phosphatase inhibitors and that these forms are indeed phosphorylated on serine and/or threonine. Furthermore, the lack of an effect of VA on Bcl-2 phosphorylation did not seem to be due to a lack of an effect of VA on protein tyrosine phosphorylation since Western blot analysis with the anti-phosphotyrosine antibodies, PT66, revealed that VA treatment did increase tyrosine phosphorylation of cellular proteins particularly in the 47.5 – 172 kDa molecular weight region (Figure 4.59C). Interestingly, LMP-1-induction did not lead to any detectable increase in the tyrosine phosphorylation of cellular proteins. However, VA treatment, led to an enhancement in the amount of Bcl-2 protein expressed in the DG75-tTA-LMP1 cells irrespective of their LMP-1 status, suggesting that tyrosine phosphorylation of protein(s) may be involved in regulating Bcl-2 expression in these cells (Fig 4.59C lane 5 in particular). The effect of VA on Bcl-2 expression did not seem to be due to an indirect effect of this agent on the expression system in these cells leading to induction of LMP-1 as revealed by Western blot analysis with anti-LMP-1 antibodies (Figure 4.3A). The lack of detectable phosphorylated forms of Bcl-2 in the cells on induction of LMP-1 is consistent with the anti-apoptotic function of the induced Bcl-2. However, the potential for phosphorylation of Bcl-2 does exist in a BL cell context despite LMP1 expression.



B.



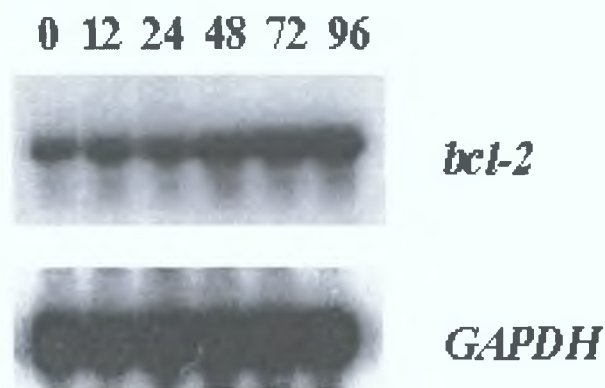
C.



**Figure 3 59 Analysis of the phosphorylation status of Bcl-2 in DG75-tTA-LMP1 cells before and after induction of LMP-1** DG75-tTA-LMP-1 cells maintained in the presence or absence of tetracycline for 96 h were treated with 1µM OA or 1mM VA or 0.025% DMSO (vehicle control) for 6 h and then lysed in a buffer containing phosphatase inhibitors. The lysates were used in Western blot analysis with antibodies to either LMP-1(A), Bcl-2 (B) or phosphotyrosine (C). The position of molecular weight markers (M) are indicated on the left-hand side of the photographs. The position of the LMP1 and Bcl-2 proteins are indicated by arrows. In panel B, the position of bands corresponding to phosphorylated forms of Bcl-2 (P-Bcl2) are indicated by smaller arrows on the photograph.

### 3.2.11.5 LMP1 upregulates steady-state levels of *bcl-2* mRNA in DG75-tTA-LMP1 cells

RPA analysis using the multiprobe set APO-2 revealed that induction of LMP-1 expression in DG75-tTA-LMP-1 cells also resulted in an upregulation of *bcl-2* mRNA levels, however, a significant upregulation was not evident until 96 h post-induction of LMP-1 upon prolonged exposure to film (Figure 3 3, result not shown). The same RNA samples used in this RPA study were subjected to Northern analysis with an antisense *bcl-2* riboprobe. As found in the RPA analysis, the upregulation of *bcl-2* mRNA on induction of LMP-1 expression followed different kinetics to *bfl-1* upregulation in that whilst *bfl-1* upregulation was detected as early as 12 h post-removal of tetracycline from the growth medium, upregulation of *bcl-2* mRNA expression was only detected at 48h. Densitometric scanning of the bands corresponding to *bcl-2* mRNA (followed by normalisation to GAPDH levels) revealed a 2-fold increase of *bcl-2* mRNA levels at 48 h which increased to approximately 3-fold by 96 h post-induction of LMP-1 (Figure 3 60). Taken together with the data of the kinetics of induction of Bcl-2 protein (Figure 3 58), it can be concluded that the upregulation of Bcl-2 protein by LMP-1 occurs earlier than that of *bcl-2* mRNA. In particular while significant upregulation of Bcl-2 protein is detected 24h post-induction of LMP-1, there was no apparent effect on *bcl-2* mRNA at this time point (compare Figures 3 58 and 3 60). These findings suggest that both transcriptional and translational mechanisms may be involved in the control of Bcl-2 expression by LMP-1.



**Figure 3.60** Northern blot analysis of *bcl-2* mRNA in DG75-tTA-LMP-1 cells upon induction of LMP-1. Total RNA prepared at various times after LMP-1 induction (indicated in hours above each lane) in DG75-tTA-LMP1 cells was used in Northern analysis with an antisense-*bcl-2* riboprobe (upper panel). Exposure to film was for 24 h. The lower panel shows the same blot stripped and reprobed with an antisense *GAPDH* probe.

### 3.2.11.6 *bcl-2* promoter studies

Studies of steady-state mRNA levels in cell lines representing different stages of B-cell development indicated that *bcl-2* is developmentally regulated within the B-cell lineage. Human pre-B cell lines express high levels of *bcl-2* mRNA, while cell lines representing more mature B-cell stages generally express *bcl-2* at low levels (Graninger *et al.*, 1987). Similar observations hold true for murine cell lines representing various stages of B-cell maturation. (Gurfinkel *et al.*, 1987). A determination of *bcl-2* mRNA half-life revealed that *bcl-2* mRNA stability did not vary between different-stage B-cell lines (Seto *et al.*, 1988). Like B-cell lines, normal B cells purified from human tonsils or peripheral blood demonstrate only low levels of *bcl-2* mRNA. However, various B-cell activating agents such as PMA, can induce *bcl-2* expression in purified human B cells (Graninger *et al.*, 1987; Reed *et al.*, 1987). However, post-transcriptional/translational regulation may also contribute to *bcl-2* expression since although resting peripheral blood B lymphocytes express Bcl-2 protein, mitogen stimulation causes a downregulation of Bcl-2 protein despite the high *bcl-2* mRNA levels (Akagi *et al.*, 1994).

Characterisation of the genomic organisation of *bcl-2* revealed that it consists of three exons with the second and third exons separated by an extremely large, 225-kb intron (Seto *et al* , 1988) Two promoter regions are responsible for the initiation of *bcl-2* transcription (Seto *et al* , 1988) The predominant promoter region, P1, where approximately 95% of transcripts initiate has been mapped to an area containing several GC boxes (Sp-1 binding sites) located between bp –1390 and –1440 upstream from the translation start site The P1 promoter lacks a TATA box and consequently transcripts initiate from several sites in the general vicinity of the GC boxes (Seto *et al* , 1988) A second promoter, P2, is located approximately 80bp upstream of the coding region and contains a TATA box, but fewer than 5% of all transcripts initiate from this region A negative regulatory element (NRE) upstream of the P2 promoter has been described previously (Young and Korsmeyer, 1993) and p53 has been shown to mediate downregulation of *bcl-2* either directly or indirectly through a 195 bp fragment (-279 to –85 relative to translation start site) of this region (Miyashita *et al* , 1994) Three  $\pi$ 1-binding sites located from 743 to 1510 bp upstream of the NRE, function as negative regulators of *bcl-2* expression in pre-B cells but are non functional in mature B cells (Chen and Boxer, 1995) A positive regulatory region located just upstream of P1 between –1661 and –1526 contains a site for binding of c-AMP response element binding protein (CREB) The CRE site has been shown to play a major role for maintenance of constitutive expression of *bcl-2* in mature B cells as well as in the induction of *bcl-2* expression during activation of mature B cells and during rescue of immature B cells from apoptosis by PMA treatment (Wilson *et al* , 1996)

A *bcl-2* promoter-luciferase construct containing bp –3934 to –8 of the 5' untranslated region of *bcl-2* was transiently transfected into DG75tTA-LMP-1 cells in which LMP-1 expression was either blocked or induced 48 h after tetracycline removal Luciferase activity was measured 48h post-transfection (= 96h post-removal of tetracycline) which is effectively the time at which a 3-fold increase in steady-state *bcl-2* mRNA levels was detected on induction of LMP-1 in these cells It was found that the activity of the *bcl-2* promoter remained unchanged on induction of LMP-1 in DG75-tTA-LMP-1 cells (results

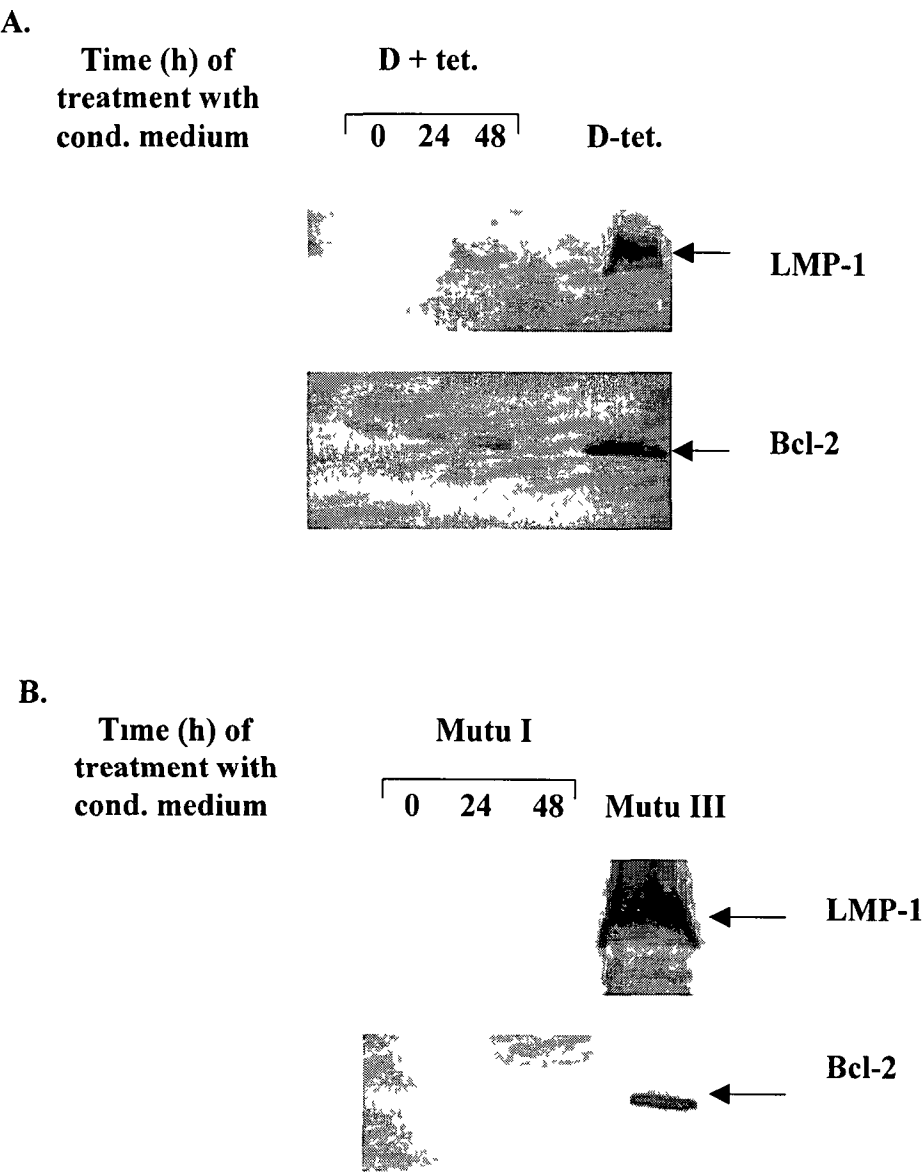
not shown) Although this region of the promoter contains the positively regulated CRE site, it also includes the negatively regulated NRE region located between the P1 and P2 promoters as well as the  $\pi 1$  binding sites which may have masked any minor positive effect of the full-length promoter (Wilson *et al* , 1996) To exclude these possibility, a promoter-reporter construct containing only the P1 promoter and upstream sequence (-3934 to -1287) or containing just the positively regulated region including the CRE site (-1640 to -1287) were used in similar transient transfection assays as the full-length *bcl-2* promoter-luciferase construct However, induction of LMP-1 did not alter promoter activity from either of these constructs as well (result not shown) Based on these results it may be concluded that LMP-1 does not alter the activity of the -3934 to -8 region of *bcl-2* promoter However, complete validation of this result would require parallel demonstration of the functionality of the ectopically expressed LMP1 which was not performed in this study

### **3.2.11.7 LMP-1-induced upregulation of expression of Bcl-2 protein does not involve factors secreted into the medium**

*In vitro*, EBV-infected BL cells exhibiting a type III phenotype and LCLs induce the secretion of a number of factors into the growth medium including cytokines such as IL-1, IL-5, IL-6, IL-8, IL-10 and IL-12 which exert several phenotypic effects on the cell in an autocrine loop (reviewed in Kaye *et al* , 1999) LMP-1 expression alone can account for the secretion of several of these cytokines and some of them such as IL10 can induce Bcl-2 expression in other cell systems (Weber-Nordt *et al* , 1996, Nakagomi *et al* , 1994 Eliopoulos *et al* , 2000)

To investigate if factors secreted into medium upon induction of LMP1 in DG75-tTA-LMP-1 cells may be responsible for the delayed response in Bcl-2 upregulation, conditioned medium was collected from DG75-tTA-LMP-1, 96 h after induction of LMP-1 and used to culture DG75-tTA-LMP-1 cells in the presence of tetracycline Cells were harvested 24 and 48 h after growth in the conditioned medium and assessed for Bcl-2 expression by Western blot analysis with anti-Bcl-2 antibodies. It can be seen from

figure 3 61A that incubation with conditioned medium from the LMP-1 expressing cells did not result in an upregulation of Bcl-2 levels in the uninduced DG75-tTA-LMP-1 cells to the same extent as in the LMP-1 – induced cells. In a similar experimental strategy, neither did conditioned medium from Mutu III induce Bcl-2 upregulation in Mutu I cells (Figure 3 61B). These experiments exclude the involvement of secreted factors as intermediaries in the LMP-1- or EBV-induced upregulation of Bcl-2 in BL cells.



**Figure 3 61 LMP-1-mediated upregulation of Bcl-2 does not involve autocrine factors secreted in the medium** **A)** DG75-tTA-LMP-1 grown in the absence of tetracycline for 72 hours were transferred to fresh medium and growth continued for 24 h. The conditioned medium was rendered cell-free, filtered (0.45µm filter) and then used to culture DG75-tTA-LMP1 cells in the presence of tetracycline. After growth in conditioned medium for 24 or 48h the cells were harvested, total cellular protein extracted and used in Western blot analysis with either anti-LMP1 antibodies (upper panel) or anti-Bcl2 antibodies (lower panel). **B)** Mutu III cells in exponential phase of growth were grown in fresh medium for 24 h. Conditioned medium prepared as in (A) was used to culture Mutu I cells. After growth in conditioned medium for 24 or 48 h, the cells were processed as in (A) for Western blot analysis with anti-LMP1 antibodies (upper panel) or anti-Bcl2 antibodies (lower panel).

Thus, LMP-1 contributes to the upregulation of Bcl-2 expression in BL cells by mechanisms that appear to involve both transcriptional and translational control, with the effect on translation predominating. Furthermore, this effect does not appear involve an increase in promoter activity (at least in the region -3934 to -8 upstream of the translation start site) and is also independent of an indirect action by secreted factors.

## **CHAPTER 4**

### **DISCUSSION**

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## Discussion

One of the main features of the biology of EBV is its ability to persist for life in healthy infected individuals and this is most likely as a latent infection in the B cell compartment (reviewed by Gregory, 1995). *In vitro*, expression of the full complement of latent viral genes in EBV-infected B cells has been found to protect the cells from apoptosis and this ability of the virus provides a mechanism for its entry into the memory B cell compartment. Of the latent genes, expression of LMP1 in several cellular contexts including B cells has been shown to confer protection against apoptotic death by both Bcl-2-dependent and Bcl-2-independent mechanisms (Milner *et al* , 1992, Fries *et al* , 1996, Laherty *et al* , 1992, Wang *et al* , 1996). The main purpose of this study was to identify other possible Bcl2-independent mechanisms triggered by EBV latent gene expression in the B cell context and to then investigate if LMP1 can account for any of these mechanisms. The results presented in this study identify a relatively new member of the *bcl-2*-family of genes with anti-apoptotic properties, *bfl-1*, as being overexpressed in EBV-infected B cells expressing the entire complement of latent genes and that LMP1 is at least one of the latent proteins that can mediate this effect. The apoptotic threshold of a cell is determined by the functional balance in the expression of both pro- and anti-apoptotic proteins and hence the ability of LMP1 to upregulate the expression of several anti-apoptotic proteins would raise the apoptotic threshold of the EBV-infected, LMP-1 expressing cell.

### 4.1 BFL-1

The human *bfl-1* (Bcl-2-related gene expressed in fetal liver) gene was isolated from a human fetal liver cDNA library and identified by computer analysis of expressed sequenced tag (EST) databases (Choi *et al* , 1995). The open reading frame of *bfl-1* encodes a protein of 175 amino acids and among the Bcl-2 related genes, it has the highest homology with murine A1 (72% amino acid identity). Bfl-1 shares highest homology with many Bcl-2-related proteins in two regions, BH (Bcl-homology)-1 and BH2 that have been shown to be required for Bcl-2 function (Figure 3.1, Choi *et al* ,

1995) Currently available data suggest that *bfl-1* and its mouse homolog *Al* exhibit differences in expression in tissues in that while the expression of the murine gene is restricted to hematopoietic tissue, expression of the human homolog would appear to have a more widespread tissue distribution (Karsan *et al* , 1996a, Choi *et al* , 1995, Lin *et al* , 1996, Zong *et al* , 1999) Expression of the *bfl-1* gene has been shown to protect against apoptosis in a variety of cell types (D'Sa Eipper and Chinnadurai, 1996, Karsan *et al* , 1996b, Zong *et al* , 1999, Wang *et al* , 1999) Furthermore, *bfl-1* expression is upregulated in cultured endothelial and leukemic cells by phorbol ester and inflammatory cytokines TNF- $\alpha$  and IL-1, suggesting a protective role for Bfl-1 during inflammation (Karsan *et al* , 1996a, 1996b, Moreb and Schweder, 1997) *bfl-1* expression is also upregulated during differentiation of leukemic cells to granulocytes and macrophages (Moreb and Schweder, 1997) In addition to protecting against apoptosis, Bfl-1 has also been shown to exhibit proliferation and transforming properties *in vitro* (D'Sa Eipper and Chinnadurai, 1998) Functional dissection of the protein suggests that its anti-apoptotic and transforming properties may be linked and map to the BH1 and BH2 domains (D'Sa Eipper and Chinnadurai, 1998) Northern analysis has revealed elevated levels of expression of *bfl-1* in a significant proportion of stomach and colon cancers (Park *et al* , 1997, Choi *et al* , 1995), however, *in situ* hybridisation studies have indicated that in tumour tissue, expression is preferentially detected in infiltrating inflammatory cells rather than in cancer cells (Jung-Ha *et al* , 1998) This discrepancy may explain the low rate of expression of *bfl-1* observed in established stomach and colon cancer cell lines (Park *et al* , 1997) However, it appears that cell lines derived from leukemias and lymphomas exhibit high levels of expression of *bfl-1* (Choi *et al* , 1995, Kenny *et al* , 1997)

#### **4.2 FUNCTIONAL SIGNIFICANCE OF UPREGULATION OF *BFL-1* EXPRESSION BY LMP1**

Initial experiments using a range of EBV-infected cell lines revealed that elevated expression of *bfl-1* and *bcl-2* is a feature of both BL and LCL cell lines expressing the full spectrum of EBV latent genes. By comparison, EBV-negative BL lines and BL lines

with a latency I phenotype express negligible or significantly lower levels of these mRNA species, thereby suggesting that expression of the entire complement of EBV latent genes upregulates *bfl-1* and *bcl-2* mRNA levels in BL lines. Upregulation of *bfl-1* mRNA thereby represents a novel function of EBV latent gene expression in BL lines and extends the list of anti-apoptotic genes whose expression is regulated by EBV (Allday, 1996; Spender *et al.*, 1999). In support of the observations with the pattern of *bcl-2* mRNA expression, a strong correlation has been established between expression of EBV latent proteins and elevated levels of Bcl-2 protein in BL lines (Henderson *et al.*, 1991; Finke *et al.*, 1992). The contribution that EBV latent gene expression makes towards the high levels of *bfl-1* mRNA expression observed in LCLs is unknown since information on the status of expression of this mRNA species in human peripheral B lymphocytes (PBLs) prior to EBV infection is lacking in the literature. Thus, in the case of EBV-infected B lymphocytes, it is possible that if resting B cells express relatively low levels of *bfl-1* mRNA, then EBV latent gene expression induces the high level of expression of this mRNA in LCLs. Alternatively, if PBLs already exhibit high levels of *bfl-1* mRNA expression, then, EBV infection may serve to maintain the level of *bfl-1* mRNA in LCLs as has been observed in the case of Bcl-2 expression (Martin *et al.*, 1993).

Using a tetracycline-regulated system for induction of LMP-1 expression in the EBV-negative BL cell line, DG75-tTA-LMP1, it was possible to demonstrate that LMP-1 upregulates expression of *bfl-1* and *bcl-2* mRNAs. However, there was a clear difference in the kinetics of induction of expression of these anti-apoptotic genes by LMP-1, in that while induction of *bfl-1* mRNA appears to be an immediate and direct effect of LMP-1 expression, induction of *bcl-2* mRNA appears to be a delayed response (Figures 3.3, 3.4, 3.60). In this respect, the induction of *bfl-1* mRNA by LMP-1 is similar to the effect of this EBV protein on induction of *A20* (Laherty *et al.*, 1992).

*In situ* hybridisation studies have detected *bfl-1* transcript in the germinal centers of the lymphoid follicles in tonsil and lymph nodes (Jung-Ha *et al.*, 1998). The germinal centers are the sites for B-cell proliferation and differentiation (Liu *et al.*, 1991;1992; Gregory,

1995). After interaction with antigens presented by follicular dendritic cells, B cells with high-affinity antigen receptors are selected for survival and differentiate into plasma cells or memory cells, whereas B cells with low-affinity antigen receptors die by apoptosis. The mechanisms for B-cell survival during the selection process have not been fully elucidated yet. Candidate mechanisms include interactions between cell surface molecules CD40 and CD40 ligand, CD21 and CD23 and LFA-1 and ICAM1 among others (Tuscano *et al.*, 1996; Bonnefoy *et al.*, 1993; Koopman *et al.*, 1993). Although many of the candidate rescue signals can induce Bcl-2 expression in germinal center B cells, immunohistochemical studies have localised Bcl-2 protein predominantly in the mantle zone and not in the germinal center region where rescue occurs (Hockenberry *et al.*, 1991; Akagi *et al.*, 1994). Recent evidence suggests that Bcl-x<sub>L</sub> is involved in centrocyte survival as centroblasts in the germinal center treated with CD40 rapidly increase Bcl-x<sub>L</sub> levels and expression of Bcl-x<sub>L</sub> rescues B cells from programmed cell death (Tuscano *et al.*, 1996; Lee *et al.*, 1999). Moreover, higher levels of Bcl-x<sub>L</sub> protein are detected in the germinal center than in the mantle zone (Krajweski *et al.*, 1994). The similarity in the pattern of expression of *bcl-x* and *bfl-1* in germinal centers suggests that like Bcl-x, Bfl-1 may also be involved in B-cell survival and differentiation. Studies in mice have shown that positive selection of immature B cells from the bone marrow into the long-lived memory B cell pool involves resistance to apoptosis and an upregulation of *A1* mRNA expression (Tomayako and Cancro, 1998).

A specific association of EBV with memory B cells has been detected in peripheral blood of healthy carriers in which the virus maintains a latent infection (Miyashita *et al.*, 1997; Babcock *et al.*, 1999). However, the infection of naive cells resulting in blast formation does not occur in the periphery but in secondary lymphoid tissue and is based on the finding of EBV-infected lymphoblasts in tonsil (Babcock *et al.*, 1999; Joseph *et al.*, 2000). It is thought that these lymphoblasts represent newly infected naïve B cells and behave like normal B blasts and remain in secondary lymphoid tissue where they follow the normal pathways of B cell activation and differentiation to gain access to the long-lived memory compartment. Since survival signals form an important component of this selection process, the ability of EBV latent gene expression to upregulate expression of

anti-apoptotic genes including *bfl-1* levels allows the EBV-infected B cell to bypass/mimic the normal selection process and facilitate its entry into the memory B cell pool

The functional significance of Bfl-1 expression in BL cells was investigated by ectopic expression of the protein in an EBV-negative cell line exhibiting type I latency, Mutu I. These cells also exhibit several phenotypic features of germinal center B cells including expression of CD10 and CD77 and a low threshold of resistance to apoptosis (Gregory *et al* , 1991, Gregory, 1995). It was found that Bfl-1 expression not only protected against apoptotic death but also exerted a proliferative effect when the cells were grown under conditions of growth factor-deprivation. Indeed, Bfl-1 has been shown to possess both anti-apoptotic and proliferative activities (D'Sa-Eipper and Chinnadurai, 1998). Bfl-1 and its mouse homolog A1 can confer resistance to a variety of apoptosis-inducers in different cells. For instance, Bfl-1 expression has been shown to protect endothelial cells against TNF- $\alpha$ -induced cytotoxicity (Karsan *et al* , 1996b) and suppress chemotherapy-induced apoptosis in a fibrosarcoma cell line (Wang *et al* , 1999). Bfl-1 can also protect against p53-mediated apoptosis and exhibits potent oncogene-cooperation activity (D'Sa Eipper *et al* , 1996, D'Sa Eipper and Chinnadurai, 1998). Mouse A1 can prolong survival of a myeloid cell line grown under conditions of growth factor deprivation (Lin *et al* , 1996). Also, neutrophils of *A1*-deficient mice exhibit increased spontaneous apoptosis relative to those from wild-type mice (Hamasaki *et al* , 1998). Also, mouse A1 can protect normal B cells from antigen-receptor ligation induced apoptosis (Grumont *et al* , 1999).

The ability of LMP-1 to upregulate the expression of several anti-apoptotic proteins is significant from the point of view that there isn't always a complete overlap in the range of apoptotic stimuli to which different anti-apoptotic proteins can respond. For instance, although Bcl-2 and A20 can independently protect cells against a number of apoptotic stimuli, Bcl-2 but not A20 is effective against glucocorticoid-induced apoptosis (reviewed in Opipari *et al* , 1992). Thus, by upregulating the levels of several anti-apoptotic proteins, LMP-1 expression can serve as one route by which the EBV-infected

host cell can protect itself against several apoptotic stimuli. The extent to which Bfl-1 and other anti-apoptotic proteins such as Bcl-2 overlap with regard to the range of apoptotic stimuli against which they protect has not been fully established.

Although the low level of DNA fragmentation observed in the Mutu I transfectant pool (Figure 3.9) could be due to the possible heterogeneity in expression of Bfl-1 between cells in the pool, it may also be that the functions of other anti-apoptotic proteins are required to confer greater protection against this apoptotic stimulus. In support of the latter interpretation, two other LMP-1-inducible anti-apoptotic proteins, A20 and Bcl-2, have been shown to independently protect against serum-deprivation-induced apoptotic death of BL cells (Laherty *et al* , 1992, Henderson *et al* , 1991). In this respect, it will be interesting in future experiments to examine extent of the protective effect of expression of A20 and Bcl-2 individually and in combination with Bfl-1 in Mutu I cells.

#### **4.3 UPREGULATION OF *BFL-1* mRNA BY LMP-1 APPEARS TO BE A B CELL EFFECT**

The absence of an effect of LMP-1 induction on *bfl-1* mRNA expression in the T cell line, Jurkat-tTA-LMP-1, and in the epithelial cell line C33A makes it tempting to speculate that LMP-1-mediated upregulation of *bfl-1* mRNA expression is a B-cell specific effect. Indeed LMP1 has been shown to exhibit cell-type specific effects in gene/protein expression. For instance, LMP-1-mediated upregulation of Bcl-2 appears to be a B-cell specific effect (Rowe *et al* , 1994). T lymphocytes and epithelial cells do exhibit considerable levels of Bcl-2 expression constitutively and so it is thought that LMP-1-mediated upregulation of Bcl-2 may be more relevant in a cellular context that is Bcl-2-negative or that has low levels of Bcl-2 expression as exists in several EBV-negative BL lines. However, the Jurkat tTALMP1 cell line and C33A epithelial cell line do not express detectable levels of *bfl-1* mRNA constitutively and hence an LMP-1-mediated upregulation of Bfl-1 would have been expected. Furthermore, expression of LMP-1 in both of these cellular contexts has been shown by others to bring about changes in gene/protein expression including activation of NF- $\kappa$ B and upregulation of

expression of A20 (Rowe *et al.*, 1994; Huen *et al.*, 1995; Floettmann *et al.*, 1998; Miller *et al.*, 1997; Devergne *et al.*, 1998). It is possible that since these cell lines were themselves derived as a result of clonal selection, they may have defects in signaling components required for upregulation of *bfl-1* mRNA. Nevertheless, it would be important in future experiments to test the functionality of LMP-1 expressed in these cell lines using parameters such as NF- $\kappa$ B activation and upregulation of markers that have been shown to be modulated by LMP-1 in these cellular contexts.

#### 4.4 UPREGULATION OF *BFL-1* mRNA STABILITY BY LMP1

The steady state level of a particular mRNA species in a cell is dependent on both the rates of transcription and decay. Thus, a coordinated balance between transcriptional and post-transcriptional events is required. Investigations into the mechanism of upregulation of *bfl-1* mRNA levels by LMP-1 revealed the importance of a role for both mRNA stabilisation and increased promoter activity. Several lines of evidence indicate that *bfl-1* and its mouse homolog *A1* are early-response genes i.e the expression of these genes is induced rapidly. For example, granulocyte-macrophage colony stimulating factor (GM-CSF) and lipopolysaccharide induce *A1* mRNA in mouse macrophages within 1 h of stimulation (Lin *et al.*, 1993). Lipopolysaccharide can induce *bfl-1* mRNA in a microvascular endothelial cell line within 3 h of stimulation (Hu *et al.*, 1998). In DG75tALMP-1 cells, the onset of upregulation of *bfl-1* mRNA was observed in parallel with the detection of LMP-1 expression (figure 3.3), suggesting that upregulation of *bfl-1* by LMP-1 is a rapid response.

The induction of expression of many early-response genes such as *c-myc* and *c-fos* is controlled at the level of mRNA stability (Chen and Shyu, 1995). Regulation of mRNA stability is so far poorly understood. However, it is known that *cis*-acting elements within the mRNA molecule can be recognised by regulatory proteins, and *cis*-elements have been demonstrated to modulate mRNA stability both positively and negatively. Numerous mechanisms exist to degrade mRNA effectively (reviewed in Ross, 1995; Beelman and Parker, 1995). For example, deadenylation triggers decapping, thus

exposing the mRNA to 5' to 3' degradation. Deadenylation can also lead to 3' to 5' degradation of mRNA independently of decapping. Alternatively, decay may be initiated independently of deadenylation by sequence-specific cleavage of the mRNA. The 5' and 3' untranslated regions (UTR) of eukaryotic mRNAs have often been experimentally demonstrated to contain sequence elements crucial for regulating the stability of the transcript (reviewed in Pesole *et al*, 1999). In particular, adenylate/uridylylate-rich elements (AREs) found in the 3' UTR of many labile mRNAs (e.g. those encoding GM-CSF, c-fos and IL-3) represent a common determinant of RNA stability in mammalian cells (Chen and Shyu, 1995). AREs range in size from 50 to 100 nucleotides and contain multiple copies of the pentanucleotide AUUUA. An examination of the nucleotide sequence of the 3' UTR of the human *bfl-1* gene failed to reveal the presence of an AUUUA motif and therefore excludes this element as a target for control of mRNA stability.

#### **4.5 UPREGULATION OF *BFL-1* PROMOTER ACTIVITY BY LMP-1**

As described in the results section of this thesis, in addition to increasing *bfl-1* mRNA stability, LMP-1 can also upregulate *bfl-1* promoter activity and that too in an NF- $\kappa$ B-dependent manner. The NF- $\kappa$ B-dependence of induction of *bfl-1* expression is in keeping with the findings of several published works. For example, whereas cell lines exhibiting normal NF- $\kappa$ B nuclear function respond to treatment with lipopolysaccharide, TNF- $\alpha$  or etoposide with upregulation of *bfl-1* mRNA, their NF- $\kappa$ B-defective counterparts (i.e. expressing a modified form of the NF- $\kappa$ B inhibitor, I $\kappa$ B $\alpha$ , that cannot be phosphorylated) are non-responsive (Hu *et al*, 1998, Zong *et al*, 1999, Wang *et al*, 1999). Also, ectopic expression of the NF- $\kappa$ B subunit proteins c-rel and p65 but not p50 can independently upregulate *bfl-1* mRNA expression in HeLa cells (Zong *et al*, 1999). In the case of TNF- $\alpha$ , induction of *bfl-1* mRNA expression was demonstrated to be due to an increase in promoter activity (Zong *et al*, 1999). Furthermore, the same study demonstrated that ectopic expression of the NF- $\kappa$ B subunit protein c-rel also led to induction of *bfl-1* promoter activity and this effect was shown to be mediated by binding

to an NF- $\kappa$ B site at position -833 of the promoter. Interestingly, expression of mouse A1 is induced in response to mitogen stimulation of primary B cells in a c-rel-dependent fashion (Grumont *et al.*, 1999).

The LMP-1-mediated effect on the induction of activity of -1374/+81 region of the *bfl-1* gene could be demonstrated in both EBV-negative BL cell lines (DG75 and BL41) and Jurkat T cells. The importance of NF- $\kappa$ B in this process was conclusively demonstrated based on several lines of evidence. Firstly, coexpression of dominant negative I $\kappa$ B $\alpha$ , inhibited LMP-1-mediated induction of *bfl-1* promoter activity which paralleled its inhibitory effect on LMP-1-mediated activation of NF- $\kappa$ B. Secondly, inhibition of TRAF2-mediated activation of NF- $\kappa$ B by LMP-1 by coexpression of a dominant-negative TRAF2 molecule or A20 expression also led to a parallel inhibition of the effect of LMP-1 on the activity of the *bfl-1* promoter. Thirdly, expression of the NF- $\kappa$ B subunit protein p65 could also moderately transactivate the *bfl-1* promoter. Fourthly, treatment with PMA, a well-known activator of NF- $\kappa$ B in many cell types, also stimulated *bfl-1* promoter activity.

In DG75 cells, titration of the amount of LMP-1 expression plasmid, pEFCXLMP-1, cotransfected with a fixed amount of the *bfl-1* promoter-luciferase construct, p-1374/+81 Bfl-1luc, revealed a dose-responsive effect on luciferase synthesis when up to 2.5  $\mu$ g of the expression plasmid was used. Usage of a higher amount (5.0  $\mu$ g) of pEFCXLMP-1 in the cotransfection studies did not induce a further increase in luciferase activity even though a higher amount of LMP-1 was expressed. A similar effect was also observed in titration experiments of pEFCXLMP-1 and an NF- $\kappa$ B-luciferase reporter. Such a saturation effect in promoter activity when high amounts of LMP-1-expression plasmid are used in transfection studies has been observed in the case of activation of the ICAM-1 promoter by LMP-1 (Mehl *et al.*, 2001) and may be a common phenomenon with many effector proteins and promoters. Nevertheless, the similar trend in responsiveness of the NF- $\kappa$ B-luc and -1374/+81Bfl-1-luc reporter constructs to expression of increasing amounts of LMP-1 protein in DG75 cells lends some support to the importance of NF- $\kappa$ B in activation of the *bfl-1* promoter.

As mentioned in the introduction section of this thesis, mutational analysis has identified two functional domains in the cytoplasmic carboxy terminus of LMP-1 CTAR1/TES1 (residues 187-231) and CTAR2/TES2 (residues 351-386) (Figure 3 18) A third activation domain, CTAR3, mapping to a region between CTAR1 and CTAR3 (residues 275-307) has been shown to mediate activation of Jak3 (Gires *et al* , 1999) Several studies have indicated that CTAR1 and CTAR2 can independently mediate activation of NF- $\kappa$ B by LMP-1, however, the extent of contribution of each of these domains to the total activation of NF- $\kappa$ B varies between cell lines (Floettmann and Rowe, 1997, Huen *et al* , 1995, Mitchell and Sugden, 1995) However, in general, CTAR2 has been found to be a stronger activator of NF- $\kappa$ B than CTAR1 The NF- $\kappa$ B-dependent upregulation of A20 expression by LMP1 maps to both the CTAR1 and CTAR2 domains and the contribution of each domain in this effect correlates with the relative extents of activation of NF- $\kappa$ B by the two domains (Miller *et al* , 1995, 1997) Usage of mutants (point mutants) of LMP-1 shown to be defective in signaling from either CTAR1 (LMP-AAA) or CTAR2 (LMP-G) or both (LMP-AAAG) revealed that, in DG75 cells CTAR2 makes a greater contribution to activation of the *bfl-1* promoter than CTAR1 and that the mutant defective in signalling from both of these domains is unable to transactivate the promoter Also, expression of these mutants resulted in the expected responses in NF- $\kappa$ B activation in that CTAR2 is the predominant contributor in this effect Based on the findings of Gires *et al* (1999), the result obtained with the double mutant would at first exclude the involvement of CTAR3 in LMP-1 mediated activation of *bfl-1* However, the results of Gires *et al* , have recently been cast in a new light with the demonstration of the dominant negative action of LMP-AAAG In coexpression studies, LMP-AAAG which contains an intact CTAR3 domain, can not only inhibit activation of NF- $\kappa$ B but also the activation of JAK3 by wild-type LMP-1 (Brennan *et al* , 2001) These observations have been interpreted to mean that there must be some cooperation between CTAR1 or CTAR2 and CTAR3, for stimulating the activity of JAK3 Thus, even though LMP AAAG is unable to activate the *bfl-1* promoter, this result does not necessarily exclude the potential involvement of JAK3 function in activation of *bfl-1* by LMP-1

In Jurkat cells, expression of LMP-1 resulted in a high level of NF- $\kappa$ B activation as revealed by luciferase assays and the extent of this effect was much higher than that in DG75 cells (Figures 3 20, 3 26 and 3 28) The difference in the extents of LMP-1-mediated NF- $\kappa$ B activation can be explained by the differences in the basal level of NF- $\kappa$ B activity between the cell lines, in that a lower basal level of NF- $\kappa$ B activation would be expected to be elevated by a greater magnitude in response to a stimulus (Floettmann *et al* , 1998) Thus, Jurkat cells which have a very low basal level of NF- $\kappa$ B activation compared to many EBV-negative BL lines, may permit a greater extent of activation of this transcription factor in response to an appropriate stimulus (Floettmann *et al* , 1998) DG75 (and BL41) exhibit higher constitutive levels of NF- $\kappa$ B activation which can only be further increased to a limited extent (Huen *et al* , 1995, Rowe *et al* , 1994) Despite the requirement for NF- $\kappa$ B in LMP-1-mediated activation of the -1374/+81 region of *bfl-1* in both Jurkat and DG75 cells, the greater extent of activation of NF- $\kappa$ B by LMP-1 in Jurkat cells relative to DG75 cells did not result in a greater extent of activation of this promoter region The basis of this difference became evident when the effect of LMP1 expression on luciferase synthesis from luciferase reporter constructs containing progressive 5' deletions of the *bfl-1* promoter in cotransfection studies was performed This analysis revealed that whereas elements within the -129/+81 region of the *bfl-1* gene were responsible for majority of the LMP-1-induced transactivation of the 'full-length' (-1374/+81) promoter region, elements located within the -1240/-367 region were more important in mediating this effect of LMP-1 in Jurkat T cells (Figure 3 36) It therefore seems likely that the differential extents of activation is related to the targeting of different transcription factor binding sites by LMP1 in the two cell types Furthermore, the greater importance of elements within the proximal region of the promoter in mediating transactivation of *bfl-1* by LMP-1 was not specific to DG75 cells in that this phenomenon was also observed when the experiment was performed in the context of another EBV negative BL line, BL41 It is thus tempting to generalise that at least in the context of BL lines, LMP-1-mediated activation of the *bfl-1* promoter involves elements residing in the proximal region of the promoter

Consistent with previous reports that the extent of CTAR1 and CTAR2-mediated signalling in LMP-1 mutants can vary with the cellular background (Huen *et al* , 1995, Floettmann *et al* , 1998), it was found that Jurkat is defective for CTAR1-mediated activation of NF- $\kappa$ B and the *bfl-1* promoter based on the studies with the LMP-1 mutant LMP G (Figure 3 20) Expression of LMP G in Jurkat T cells resulted in retention of approximately 5% of *bfl-1* promoter activity and NF- $\kappa$ B activation by wild-type LMP1 and in DG75 cells this value was approximately 25% Although this result on its own would predict a sole role for CTAR2 in mediating the effects of LMP-1 on activation of NF- $\kappa$ B and the *bfl-1* promoter in Jurkat cells, this did not appear to totally be the case since experiments with the LMP AAA mutant revealed that the function of CTAR2 with respect to these parameters was still partially impaired (retention of approximately 40% and 30% of wild-type LMP1 transactivation of the *bfl-1* promoter and NF- $\kappa$ B activation), albeit to a lesser extent than CTAR1 in these cells These results can be explained by the recent findings that functional activation of LMP-1 requires oligomerisation in the absence of ligand binding and also requires cooperation between CTAR1 and CTAR2 within a heterooligomeric LMP-1 complex (Floettmann *et al* , 1998, Floettmann and Rowe, 1997, Gires *et al* , 1997) Furthermore, this cooperation between CTAR1 and CTAR2 results in a signalling event that can be qualitatively distinct from the signals generated by CTAR1 and CTAR2 independently (Floettmann *et al* , 1998) The finding that the activation of NF- $\kappa$ B and induction of *bfl-1* promoter activity by LMP-1 in Jurkat cells was not necessarily the additive effect of independent signals from CTAR1 and CTAR2 is in keeping with the necessity for cooperation between these two domains for a fully functional signal to be generated by LMP-1

Expression of the LMP-1 mutant, LMP AAAG, that is defective in signalling from both CTAR1 and 2 was nonfunctional with respect to activation of not only NF- $\kappa$ B but also the *bfl-1* promoter in both DG75 and Jurkat cells These findings suggest the crucial involvement of signalling from both of these domains in these LMP1-mediated effects In fact, the LMP AAAG mutant has recently been demonstrated to function as a dominant negative mutant of LMP1 and was shown to efficiently inhibit wild-type LMP-1 activation of NF- $\kappa$ B, JAK3 and Jun transcriptional activity (Brennan *et al* , 2001) In

keeping with these findings it was possible to demonstrate in cotransfection experiments that LMP AAAG can efficiently inhibit wild-type LMP-1-mediated activation of not only NF- $\kappa$ B but also of the *bfl-1* promoter in DG75 cells (Figure 3 21) This mutant functions as a dominant negative by interacting with wild-type LMP-1 and interfering with its ability to bind TRAF2 (Brennan *et al* , 2001) The requirement of cooperation between CTAR1 and CTAR2 present on different LMP1 molecules within the same hetero-oligomeric complex forms the basis of the action of LMP AAAG The high efficiency with which LMPAAAG was found to inhibit LMP1-mediated signaling might be related to the possibility that only one LMPAAAG molecule within the oligomeric complex (at least trimeric) may be required to alter signaling from the complex (Floettmann and Rowe, 1997, Floettmann *et al* , 1998)

The existence of a cooperative function between CTAR1 and CTAR2 of LMP1 cautions against over-interpretation of data obtained with mutants non-functional for CTAR1 or CTAR2, but particularly with mutants containing large deletions that may adversely affect physical cooperation between CTAR1 and CTAR2 In agreement with this, there is evidence that differences in signalling from individual CTAR1/2 domains and full length LMP1 might result from changes in the components of the TRAF complexes bound to LMP1 Thus, it has been found that aminoacid residues 187-386 of LMP1 bind less TRAF1 and 2 than deletion mutants encoding aminoacids 187-231 only (Devergne *et al* , 1996, Sandberg *et al* , 1997)

Much insight into the early events in signalling from LMP1 has been obtained over the last 6 years with the discovery that LMP-1 engages signalling proteins for TNFR family including TRAFs and TRADD (Mosialos *et al* , 1995, Devergne *et al* , 1996, Brodeur *et al* , 1997, Sandberg *et al* , 1997, Izumi *et al* , 1997, Eliopoulos *et al* , 1999) TRAF2 has been shown to play a key role in mediating activation of NF- $\kappa$ B by LMP-1 Whereas TRAF2 has been shown to bind directly to CTAR1, it requires TRADD as a bridging protein to bind to CTAR2 and dominant negative TRAF2 mutants have been shown to inhibit NF- $\kappa$ B activation from both CTAR1 and CTAR2 (Devergne *et al* , 1996, Kaye *et al* , 1996, Izumi and Kieff, 1997; Sylla *et al* , 1998) In this thesis, the LMP-1 mediated

transactivation of the *bfl-1* promoter in both DG75 and Jurkat cells was shown to involve a TRAF2-dependent mechanism and was demonstrated by usage of a TRAF2 dominant negative mutant, TRAF2 $\Delta$ (6-86) (Figures 3 22 and 3 24), and the TRAF2-binding zinc-finger protein, A20 (Figures 3 23 and 3 25), in independent reporter assays. However, of the two, A20 appeared to be more efficient at inhibiting both the activation of the *bfl-1* promoter and NF- $\kappa$ B by LMP-1. Similar findings with these TRAF2-inhibitors have been reported in literature by others working on LMP-1 and TNF- $\alpha$  (Kaye *et al* , 1996, Hsu *et al* , 1996, Reinhard *et al* , 1997, Ehopoulos *et al* , 1997, 1999). In the case of LMP-1, this TRAF2 mutant has been shown by others to only partially block activation of NF- $\kappa$ B by CTAR2 (40%) whilst almost completely blocking that mediated by CTAR1 (>75%) (Kaye *et al* , 1996). The data on the effect of expression of the TRAF2 $\Delta$  (6-86) mutant on LMP-1 mediated activation of *bfl-1*, may be interpreted to mean that although TRAF2 is a component of the signaling pathway involved in the activation of *bfl-1* by LMP-1, additional contributions from other CTAR1/2-associated proteins may also be involved such as other TRAFs (Devergne *et al* , 1996). In this regard, it is also interesting to note that the receptor interacting protein (RIP) has also been shown to interact directly with CTAR2 but does not mediate activation of NF- $\kappa$ B and is therefore not a likely candidate (Izumi *et al* , 1999). Alternatively, it is possible that TRAF2 is bound in a stable complex with other proteins and that large amounts or prolonged incubations following transfection are required for TRAF2 $\Delta$ (6-86) to displace endogenous wild-type TRAF2. Indeed a number of TRAF2-interacting proteins have been identified, such as TRAF1, TANK/I-TRAF and cellular inhibitors of apoptosis (c-IAPs), which may influence TRAF2 heterocomplex stability and signaling (reviewed in Eliopoulos *et al* , 1999). Consistent with this possibility is the observation that while CTAR1 can directly bind TRAF2, CTAR2 binds TRAF2 indirectly via TRADD, and hence the differences in the stoichiometry of binding of TRAF2 to these two domains of LMP1 may account for the inefficiency of TRAF2 $\Delta$ (6-86) to inhibit TRAF2-mediated signaling from the predominant signaling domain, CTAR2, relative to CTAR1. The A20 protein, a zinc finger protein, that is itself inducible by LMP-1 in an NF- $\kappa$ B-dependent manner, can inhibit both LMP1-induced NF- $\kappa$ B and JNK activation thereby acting in a negative feedback loop to control LMP-1 signalling. Such control over the extent of LMP-1

signaling may be important in that overexpression of LMP1 can have toxic effects on the cell (Hammerschmidt *et al* , 1989, Martin *et al* , 1993, Fries *et al* , 1999, Eliopoulos *et al* , 1999) Although this activity of A20 was initially presumed to depend on its interaction with only TRAF2 (Eliopoulos *et al* , 1999), more detailed studies suggest that A20 can function as a promiscuous inhibitor of the activities of other TRAFs and TRADD (Fries *et al* , 1999) In this context the data presented in this thesis demonstrating that A20 expression suppresses both LMP-1-induced NF- $\kappa$ B and *bfl-1* promoter activity while dominant-negative TRAF2 has only a partial effect may indicate an additional role for other LMP-1-interacting signaling molecules (such as other TRAFs or TRADD) in mediating activation of the *bfl-1* promoter

LMP-1 has also been demonstrated to activate the p38 mitogen-activated protein kinase (MAPK) pathway and both CTAR1 and CTAR2 have been demonstrated to mediate this effect in a TRAF-2-dependent mechanism with CTAR2 playing the predominant role (Eliopoulos *et al* , 1999b) Activation of p38MAPK has been shown to be involved in the coregulation of IL6 and IL8 production by LMP1 (Eliopoulos *et al* , 1999b) The downstream targets of activated p38 MAPK include a number of transcription factors including ATF2 (reviewed in Nebreda and Porras, 2000) ATF2 can homodimerise but can also form heterodimers with Jun and bind to AP1 motifs in the promoters of several genes Indeed transactivation of the IL8 promoter by LMP1 was shown to require p38 activation and LMP-1 induced binding of ATF2-Jun dimers to an AP1 motif in the promoter was also demonstrated (Eliopoulos *et al* , 2000) Given these findings and also the findings in this thesis that the pattern of the extent of contribution of CTAR1 and CTAR2 to activation of the *bfl-1* promoter is similar to that reported for p38 activation, it seemed reasonable to hypothesise that the activation of p38 may also contribute to the activation of the *bfl-1* promoter by LMP-1 This is further strengthened by the existence of potential AP-1-binding sites in the *bfl-1* promoter region However, in a preliminary experiment, a well-established and specific inhibitor of p38 activation, SB203580, did not affect transactivation of the *bfl-1* promoter by LMP-1 in DG75 cells (result not shown) The ability of LMP1 to activate p38 and the functionality of the inhibitor were not established in this experiment, therefore questioning the validity of this result The former

can be easily tested by performing *in vitro* kinase assays which would involve testing the ability of LMP-1 to phosphorylate a downstream substrate (such as ATF2) of p38 MAPK. Furthermore, it should be possible to also monitor the functionality of the inhibitor by measuring its effect on LMP-1-mediated upregulation of IL6 and IL8 production by ELISA. Several of the other members of the TNFR family including CD40, TNFR and IL-1 can also activate p38 and activated p38 isoforms have been shown to play essential roles in not only development, differentiation, proliferation but also cell survival (reviewed in Nebreda and Porras, 2000). An important role for p38 kinases in post-transcriptional regulation of gene expression has been demonstrated (Lee and Young, 1999, Roulston *et al* , 1998). Hence, a role for activation of p38 in mediating induction of *bfl-1* expression by LMP1 at the post-transcriptional level cannot be excluded.

The importance of NF- $\kappa$ B activation in mediating the transactivation of the *bfl-1* promoter by LMP-1 was conclusively demonstrated by using a dominant negative I $\kappa$ B $\alpha$  mutant that contains mutations in two critical residues (serine 32 and serine 36) that are required for phosphorylation and subsequent proteolysis of the molecule (Liljeholm *et al* , 1998). Phosphorylation of I $\kappa$ B $\alpha$  on these residues is required for release of NF- $\kappa$ B dimers from NF- $\kappa$ B-I $\kappa$ B $\alpha$  complexes, thus allowing the dimers to translocate to the nucleus and activate transcription. Binding of NF- $\kappa$ B dimers to the non-phosphorylatable I $\kappa$ B $\alpha$  mutant thus prevents their activation. The I $\kappa$ B $\alpha$  dominant negative mutant therefore targets a more downstream event in an NF- $\kappa$ B signalling pathway and is hence a useful tool in examining NF- $\kappa$ B-dependence of gene expression. Indeed this mutant inhibited both LMP-1-induced activation of NF $\kappa$ B and the *bfl-1* promoter in DG75, BL41 and Jurkat T cells (Figures 3 26, 3 27 and 3 28). However, expression of higher amounts of this I $\kappa$ B $\alpha$  mutant were required to achieve equivalent extents of inhibition of these LMP-1 mediated effects in DG75 cells compared to the other two cell lines. This may be related to the higher basal levels of NF- $\kappa$ B activation known to be present in the DG75 cell line (Huen *et al* , 1995, Rowe *et al* , 1994).

The NF- $\kappa$ B-responsiveness of the *bfl-1* promoter was also confirmed by demonstrating that PMA treatment or ectopic expression of the NF- $\kappa$ B subunit protein p65 could transactivate the *bfl-1* promoter. PMA is a well known chemical activator of NF- $\kappa$ B (reviewed in Wilkinson and Hallam, 1994) and in both DG75 and Jurkat cells stimulation of the *bfl-1* promoter by PMA was inhibited by expression of the dominant negative mutant I $\kappa$ B $\alpha$  $\Delta$ N, suggesting that in both of these cell types PMA-mediated activation of the *bfl-1* promoter is NF- $\kappa$ B-dependent (Figures 3.31 and 3.41). PMA has been shown to upregulate *bfl-1* mRNA expression in several leukemic cell lines (Moreb and Schweder, 1997). Activation of Jurkat T cells by treatment with PMA and ionomycin has been shown by others to result in an upregulation of steady-state *bfl-1* mRNA levels (Zong *et al*, 1999) and results presented in this thesis demonstrate that transactivation of the promoter via an NF- $\kappa$ B-dependent mechanism contributes to this effect (Figure 3.41). While ionomycin, a calcium ionophore that stimulates the calcium-dependent component of lymphocyte activation, caused only minimal activation of the *bfl-1* promoter on its own, it exerted a synergistic effect with PMA on *bfl-1* transactivation. Such a synergistic effect of PMA and ionomycin on promoter activation has been demonstrated in the case of the NF- $\kappa$ B-inducible A20 promoter (Laherty *et al*, 1993). However, NF- $\kappa$ B activation may not necessarily be the only transcription factor whose activation by PMA leads to transactivation of *bfl-1* since this chemical agent can also activate several other transcription factors including AP-1 and AP-1 responsive elements have been identified in the *bfl-1* promoter. With respect to the possibility that NF- $\kappa$ B-independent mechanisms may also be involved in PMA (or PMA/ionomycin)-mediated activation of the *bfl-1* promoter it is relevant to note that although LMP-1 appears to be a stronger inducer of NF- $\kappa$ B activity than PMA (or PMA/ionomycin) in Jurkat cells, PMA (and PMA/ionomycin) exerted a much greater effect on *bfl-1* promoter transactivation than LMP-1. However, this result may also be explained by the possible difference in the subunit composition of NF- $\kappa$ B activated by the two different effectors/stimuli.

One of the biochemical consequences of PMA treatment is activation of the protein kinase C (PKC) pathway. PKC is a widespread family of kinases responsible for many

diverse and critical cellular functions including aspects of cellular growth and metabolism (reviewed in Wilkinson and Hallam, 1994). One of the substrates of activated PKC is I $\kappa$ B $\alpha$  and phosphorylation of I $\kappa$ B $\alpha$  by PKC thus releases NF $\kappa$ B which can then translocate to the nucleus and activate transcription. Many of the PKC isoforms can be activated by a calcium-dependent mechanism (reviewed in Wilkinson and Hallam, 1994) and since ectopic expression of LMP-1 in BL cells coincides with increased intracellular calcium levels (Wang *et al* , 1988), it is tempting to speculate that PKC may be a downstream target of LMP1. In this respect it is interesting to note that PMA has been shown to upregulate *bcl-2* expression in EBV-negative BL lines (Genestier *et al* , 1995), so an intact PKC-pathway is potentially available in BL cells. However, to date there is no evidence that LMP-1 activates PKC.

More direct confirmation of the NF- $\kappa$ B-responsiveness of the *bfl-1* promoter was obtained with the demonstration that ectopic expression of p65 transactivated the *bfl-1* promoter in DG75 cells (Figures 3.33, 3.39). However, the extent of transactivation of the *bfl-1* promoter or even the control NF- $\kappa$ B-reporter construct (3 x enh $\kappa$ B luc) by p65 was much lower than that obtained when LMP-1 was used as the effector. It is possible that much higher levels of p65 expression are required to obtain equivalent levels of transactivation as LMP-1. Studies have also indicated that coexpression of two different NF- $\kappa$ B subunit proteins (such as p65 and p50) can have a greater than additive effect on transactivation of NF- $\kappa$ B-responsive elements than expression of the individual proteins (Perkins *et al* , 1992, Laherty *et al* , 1993) so it is possible that cotransfection of p65 and a heterodimeric partner such as p50 may activate the *bfl-1* promoter and the control NF- $\kappa$ B-reporter construct to a greater extent than that achieved with expression of p65 alone and this possibility needs to be tested. The control NF- $\kappa$ B-luc construct contains three copies of the  $\kappa$ B-responsive element in the Ig  $\kappa$  light chain enhancer region. This element has been shown to have a greater affinity for p65/p50 heterodimers than p65 homodimers and since the specificity and extent of NF- $\kappa$ B-mediated transcriptional activation is determined by the combinatorial associations of the subunits and the sequence of the  $\kappa$ B site, it would be expected that p65/p50 dimers would result in a higher level of transactivation of this NF- $\kappa$ B-responsive element than p65 homodimers.

(Perkins *et al* , 1992) p65/p50 but not p65 homodimers appear to be one of the dominant NF- $\kappa$ B proteins activated by LMP-1 in EBV-negative BL lines (Herrero *et al* , 1995) and this may also account for the observed differences in extents of activation of the control NF- $\kappa$ B-luc reporter construct between LMP-1 and p65 (assuming that transfection of p65 results in a predominance of p65 homodimers in the cell) It would be interesting in future experiments to analyse the effect of expression of other NF- $\kappa$ B subunit proteins and dual combinations thereof on activation of the *bfl-1* promoter

The demonstration of the importance of NF- $\kappa$ B in transactivation of the *bfl-1* promoter prompted a search for potential NF- $\kappa$ B binding sites in the promoter region of *bfl-1* Using the TESS program, eight potential NF- $\kappa$ B-like sites were identified One of these sites, which has the sequence GGGGATTTACC is located at bp –833 to –823 of the *bfl-1* promoter region (Figure 3 29) (Zong *et al* , 1999) However, inactivation of this site by mutation (Zong *et al* , 1999) did not affect activation of the promoter in either DG75 or Jurkat cells (Figure 3 30) This site has been shown to mediate c-rel-mediated transactivation of the *bfl-1* promoter in the HeLa epithelial cell context and although LMP-1 activates different subsets of NF- $\kappa$ B proteins in a cell-type dependent manner (Paine *et al* , 1995, Herrero *et al* , 1995, Chien and Hammarskjold, 2000), c-rel has been shown by others to be activated by LMP-1 in DG75 and Jurkat T cells (Liljeholm *et al* , 1998, Chien and Hammarskjold, 2000) This would argue against the lack of involvement of the NF- $\kappa$ B site at –833 of the *bfl-1* promoter in LMP-1-mediated transactivation being a result of the lack of LMP-1-induced activation of c-rel in the lymphocyte cellular context Furthermore, the NF- $\kappa$ B site at position –833 was also non-responsive in PMA-or p65-mediated activation of the promoter in DG75 cells (Figures 3 32 and 3 33), thereby indicating that the lack of involvement of this site in transactivation of the *bfl-1* promoter is not restricted to LMP-1 These findings would suggest that the lack of involvement of the NF- $\kappa$ B site at –833 in transactivation of *bfl-1* may be a lymphocyte-specific effect irrespective of the stimulus used for transactivation It is possible that access to this site may be obstructed by lymphocyte-specific nuclear proteins binding in the vicinity of this site In this respect it would be interesting to

examine if LMP-1 can activate *bfl-1* promoter activity in an epithelial cell context such as HeLa and test the responsiveness of this NF- $\kappa$ B site.

Deletion analysis revealed that LMP-1 exerts differential effects on the regions of the *bfl-1* promoter targeted for mediating transactivation in BL lines and Jurkat cells. Thus, whereas the -129/+81 region of the *bfl-1* gene accounted for the majority (64-69%) of the transactivation of the full-length promoter by LMP-1 in DG75 and BL41 cells, in Jurkat cells this region accounted for only 23% of the LMP-1-induced transactivation of the 'full-length' promoter and elements within the -1240/-367 region appeared to be targeted by LMP-1 to a greater extent in this cell line (Figure 3.36). However, even though the proximal region on its own mediates a minor fraction of the transactivation of the *bfl-1* promoter by LMP-1 in Jurkat cells, LMP-1-activated nuclear factors binding to elements within the -1240/-367 region would require interaction with proteins (at least with the basal transcription apparatus) binding to the proximal region to effectively and optimally transactivate the promoter. In DG75 cells, transactivation of the -129/+81 region of *bfl-1* was conclusively demonstrated to be NF- $\kappa$ B-dependent and involved a TRAF2-dependent mechanism. Although the mechanism underlying the low level of activation of the -129/+81 region of *bfl-1* by LMP-1 in Jurkat cells was not formally investigated, it was possible to speculate on a role for NF- $\kappa$ B based on the results obtained in experiments with PMA and PMA/ionomycin. PMA or PMA/ionomycin treatment of Jurkat cells could activate the -129/+81 region of *bfl-1* albeit to a lesser extent than the 'full-length' promoter region but more effectively than when LMP-1 was used as the stimulus. Furthermore, PMA or PMA/ionomycin-mediated activation of the -129/+81 region of *bfl-1* could be demonstrated to be NF- $\kappa$ B-dependent. Therefore, this region is potentially NF- $\kappa$ B-responsive in Jurkat cells. It is possible then that the differences in the extents of activation of the -129/+81 region of *bfl-1* by PMA (or PMA/ionomycin) and LMP-1 may be due to differences in the subunit composition of the NF- $\kappa$ B protein involved if LMP-1-mediated activation of this region is NF- $\kappa$ B-dependent and the target of NF- $\kappa$ B is the same as that of PMA (or PMA/ionomycin) in Jurkat cells. As pointed out earlier, the subunit composition of the NF- $\kappa$ B protein can greatly influence not only its ability to bind to a particular site but also the extent of transactivation (Perkins *et al.*,

1992) However, the possibility that PMA or PMA/ionomycin may be having an effect on a cryptic site in the reporter plasmid cannot be excluded owing to the exceptionally high level of activation of the *bfl-1* promoter-luciferase construct in Jurkat T cells and this possibility needs to be investigated by analysing the effect of PMA or PMA/ionomycin on the basic reporter construct containing a minimal promoter region (that binds the basic transcriptional apparatus)

#### 4.6 ELECTROPHORETIC MOBILITY SHIFT ANALYSIS (EMSA)

The DG75tALMP-1 tet-off system was used to further investigate the role of NF- $\kappa$ B in the transactivation of the -129/+81 region of *bfl-1* by EMSA primarily because this system allows testing the effects of LMP-1 on gene expression in a more controlled fashion (i.e. excluding the possibility of any effects resulting from clonal variation) and induction of LMP-1 in this cell system could be shown to result in an upregulation of *bfl-1* mRNA levels (Figure 3.3). In agreement with the high constitutive levels of NF- $\kappa$ B activation known to be present in the DG75 cellular context (Rowe *et al* , 1994, Huen *et al* , 1995), EMSA analysis with a 22 bp oligonucleotide containing the  $\kappa$ B enhancer element from immunoglobulin- $\kappa$ -light chain gene, detected the formation of two specific p65-containing complexes with nuclear proteins from the cells in the absence of LMP-1 induction. Induction of LMP-1 led to an increase in the extent of formation of these two complexes and did not lead to the formation of any new complex (Figure 3.45). Supershift studies also revealed that p65 was not dimerised to either p50 or c-rel in the two specific complexes (Figure 3.46). This result was surprising since LMP1 has been shown to induce the formation of p65/p50 and p50/p50 as well as p65/p52 NF- $\kappa$ B complexes in an EBV-negative BL cell context (Herrero *et al* , 1995). Also reporter assays have demonstrated that LMP-1 can transactivate both p65/p50-responsive and c-rel-responsive elements in DG75 cells (Liljeholm *et al* , 1998). At least in the case of p50, the possibility that the antibody to p50 is unable to bind its target and therefore function in supershifting was ruled out since this antibody could supershift a p50 homodimer complex formed between the same oligonucleotide and nuclear proteins from an LCL (IARC290B) (result not shown). In the case of c-rel, it is possible that the

antibody does not function in supershiftmg or that c-rel is truly not activated in this clonal cell line of DG75 even in the absence of LMP-1 expression. Also, it is not known at the time of writing this thesis if the p50 and c-rel antibodies used exhibit preferences for supershifting NF- $\kappa$ B complexes with specific subunit composition. Candidate partners for p65 in the two NF- $\kappa$ B complexes formed with the consensus  $\kappa$ B element in EMSA include p65 itself (homodimers) and p52. The possibility that p52 may be contained in one of the complexes can be tested by performing supershift analysis with an appropriate antibody.

A transcription factor binding site search of the -129/+81 region of *bfl-1* revealed the presence of a NF- $\kappa$ B-like site at position -52 to -43 (5'-AGAAATTCCA-3' on the sense strand, or 5'-TGGAATTTCT-3' on the antisense strand) (Figure 3.44). NF- $\kappa$ B-like sites in both forward and reverse orientations that diverge from consensus NF- $\kappa$ B binding sites at one or more positions have been identified in promoters of several genes including apoptosis-related genes such as *bcl-x*, *c-IAP-1*, *c-IAP2* and mouse *A1* as well as cytokine genes such as *IL-8* and functionality of these sites in promoter activation has been demonstrated in a few cases (Chen *et al*, 1999, Hong *et al*, 2000, Okamoto *et al*, 1994). Classic NF- $\kappa$ B (p65/p50) binds the sequence 5'-GGGRNNYYCC-3' whereas the RelA/c-Rel dimer binds to a sequence 5'-HGGARNYYCC-3' [H indicates A, C or T, R is purine (A or G), Y is pyrimidine (T or C)] (Baldwin, 1996). The  $\kappa$ B-site at position -52 of *bfl-1* in forward orientation diverges from the p65/p50 and p65/c-rel consensus binding sites at 3 and 2 nucleotide positions respectively, but in reverse orientation differs from the consensus p65/p50 binding site at 2 positions and from the p65/c-rel binding site at only one position (Table 4.1).

**Table 4 1 Degree of homology of the NF-κB-like site at position -52 (-52/-43) of *bfl-1* in forward and reverse orientations to the p65/p50 and p65/c-rel consensus binding sites**

	Consensus p65/p50 site GGGRNNYYCC	Consensus p65/c-rel site HGGARNYYCC
-52/-43 <i>bfl-1</i> κB-like site FORWARD	<u>AG</u> <u>AA</u> ATTCCA (3)	AG <u>AA</u> ATTCCA (2)
REVERSE	TGGAATT <u>TTCT</u> (2)	TGGAATT <u>TTCT</u> (1)

R= purine (A/G), N= any nucleotide, Y= pyrimidine (T/C), H= A, C or T  
The nucleotide positions that diverge from the consensus sequence are underlined  
The number of 'divergent positions' in the *bfl-1* κB-like site are given in brackets  
Forward and reverse refer to the orientations of the sequence as found on the sense and antisense strand respectively of the promoter region

Three potential κB-like sites have been identified in A1, the mouse homolog of *bfl-1*, and one of these, AGAAATTCTA differs from the κB-like site at position -52 (forward/sense orientation) of *bfl-1* at one nucleotide position (Chen *et al* , 1999) Also, the promoter of the human c-IAP-2 gene contains a homologous potential κB-like site and has the sequence AGAAATTTCA (Chen *et al* , 1999) (Table 4 2) However it is not known if both of these NF-κB-like motifs are functional In the case of mouse A1, a functional c-rel-responsive element has been identified and has the sequence AGGGATCCC (Grumont *et al* , 1999) The AGAAATTCCA κB-like site of *bfl-1* bears 100% homology to a CD28-responsive κB site in the IL-2 promoter (Civil *et al* , 1996, 1999, Verweij *et al* , 1991) CD28-mediated regulation of the IL2 κB-like site was shown to involve c-Rel and p65 (Ghosh *et al* , 1993)

**Table 4 2 The κB-like site at position -52 (-52 to -43) of *bfl-1* differs from κB-like sites in the promoters of mouse A1 and human c-IAP-2 at one nucleotide position**

<i>Bfl-1</i>	AGAAATTCCA
<i>A1</i>	AGAAATTCT <u>A</u>
<i>c-IAP-2</i>	AGAAATT <u>T</u> CA

Divergent nucleotide positions are underlined

Induction of LMP-1 in the DG75tALMP-1 cells resulted in the formation of a novel complex when nuclear extracts were incubated with a 14 bp oligonucleotide containing the NF- $\kappa$ B-like site of the -129/+81 region of *bfl-1* and the intensity of this complex was diminished when extracts were pretreated with a p65 antibody (Figure 3 47). However, the unlabelled 14 bp oligonucleotide itself or the consensus NF- $\kappa$ B oligonucleotide did not compete out the formation of this complex with the labelled 14 bp oligonucleotide in EMSA (Figure 3 48), suggesting that this complex was non-specific. These findings suggest the need to exert caution when interpreting results obtained with EMSA and the need to base the interpretation on a combination of supershift and competition studies as far as possible. The size of the oligonucleotide used as a probe in EMSA, particularly in relation to the amount of sequence flanking the  $\kappa$ B-site that is included in the oligonucleotide, may have had some bearing on the failure to detect formation of a specific NF- $\kappa$ B-containing complex irrespective of LMP-1-expression. While it is useful to limit the size of the oligonucleotide to about the size of the binding site under study (to exclude non specific binding of other proteins), it has now become clear, particularly in the case of non-consensus/ $\kappa$ B-like sites, that sequences in the 5' and/or 3' flanking regions may play a role in stabilising Rel protein binding and hence participate in directing transactivation as has been demonstrated in the case of a variant  $\kappa$ B-site in the *ICAM-1* promoter (Paxton *et al* , 1997). Hence, it is possible that if NF- $\kappa$ B-binding at the  $\kappa$ B-like site at position -52 of *bfl-1* does occur *in vivo*, this may not have been detected *in vitro* due to lack of sufficient amount of flanking sequence included in the oligonucleotide. The amount of flanking sequence should be taken into account in the design of an oligonucleotide in future EMSA experiments to establish the role of the NF- $\kappa$ B site at position -52 of *bfl-1*. It might also be more useful in future studies to investigate the role of this site in the context of the 'full-length' promoter by mutational analysis.

EMSA studies using DNA probes encompassing the entire -129/+81 region or different portions thereof were inconclusive with respect to a role for NF- $\kappa$ B in mediating activation of this region of the *bfl-1* promoter by LMP1. Even though complex

formation was detected with these fragments, there did not appear to be any consistency in the pattern of complex formation with nuclear extracts from DG75 tTALMP1 cells irrespective of LMP1 expression. There was also no obvious difference in the pattern or intensity of complexes between nuclear proteins extracted from DG75tTALMP-1 cells before and after induction of LMP1 in any experiment. There are several factors that could account for the inconsistency observed in these EMSA studies. Firstly, the quality of the extracts may have diminished after recovery from storage, most likely during the binding reaction. Degradation of the proteins during the binding reaction could have occurred due to protease contaminants in any of the components of the binding reaction including the probe preparations. Two protease inhibitors, PMSF and leupeptin, were included in the lysis and binding buffers. However, the action of these inhibitors is limited to a few proteases (Sambrook *et al*, 1989) and an improvement in the experimental protocol in this respect would be to include a cocktail of protease inhibitors in the buffers used to extract the nuclear proteins and in the binding buffers. Secondly, if the quality of the extracts were optimum and binding did occur, the electrophoresis conditions may have been suboptimal to the extent of altering the stability of the complexes. Two contributing factors in this case are buffer composition and the temperature at which electrophoresis is performed. In addition to affecting the stability of the complexes (particularly to those of slower mobility) these factors may have also contributed to the smearing of some of the bands detected in these experiments (Figure 3.51). Increase in temperature of the gel or buffer during electrophoresis can be controlled by performing the electrophoresis at 4°C. Smearing of bands can also occur if detergent residues are left on the gel plates and in this regard it is known that NF-κB is very sensitive to detergents (Promega Technical Bulletin No. 110). Also, suboptimal salt or glycerol concentrations in the binding buffer can contribute to band smearing.

Given that the reporter assays proved that LMP-1-mediated activation of the -129/+81 region of *bfl-1* is NF-κB-dependent and EMSA analyses suggest a lack of an LMP-1-induced increase in the extent of nuclear protein complex formation (or formation of new complexes) with a probe encompassing this region, two speculative mechanisms can be arrived at regarding the role of NF-κB in mediating activation of the -129/+81 region of

*bfl-1* Firstly, the involvement of NF- $\kappa$ B may be indirect in that being a transcription factor, it induces the expression of another transcription factor which then directly contacts the promoter DNA and activates transcription. Secondly, the transactivation potential of a p65-containing complex bound to the NF- $\kappa$ B site at -52 can be stimulated by LMP1-mediated phosphorylation of the activation domain of p65. If the first scenario is true then it would mean that the binding conditions used in the EMSA analysis were not suitable for the binding of this transcription factor to the -129/+81 region of *bfl-1*. Since this scenario would require intermediate protein synthesis, it would be possible to test this out by treating cells with a protein synthesis inhibitor such as cycloheximide prior to examining the inducibility of *bfl-1* mRNA by LMP-1. In this respect it is interesting to note that CD40-mediated upregulation of *bfl-1* mRNA levels has been shown by others to be partially dependent on protein synthesis (Lee *et al* , 1999). Cell systems in which LMP-1 signaling can be induced in a manner that is unaffected by a protein synthesis inhibitor are available and are based on the fact that the carboxy cytoplasmic region of LMP-1 encodes the signaling functions of the molecule and that activation requires oligomerisation. One example of such a system involves the inducible activation of LMP1 function from a fusion protein of the extracellular domain of Nerve Growth Factor and the carboxy cytoplasmic domain of LMP-1 by addition of NGF into the growth medium (Schwenger *et al* , 1998, Gires *et al* , 1998). The second scenario is dependent on successful demonstration that NF- $\kappa$ B binds to the  $\kappa$ B site at position -52 and that one of the complexes formed with the -129/+81 fragment contains p65 in EMSA analysis using extracts from the DG75tALMP-1 cells. This scenario is also compatible with a lack of an LMP-1-induced increase in the extent of complex formation with the -129/+81 promoter region in EMSA. It has recently been demonstrated that Akt (or protein kinase B), a serine/threonine kinase, can suppress apoptosis by not only directly phosphorylating and inactivating proapoptotic proteins including Bad and procaspase 9 but also stimulating the transactivation potential of the NF- $\kappa$ B subunit p65 by targeting it for phosphorylation (Madrid *et al* , 2000). Furthermore, IL-1 which is a member of the TNFR superfamily can increase the transactivation potential of p65 by inducing phosphorylation of the transactivation domain in a PI3-kinase- Akt-dependent pathway (Sizemore *et al* , 1999). LMP-1 has recently been shown to activate Akt in a

phosphoinositide-3-OH kinase (PI3K) –dependent pathway (Young *et al* , 2000) and since LMP-1 activates *bfl-1* expression in an NF- $\kappa$ B-dependent manner it is possible that LMP-1-mediated transactivation of the –129/+81 region of *bfl-1* in the DG75 cell context involves a mechanism in which LMP1-induced activation of Akt leads to phosphorylation of the p65 subunit of an NF- $\kappa$ B-containing complex bound to the  $\kappa$ B site at position –52 in the *bfl-1* promoter. It may be relevant that the constitutive activity of this region of the promoter is also NF- $\kappa$ B-dependent and hence the  $\kappa$ B site at position –52 may already be occupied by NF- $\kappa$ B proteins in the absence of LMP-1 expression. The fact that this NF- $\kappa$ B site is not a consensus site may make it a weak transactivation site (contributing to basal levels of activation of the promoter) in the absence of phosphorylation of a bound p65-containing complex.

#### **4.7 A ROLE FOR LMP1 IN MAINTAINING ACTIVATION OF THE –129/+81 REGION OF THE *BFL-1* PROMOTER IN LCLS**

LCLs express high levels of *bfl-1* mRNA and analysis of the activity of the promoter region of the gene by promoter-luciferase assays conducted in two LCLs (IARC290B and IB4) suggests that transcriptional activation of the promoter contributes to the steady-state mRNA levels of *bfl-1* observed in these cells. Deletion analysis of the promoter region revealed that the –129/+81 region is optimal for transactivation of the *bfl-1* promoter in both of the LCLs. LCLs, like BL lines expressing a type III latency phenotype, express all of the latent proteins including LMP-1. Since LMP-1 expression can mimic several of the changes in gene/protein expression and phenotype induced by EBV on infection of primary B lymphocytes and LMP-1 can upregulate *bfl-1* expression at the transcriptional level, it seemed reasonable to hypothesize that LMP-1 may contribute to the high constitutive activity of the *bfl-1* promoter in LCLs. Further support for this hypothesis comes from the observations that induction of expression of LMP1 has been shown to result in activation of NF- $\kappa$ B in resting B cells (Spender *et al* , 1999, K lger *et al* , 1998) and inhibition of NF- $\kappa$ B activation in an LCL has been shown to result in a downregulation of *bfl-1* mRNA levels (Cahir-McFarland *et al* , 2000). Indeed it was found that transfection of a dominant negative LMP1 molecule, LMP1-AAAG,

could downregulate *bfl-1* promoter activity in IB4 and IARC290B by approximately 50-65%. Nevertheless, the inhibition of *bfl-1* promoter activity, albeit partial, by LMP-AAAG in LCLs would suggest the requirement of CTAR1 and/or CTAR2 domains of LMP1 for maintaining the constitutive levels of *bfl-1* promoter activity in LCLs. Although this result might at first hand implicate the possible involvement of other EBV latent proteins in also regulating *bfl-1* promoter activity in LCLs, the mechanism of action/efficiency of LMP-AAAG as a dominant negative has to be considered before interpreting the result. LMP-AAAG may be acting by directly displacing wild-type LMP-1 in oligomeric complexes. However, there isn't any evidence of any investigation in the literature addressing this possibility. LMP-AAAG may also be acting by interacting with newly synthesised LMP1 molecules. The active form of LMP1 is the cytoskeletally-associated form and comprises at least half of the bulk LMP1 protein in the cell. Although, it is generally accepted that the nascent protein has a shorter turnover rate than the cytoskeletally-associated form, there is variability in the half-lives of each of the two forms (half-life of nascent protein, less than 5 h, cytoskeletally-associated form, 3 –15h) depending on the cell-type and detergent used for solubilisation (Kieff, 1996, Baichwal and Sugden, 1987, Martin and Sugden, 1987). In LCLs, the nascent protein has a half-life of less than 30 min but the bulk protein has a half-life of less than 2 hours (Mann and Thorley Lawson, 1987) and therefore if the dominant negative action of LMP-AAAG requires it to oligomerise with newly-synthesised LMP-1 then a greater effect may have been realised if cells were harvested for analysis at a later timepoint. Furthermore, LMP-AAAG can itself homo-oligomerise, thereby reducing its availability to interact with wild-type LMP1. However, given that as with other TNFR family members, oligomerisation of LMP1 molecules occurs as at least trimers (Floettmann and Rowe, 1997), it is possible that only one LMP-AAAG molecule within the oligomeric complex may be required to disrupt signaling. This seems likely based upon the results obtained with the study assessing the efficiency of LMP-AAAG in inhibiting signaling from wild-type LMP1 in cotransfection assays. In this study cotransfection of expression plasmids for wild-type LMP-1 and LMP-AAAG in a 2:1 ratio resulted in an approximately 70% inhibition of wild-type LMP-1 signaling, while transfection of the plasmids in a 1:1 ratio led to a 90% inhibition. It would be useful in future experiments to support these results.

by using another approach such as transfection of an antisense LMP1 construct to downregulate expression of LMP-1. LMP-AAAG expression also inhibited the constitutive levels of NF- $\kappa$ B activation in both of the LCLs tested in keeping with the observations that induction of LMP1 expression in the context of primary B lymphocytes stimulates activation of NF- $\kappa$ B (Spender *et al.*, 1999; Kilger *et al.*, 1998). However, inhibition of NF- $\kappa$ B activation by expression of a dominant negative I $\kappa$ B $\alpha$  molecule exerted different effects on constitutive *bfl-1* promoter activation in the two LCLs. Thus, it was found that whereas expression of dominant negative I $\kappa$ B $\alpha$  decreased *bfl-1* promoter activity in the IB4 cell line, its expression did not significantly alter the activity of the promoter in IARC290B. Also, neither did ectopic expression of the dominant negative TRAF2 molecule, TRAF2 $\Delta$ (6-86), alter *bfl-1* promoter activity in IARC290B. The latter finding may be related to the observation by others that most of LMP1 in LCLs is associated with TRAF1 and TRAF3 and very little with TRAF2 (Devergne *et al.*, 1996). The finding that the activation of the *bfl-1* promoter in IB4 cells is NF- $\kappa$ B-dependent is very significant since it supports the results of Cahir-McFarland *et al* (2000) who found that inhibition of NF- $\kappa$ B activation in this particular cell line resulted in a downregulation of *bfl-1* mRNA levels. The results relating to the control of *bfl-1* expression in LCLs presented in this thesis would suggest that different signalling mechanisms may be engaged by LMP1 in different LCLs to regulate expression of a particular gene. In this regard, it is known that LMP-1 can also activate other signal transduction pathways including the JNK pathway (leading to AP1 activation) (Kilger *et al.*, 1998) and several potential AP-1 sites are present in the *bfl-1* promoter. The usage of different signalling mechanisms to regulate gene expression in different LCLs may be related either to cell-type specific factors present in different LCLs either because of their origin or EBV strain-dependent variation in LMP-1 sequence since while IB4 is derived from infection of fetal cord blood B lymphocytes with the B95.8 strain (King *et al.*, 1980) (LMP-AAAG was derived by mutating the LMP1 sequence from this strain), IARC290B is a spontaneous LCL derived from adult peripheral blood (Lenoir *et al.*, 1985). It would be interesting in future experiments to examine the role of NF- $\kappa$ B and LMP1 in activating the *bfl-1* promoter in an extended panel of LCLs by using the dominant negative LMP mutant, LMP-AAAG, and the dominant negative I $\kappa$ B $\alpha$  mutant.

#### 4.8 CD40 UPREGULATES *BFL-1* PROMOTER ACTIVITY

Lee *et al* (1999) have recently reported that CD40 activation can transiently upregulate *bfl-1* mRNA levels in BL lines. The results presented in this thesis support the results of Lee *et al* and also extend their study by 1) excluding mRNA stability as a possible level of control by CD40 and 11) demonstrating that CD40 activates the *bfl-1* promoter in an NF- $\kappa$ B-dependent manner in this cellular context. The lack of an effect of CD40 ligation on the stability of *bfl-1* mRNA is surprising since regulation of mRNA stability is a common method of regulation of expression of genes displaying a quick response. Since the mRNA stability studies were performed in only one cell line namely, BJABtTA, it is possible that a lack of an effect of CD40 activation on *bfl-1* mRNA stability may be specific to this cell line. An important aspect of CD40 signalling in B cells is its ability to control apoptosis (reviewed in Gregory, 1995). Isolated germinal centre cells, which spontaneously undergo apoptosis in culture, are capable of surviving for extended periods when treated with anti-CD40 antibodies or CD40 ligand. In addition, CD40 is known to rescue immature B cells and mature B cells from surface IgM and Fas-mediated apoptosis (reviewed in Gregory, 1995). Many studies indicate that the anti-apoptotic function of CD40 is mediated by upregulation of Bcl-xL and A20 (Tuscano *et al*, 1996, Lee *et al*, 1999, Sarma *et al*, 1995). The results presented in thesis and the study of Lee *et al* (1999), extend this list to include *bfl-1*.

Both CD40 and LMP-1 belong to the TNFR superfamily and in many respects, LMP1 is a constitutively active CD40 receptor (Baker and Reddy, 1996). However, while CD40 is dependent on ligand-binding for oligomerisation and signalling, LMP1 oligomerises independent of ligand. Similarities between CD40 and LMP1 functions include their ability to bind TRAFs through a PxQxT motif, and to activate the NF- $\kappa$ B, JNK and p38MAPK pathways (Kehry, 1996, Aicher *et al*, 1999, Kieser *et al*, 1997, Ehoupoulos *et al*, 1999, Hatzivassiliou *et al*, 1998). Furthermore, LMP1 and CD40 show remarkable similarities in their downstream effects upon cellular phenotype, including upregulation of a similar set of cell surface (e.g. CD54, CD23 and LFA-1) and anti-apoptotic proteins

(e.g. A20) and in their effects upon cell differentiation, proliferation and apoptosis (Eliopoulos *et al* , 1997, Kilger *et al* , 1998, van Kooten and Banchereau, 1997, Zimmer-Strobl *et al* , 1996, Kehry, 1996) However, some differences in signaling by these two molecules do exist, since as demonstrated in this thesis, LMP1 but not CD40 can upregulate *bfl-1* mRNA stability Also, although both LMP1 and CD40 have been shown to bind several of the same TRAF molecules (namely TRAF-2,-3 and -5), TRAF6 binds to CD40 but not to LMP1 and TRAF1 and TRADD have been reported to bind to LMP-1 but do not bind CD40 directly (reviewed in Busch and Bishop, 1999)

At the steady state mRNA level, dual signaling from CD40 and LMP1 in DG75tTALMP-1 cells appeared to lead to an upregulation of *bfl-1* mRNA levels, the extent of which appeared to be greater than that expected of an additive effect of the independent signals (Figure 3 54) At the promoter level, CD40 activation and LMP1 expression individually transactivated the *bfl-1* promoter in an NF- $\kappa$ B-dependent manner in DG75 cells and although dual signaling from the two molecules had a greater effect on *bfl-1* promoter transactivation than each of the individual signals the overall effect was less than that expected of an additive effect of the individual signals (Figure 3 56) The discrepancy between the results of the studies at the promoter level and at steady-state mRNA level may be a reflection of the fact that the parental cell line DG75 was used in the case of the former, while a subclone DG75tTALMP1 was used in the case of the latter In fact, uninduced DG75tTALMP-1 cells have much lower levels of CD40 expression than DG75 arguing the case for the possibility of clonal variation accounting for the discrepancy Alternatively, there may be elements outside of the -1374/+81 region of *bfl-1*, that may also be involved in transactivation of the promoter by LMP1 and CD40 and hence the promoter studies conducted in this thesis using the -1374/+81 region of *bfl-1* may not be a complete reflection of the effect of these molecules on the endogenous *bfl-1* promoter Arriving at a firm conclusion about the question of cooperativity between LMP-1 and CD40 in upregulating *bfl-1* expression is further complicated by the fact that LMP1 can upregulate CD40 expression in BL lines and that in the case of interpreting the results of Northern analysis, LMP-1 may increase the stability of the CD40-induced *bfl-1* mRNA The results of the promoter studies are easier to interpret in this regard If LMP1

and CD40 utilise different signaling pathways to transactivate the *bfl-1* promoter then at least an additive effect would be expected. The finding of a less than additive effect but greater than an individual effect of signaling from the two membrane molecules on *bfl-1* transactivation can be interpreted to mean that both LMP1 and CD40 are using the same signaling pathway in this effect. Furthermore, although the signaling components would appear to be non-limiting even after engagement by either LMP1 or CD40 individually, they do become limiting when both molecules are activated to less than the capacity required of an additive signal.

LMP1 and CD40 have been shown to cooperate in an additive or synergistic manner in upregulating cell surface molecules such as B7-1 as well as in inducing IL-6 secretion in mouse B cell lines in which LMP-1 does not affect endogenous CD40 levels (Busch and Bishop, 1999). However, this does not apply to all of the effects on gene/protein expression that these molecules share. For example, although both CD40 and LMP-1 could individually induce ICAM-1 and LFA-1 expression a clear cooperative effect was not demonstrated. The studies of Busch and Bishop were conducted at the protein level and hence the possibility that CD40 and LMP-1 may exert a cooperative effect in upregulating *bfl-1* expression at the post-transcriptional/translational level cannot be excluded. In BL cell lines, dual signaling from CD40 and LMP1 failed to result in a cooperative effect on induction of an NF- $\kappa$ B reporter construct in transient transfection assays (Floettmann *et al*, 1998). Thus, cooperativity between CD40 and LMP-1 in regulating gene/protein expression may not be a global phenomenon.

It is well documented that BL lines express CD40 endogenously and LMP1 upregulates CD40 expression in BL lines (reviewed in Gregory, 1995). Hence, it would be expected that when LMP1 and CD40 signals are given concurrently, the B cell responds more robustly than it does to either signal alone. *In vivo*, latently infected B cells expressing LMP1 can be stimulated through both CD40 and LMP1, perhaps resulting in a superactivated state predisposing the cell to a second event resulting in transformation. However, there may also be circumstances where cooperativity in signaling by LMP1 and CD40 leading to overexpression of particular proteins may be deleterious to the cell.

such as in the upregulation of Fas. There may also be yet other circumstances where cells are defective in CD40 activation but are responsive to the LMP1 expression. This may be relevant *in vivo*, in that since reception of a CD40-mediated signal by B cells in the absence of cognate antigen can lead to cell elimination (reviewed in Busch and Bishop, 1999), then the selection of cells with a defect in the CD40 pathway may in some cases be a contributory step in BL pathogenesis. The finding that Rael, which is a type I BL line, is defective in responding to CD40 activation with upregulation of *bfl-1* mRNA is in accordance with the findings of others that this cell line is non-responsive to CD40 activation with respect to other parameters as well (Henriquez *et al.*, 1999). In fact, Rael is one amongst a group of BL lines that are non-responsive to CD40 activation but respond to LMP1 expression (in terms of upregulation of ICAM-1 expression as well as NF- $\kappa$ B activation). Furthermore, ectopic expression of functional CD40 into Rael cells did not confer responsiveness to CD40 ligand, thereby excluding that the lack of responsiveness to CD40-mediated effects in this cell line may be due to a defective endogenous CD40 receptor itself (Henriquez *et al.*, 1999). These observations would indicate the use of different signaling pathways or different components of the same signaling pathway by CD40 and LMP1, which usually converge to effect similar downstream functions. Therefore, in this case if one subscribes to the view that LMP1 mimics CD40 this would give LMP1 the advantage to remain fully functional in cellular backgrounds in which CD40 signaling is impaired.

#### **4.9 MECHANISM UNDERLYING LMP-1-MEDIATED UPREGULATION OF BCL-2 EXPRESSION**

The results presented in this thesis support the findings of others that LMP1 upregulates Bcl-2 expression in BL cells (Henderson *et al.*, 1991; Rowe *et al.*, 1994; Wang *et al.*, 1996). In several of the cell systems used in these studies, even though upregulation of Bcl-2 protein was detected by 24–48 h post-induction of LMP-1 expression, maximum upregulation was not detected until after 72h, suggesting that the induction of Bcl-2 protein expression by LMP1 is a delayed response. The results presented in this thesis are in agreement with the induction of *bcl-2* expression by LMP1 as being a delayed

response particularly at the steady-state mRNA level and to a lesser extent at the protein level. There appeared to be some discordance between extents of upregulation of *bcl-2* expression at the mRNA and protein levels by LMP1 in DG75tTALMP1 cells particularly at 24 h post induction of LMP1, suggesting that the control on upregulation of *bcl-2* expression by LMP-1 occurs at both the transcriptional and translational levels with the latter exerting a stronger effect (by 24 h post-induction of LMP1). It is possible that this effect on induction of *bcl-2* expression by LMP1 may be specific to DG75 tTALMP1 cells. However, there is evidence for the action of both of these mechanisms in the control of *bcl-2* expression during normal B-cell development and differentiation. Changes in the rate of transcription have been found to regulate *bcl-2* mRNA levels during B-cell development (Seto *et al* , 1988, Young and Korsmeyer, 1993). Also, in hemopoietic tissue *in vivo*, although Bcl-2 protein can be detected in the mantle zone but not in the germinal centre, its mRNA can be detected in both of these regions, implying that translational mechanisms can also contribute to the regulation of *bcl-2* expression (Akagi *et al* , 1994, Chleq-Deschamps *et al* , 1993). In addition a cis-acting element within the 5' untranslated region of the *bcl-2* gene has been shown to be necessary for regulating *bcl-2* expression at the level of translation (Harigai *et al* , 1996).

A cAMP responsive element (CRE) has been shown to play a positive role in constitutive activity of the *bcl-2* promoter in mature B cells and also in mediating PMA-induced activation of the *bcl-2* promoter in immature and mature B cells (Wilson *et al* , 1996). Unfortunately, studies presented in thesis suggested the lack of a demonstrable effect of LMP1 induction on activation of a *bcl-2* promoter-luciferase construct in which the promoter region included the CRE site by transient transfection assays. It was expected that since LMP-1 has been shown to activate a calcium/calmodulin-dependent kinase (Mosialos *et al* , 1994) and that these enzymes can in turn activate CRE binding proteins (CREBs), LMP-1 may activate the *bcl-2* promoter via the CRE site. Nevertheless, if LMP-1 does affect *bcl-2* promoter activity it is likely not to involve elements within the – 3934 to –8 region upstream of the translation start site.

Phosphorylation represents a further level of control of Bcl-2 function. Bcl-2 phosphorylation can be induced by various agents including IL-3, erythropoietin, the

PKC activator bryostatin, the phosphatase inhibitor okadaic acid, the chemotherapeutic agent taxol and the microtubule agent vincristine (Haldar *et al* , 1995, Ito *et al* , 1997, Srivastava *et al* , 1998) While Bcl-2 phosphorylation is associated with apoptosis in some cell lines (Haldar *et al* , 1995, Srivastava *et al* , 1998), it is associated with an anti-apoptotic function in others (Ito *et al* , 1997) Phosphorylation of Bcl-2 occurs on serine residues and there are 17 such residues in the protein that serve as candidate sites for phosphorylation (Ito *et al* , 1997) In the case of some but not all stimuli, the serine residues whose phosphorylation is critical to Bcl-2 function have been identified (Ito *et al* , 1997) Thus, phosphorylation of serine 70 by bryostatin-1 has been shown to be the critical residue required for the anti-apoptotic function of Bcl-2 (Ito *et al* , 1997) In several lymphoid cell lines phosphorylation of Bcl-2 by the serine phosphatase inhibitor, okadaic acid (OA), is associated with cell death (Haldar *et al* , 1995) However, in an EBV-immortalised cell line examined in this study by Haldar *et al* , OA treatment did not induce apoptosis or phosphorylation of Bcl-2 This result was interpreted to mean that the putative kinase responsible for phosphorylation of Bcl-2 is not activated in this cell line However, the possibility that OA may not have been able to permeate this cell line was not considered in this study The finding in this thesis that a large fraction of the Bcl-2 protein induced by LMP-1 in DG75tTA-LMP1 cells appears to be unphosphorylated but that the potential for phosphorylation does exist would suggest that the cells may be primed for apoptosis However, at least for the duration of the experiment (6h), there appeared to be no increase in the number of trypan-blue stained cells after treatment with OA either in the absence or presence of LMP1 expression (result not shown) Although this might question the importance of Bcl-2 phosphorylation in controlling apoptosis in this cell context, it is possible that cell death may have occurred on prolonged treatment This needs to be examined in future experiments and perhaps using methods that detect early markers of apoptosis such as measuring cytochrome c release (in response to an apoptotic stimulus cytochrome c is released from mitochondria into the cytoplasm where it activates dormant killer proteases) (Wetzel and Green, 2000a) or annexin V labeling [annexin V (fluorescein-labeled) binds phosphatidyl serine that translocates from the cytoplasmic face of the membrane to the cell surface in response to an apoptotic stimulus] (Wetzel and Green, 2000b)

#### 4.10 FUTURE WORK

A few of the conclusions arrived at in this thesis would be strengthened by additional experiments and these have been pointed out either in the results chapter or in earlier sections of this chapter. Some of the unanswered questions include a) Does LMP-1 upregulate *bfl-1* mRNA in T-lymphocyte and epithelial cell contexts? This is particularly relevant to the T cell context since induction of LMP1 expression in Jurkat tTALMP1 did not lead to *bfl-1* mRNA upregulation but LMP1 expression did result in transactivation of the *bfl-1* promoter in the parental Jurkat T cells. b) Does Bfl-1 expression confer protection against other apoptotic stimuli relevant to BL lines such as calcium ionophore and anti- $\mu$  antibody? How does Bfl-1 compare with other anti-apoptotic proteins such as Bcl-2 and A20 in protecting BL cells against apoptosis? c) Does JAK3 activation contribute to upregulation of *bfl-1* promoter activity and mRNA by LMP1? If so, does this effect map solely to CTAR3 or is there an additional requirement for CTAR1 and/or CTAR2 function? d) Is the lack of responsiveness of the NF- $\kappa$ B site at position -833 to LMP1 signalling a lymphocyte-specific effect? e) Does LMP-1-mediated activation of the PI3-kinase-Akt pathway contribute to activation of the *bfl-1* promoter?

The major findings of the investigations detailed in this report suggest a number of potentially useful further experiments. As already mentioned it will be important to elucidate the mechanism of *bfl-1* mRNA stabilisation by LMP1. As a first step it would be interesting to investigate if inhibition of *de novo* protein synthesis, by using inhibitors such as cycloheximide, can stabilise *bfl-1* mRNA. The mouse *A1* mRNA can be rapidly induced by cycloheximide treatment of bone marrow macrophages as would be expected of an early-response gene (Lin *et al* , 1993). Cycloheximide can also induce *bfl-1* mRNA in endothelial cells (Hu *et al* , 1998). Two mechanisms have been proposed to explain how inhibition of protein synthesis can stabilise mRNAs (reviewed in Ragheb *et al* , 1999a). The first is that mRNA degradation is coupled to translation and thus blocking translation prolongs mRNA half-life. The second is that degradation is dependent on a

labile protein whose synthesis is blocked by the translation inhibitor. In addition some translational inhibitors such as cycloheximide, cause ribosomes to stall on the mRNA potentially shielding it from degradation by cytoplasmic RNases.

Owing to the difficulties encountered with EMSA it may be better to initially approach the investigation of the role of candidate elements in LMP-1-mediated activation of the *bfl-1* promoter by mutation/deletion analysis. This will allow the identification of the role of these elements in the context of the full-length promoter. This approach should apply to not only the NF- $\kappa$ B site in the -129/+81 region of the promoter in BL cells and LCLs but also the four NF- $\kappa$ B elements (excluding the site at -833) in the -1240/-367 region in Jurkat cells. Although NF- $\kappa$ B activation is required for several of the stimulatory effects of LMP-1 on gene (*IL6*, *A20*, *ICAM-1*, *MMP-9*) and protein expression (LFA-1, CD40) (Eliopoulos *et al*, 1997, Laherty *et al*, 1992, Liljeholm *et al*, 1998, Yoshizaki *et al*, 1998, Devergne *et al*, 1998), these effects may also be mediated by other LMP-1-activated transcription factors including AP-1 (Eliopoulos and Young, 1998). Indeed, it has been demonstrated that both AP-1 and NF- $\kappa$ B mediate the activation of the *MMP-9* promoter by LMP-1 (Yoshizaki *et al*, 1998). The presence of several potential AP-1 sites in the *bfl-1* promoter, two of which lie in the -129/+81 region of *bfl-1* (Figure 3.44), makes the investigation of a potential role for AP-1 activation in LMP-1 mediated activation of *bfl-1* feasible, perhaps by using a dominant negative approach (for instance using dominant negative SEK1 that is targeted by LMP-1 to activate the JNK pathway).

The absence of suitable antibodies to Bfl-1 has made it difficult to extend the observations made in this study at the mRNA level to the protein level. Antibodies that detect Bfl-1 protein either by Western blotting or immunohistochemistry would also prove useful in examining normal and tumour tissue material. In the case of normal tissue material it would be interesting to correlate the pattern of expression of *bfl-1* in the germinal centers as determined by *in situ* hybridisation studies (Jung-Ha *et al*, 1998) with immunohistochemical analysis of Bfl-1 protein expression. In the case of tumour material it will be useful to correlate expression of Bfl-1 with expression of LMP1 in

those EBV-associated cancers such as PTLD and NPC which are known to have a high association with LMP-1 expression (Young *et al* , 1989, Thomas *et al* , 1990)

It will be useful to map the domains of CD40 required for upregulating *bfl-1* mRNA levels by using various CD40 mutants that effect known signaling functions of the molecule. Like LMP-1, CD40-mediated signaling results in activation of NF- $\kappa$ B, JNK and p38 and several of these effects are mediated by TRAF interactions with two regions within the cytoplasmic tail of the CD40 receptor (Pullen *et al* , 1999). The membrane proximal TRAF-binding region of CD40 binds TRAF6 whilst the membrane distal TRAF-binding region binds TRAF1, TRAF2 and TRAF3. Mutational analysis has shown that both of the TRAF binding sites are required for optimal NF- $\kappa$ B and JNK activation, while activation of p38 is dependent solely on TRAF6 binding (Pullen *et al* , 1999). This analysis could be supported by identification of the regions of the *bfl-1* promoter targeted by CD40 and the identification of target promoter elements mediating *bfl-1* transactivation.

An understanding of the translational mechanisms underlying the LMP-1 induced upregulation of Bcl-2 is also worthy of future investigation, in particular, the potential role of LMP-1 in modulating the negative regulatory element (NRE) in the 5' UTR of *bcl-2* (Harigai *et al* , 1996). This NRE is located at -119/-84 bp upstream of the translation start site. The functional significance of this element was established by performing transient transfection assays in cell lines using Bcl2-CAT reporter constructs containing the 5' region of *bcl-2* [region -1649/-1 containing the major promoter (P1), the 5'UTR (containing the NRE) and minor promoter (P2)] either in a wild-type format or with point/deletion mutations of the NRE in transfection assays followed by measuring CAT mRNA levels and CAT activity. It was found that whilst the CAT mRNA levels in the transfectants were similar, the CAT activity was elevated when the NRE was mutated. Thus, by transfecting a series of similar constructs as that used in the study of Harigai *et al* in transfection studies of DG75tTALMP-1 cells before and after induction of LMP-1 (perhaps 24h post-induction of LMP-1 to avoid the effect on *bcl-2*

transcription) it should be possible to determine if this NRE is the control point in the LMP-1-mediated upregulation of Bcl-2 protein

Although resting B cells already express a high level of Bcl-2, which is only slightly elevated following infection with EBV (Martin *et al* , 1993), the Bfl-1 status of these cells before and after infection remains unknown and therefore merits investigation. Since mitogenic activation of lymphocytes *in vitro* results in decreased Bcl-2 protein expression (Aiello *et al* , 1992) it is possible that depending on the type of B cell that the virus infects, the expression of LMP-1 may serve to transiently upregulate or maintain Bfl-1 and Bcl-2 levels during the EBV-associated mitogenic stimulation that cells undergo upon infection and during the critical period prior to entry of the virus-infected cell into the long-lived memory B cell pool.

## **CHAPTER 5**

### **SUMMARY**

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## SUMMARY

Control over apoptosis is an important feature of the life cycle of EBV in both the latent and lytic phases (reviewed by Allday, 1995). *In vitro*, latent infection of B-lymphocytes involving the expression of all of the EBV latent proteins is accompanied by an increased resistance to apoptosis. In this thesis, an investigation was carried out to identify EBV-associated changes in the level of expression of apoptosis-related genes. Using a multiprobe set specific for a subset of apoptosis-related genes (both pro- and anti-apoptotic genes) in ribonuclease protection assays, the steady-state levels of mRNA transcripts of each of these genes was assessed in the context of a range of EBV-related cell lines. Of the genes under study, which included *bclx<sub>L/S</sub>*, *bfl-1*, *bik*, *bak*, *bax*, *bcl-2* and *mcl-1*, the most significant change was observed in the level of *bfl-1* mRNA which was markedly upregulated on transition from EBV latency type I to type III in EBV-positive BL cells. EBV-negative BL cells like type I BL cells were found to express negligible or very low levels of *bfl-1* mRNA, while EBV-immortalised LCL lines exhibited elevated levels of expression of *bfl-1* mRNA. Functional assessment of ectopic expression of Bfl-1 in the context of an EBV-positive type I BL line revealed that Bfl-1 expression can confer resistance to apoptosis induced by serum-deprivation and also exert a proliferative effect under such growth conditions.

Using a tetracycline-regulated gene expression system in an EBV-negative BL line, DG75tTALMP-1, in which LMP-1 is expressed as the sole EBV latent protein, it was possible to show that LMP-1 can contribute to the upregulation of *bfl-1* mRNA. Levels of *bfl-1* mRNA were barely detectable in a T cell line, Jurkat-tTA-LMP-1, or an epithelial cell line, C33A, and expression of LMP1 in either cellular context had no apparent effect on *bfl-1* mRNA levels. Although these results would suggest that LMP1-mediated upregulation of *bfl-1* mRNA levels is a B-cell effect, the absence of a similar effect in the T and epithelial cell lines may be the result of clonal variation.

Further investigations into the mechanism of upregulation revealed that stabilisation of

*bfl-1* mRNA contributed to the observed LMP1-mediated effect. An LMP1-induced increase in activity of the *bfl-1* promoter could also be demonstrated in transient transfection reporter assays in two EBV-negative BL lines (DG75 and BL41) and a T cell line (Jurkat) with the CTAR2 domain of LMP1 exerting the predominant effect. However, the regions of the promoter targeted for activation by LMP1 differed between DG75 and Jurkat T cells. Thus it was found that whereas elements in the proximal region (bp -129 to +81 relative to the transcription start site) of the promoter appeared to play a greater role in the LMP1-mediated effect in DG75 cells, elements in the -1240/-367 region of the promoter were targeted for activation of the *bfl-1* promoter by LMP1 in Jurkat cells. However, in both cell types NF- $\kappa$ B was demonstrated to play an important role in the LMP1-mediated effect on the *bfl-1* promoter and that too in a TRAF-2-dependent mechanism. This was based on observations of the inhibitory effects of a dominant negative I $\kappa$ B $\alpha$  mutant or a dominant negative TRAF2 or a TRAF-interacting protein A20 on the LMP1-mediated effect. Ectopic expression of p65 or PMA treatment could also activate the *bfl-1* promoter in transient transfection reporter assays with DG75 and Jurkat cells thereby demonstrating the NF- $\kappa$ B-responsiveness of the promoter. Despite the importance of NF- $\kappa$ B in mediating the activation of the -129/+81 region of *bfl-1*, it was not possible to demonstrate binding of NF- $\kappa$ B to this region.

In LCLs, the -129/+81 region of the promoter was found to be optimal for constitutive activation of the gene. Furthermore, by using a dominant negative mutant of LMP1 that is defective in NF- $\kappa$ B-signaling from both CTAR1 and CTAR2, it was possible to show that this EBV latent protein contributes to the constitutive level of activation of the promoter in LCLs. However, NF- $\kappa$ B may not be the only mechanism responsible for activating the *bfl-1* promoter in different LCLs as revealed by transient transfection studies with a dominant negative I $\kappa$ B $\alpha$  mutant.

Activation of CD40 was also found upregulate *bfl-1* mRNA levels in BL cells, however although both CD40 and LMP1 are members of the TNFR superfamily, the effect of

CD40 was transient and unlike LMP1, CD40 activation did not result in an increased stabilisation of *bfl-1* mRNA. Like LMP1, CD40-induced activation of the *bfl-1* promoter was also found to be mediated by NF- $\kappa$ B. Coactivation of CD40 and LMP1 in DG75 cells resulted in a greater effect on the *bfl-1* promoter than coactivation of either molecule alone, however the effect was less than additive suggesting that both molecules must engage common components of the same signaling pathway to activate the *bfl-1* promoter.

A perhaps minor but significant finding of the results presented in this thesis is the involvement of both transcriptional and translational mechanisms in the LMP1-mediated effect on Bcl-2 expression in DG75tALMP-1 cells with the effect on translation appearing to be more significant. Furthermore, it was possible to demonstrate that autocrine/paracrine factors are not involved in this effect of LMP1.

The induction of *bfl-1* expression by LMP1 in B lymphocytes has implications for the biology of EBV. LMP1-mediated upregulation of *bfl-1* may contribute to the survival of EBV-infected B cells, since cells similar to LCLs are present in the circulation during primary infection by EBV (Qu and Rowe, 1992, Tierney et al, 1994). Additionally, the effect on *bfl-1* may contribute to the development of EBV-associated B-cell malignancies such as posttransplant lymphoproliferative disorders and BL tumour metastases in which LMP1 is expressed. Mechanistic studies of the contribution of Bfl-1 to cell survival will provide important information about normal B-cell development and potential routes to B-cell and non-B-cell malignancy.

## **CHAPTER 6**

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## APPENDIX

## **SOLUTIONS FOR DNA MANIPULATION**

### **TE buffer**

10 mM	Tris-Cl
1 mM	EDTA pH 8.0

### **Solutions for mini-preparation of plasmid DNA**

#### **Solution I**

50 mM	Glucose
25 mM	Tris Hcl (pH 8.0)
10 mM	EDTA (pH 8.0)

#### **Solution II (Prepared fresh)**

0.2 M	NaOH
1 % (w/v)	SDS

#### **Solution III**

60 ml	5 M potassium acetate
11.5 ml	Glacial acetic acid
28.5 ml	Distilled water

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

### **DNase-free RNase**

RNase A (1 mg/ml) in sterile water

Heat to 100°C for 30 min. Cool slowly and store -20°C

### **Solutions for Maxipreparations of DNA – Qiagen Buffers**

#### **Buffer PI (Resuspension buffer)**

50 mM	Tris-cl, pH 8.0
-------	-----------------

10 mM                      EDTA  
100 µg                     RNase A  
Store at 4<sup>0</sup>C after the addition of RNase A

**Buffer P2 (Lysis buffer)**

200 mM                    Sodium Hydroxide  
1% (w/v)                 SDS  
Prepared fresh and stored at room temperature

**Buffer P3 (Neutralization buffer)**

3.0 M                      Potassium acetate pH 5.5  
Stored at 4<sup>0</sup>C

**Buffer QBT (Equilibration buffer)**

750 mM                    NaCl  
50 mM                     MOPS pH 7.0  
15% (v/v)                 Isopropanol  
0.15% (v/v)              Titon X-100  
Stored at room temperature

**Buffer QC (Wash buffer)**

1.0 M                      NaCl  
50 mM                     MOPS pH 7.0  
15% (v/v)                 Isopropanol  
Stored at room temperature

**50% (v/v) Glycerol**

25 ml                      Distilled  
25 ml                      Glycerol  
Autoclaved and stored at room temperature

### **0.5 M EDTA**

186.1 g	EDTA
800 ml	Distilled water
6 g	NaOH pellets

pH to 8.0 with 5 M NaOH    Volume was adjusted to 1 L with water

### **50X TAE**

242 g	Tris
57.1 ml	Acetic acid
100 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water

### **10X TBE**

108 g	Tris
50 g	Boric acid
40 ml	0.5 M EDTA pH 8.0

Adjusted to 1L with water

### **Ethidium bromide**

0.1 g/10 ml water (10 mg/ml)  
Stored in dark at room temperature

### **Agarose gel loading dye**

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

## **BACTERIAL GROWTH MEDIA**

### **LB agar**

10 g	Tryptone
5 g	Yeast extract
5 g	NaCl

15 g                      Agar technical

Autoclaved and plates stored at 4<sup>0</sup>C

LB agar plus ampicillin

Ampicillin was added to a final concentration of 100 µg/ml to LB agar (50<sup>0</sup>C)

Plates were stored at 4<sup>0</sup>C

### **LB broth (per L)**

10 g                      Bacto-tryptone

5 g                        Yeast extract

5 g                        NaCl

Autoclaved and stored at 4<sup>0</sup>C

### **LB Ampicillin broth**

Ampicillin was added to LB broth to a final concentration of 100 µg/ml from stock solutions (100 mg/ml in dH<sub>2</sub>O, stored at -20<sup>0</sup>C)    Stored at 4<sup>0</sup>C

### **SOB medium (per L)**

20 g                      Tryptone

5 g                        Yeast extract

0.5 g                      NaCl

10 ml                     KCl (250 mM)

Adjusted pH to 7.0 with 5 M NaOH

Autoclaved, cooled to -5<sup>0</sup>C and added

10 ml    1 M MgCl<sub>2</sub>

Stored at 4<sup>0</sup>C

### **SOC medium (per L)**

1 L                        SOB

7.5 ml                    50% glucose (filter sterilised)

Stored at 4<sup>0</sup>C

**Ampicillin stock solution (50 mg/ml)**

50 mg of ampicillin per ml of sterile H<sub>2</sub>O

Filter sterilised and stored at -20°C

**CELL CULTURE MEDIA/SOLUTIONS****Supplemented RPMI (200 ml)**

176 ml	RPMI 1640
20 ml	Foetal calf Serum (Decomplemented -50°C for 30 min)
2 ml	200 mM L-glutamine
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 µg/ml)

**Supplemented DMEM High Glucose**

178 ml	DMEM high glucose with L-glutamine
20 ml	Foetal calf Serum (Decomplemented, 50°C for 30 min)
2 ml	Penicillin/Streptomycin (1000 U/ml-1000 µg/ml)

**10X Phosphate Buffered Saline (PBS)**

14.24 g	Na <sub>2</sub> HPO <sub>4</sub> 2H <sub>2</sub> O (8 mM)
2.04 g	KH <sub>2</sub> PO <sub>4</sub> (1.5 mM)
80.0 g	NaCl (137 mM)
2.0 g	KCl (2.7 mM)

pH 7.5 and make up to 1 litre

Diluted 1 in 10 in sterile distilled water and used at a 1 X working concentration

**Thiol supplements**

The following were added to 200 ml of supplemented media

200 µl	α-Thioglycerol
2 ml	Sodium pyruvate
2 ml	HEPES

**Bathocuproine disulfonic acid (BCS – 10 mM stock solution)**

36.4 mg                      BCS

10 ml                        1 X PBS

Dissolved by vortexing, filter sterilised using a 0.2 micron filter

Aliquoted and stored at  $-20^{\circ}\text{C}$

 **$\alpha$ -Thioglycerol**

A stock solution of 50 mM in PBS containing 20  $\mu\text{M}$  BCS was prepared

20  $\mu\text{l}$                         10 mM BCS

10 ml                        1 X PBS

43.3  $\mu\text{l}$  100%  $\alpha$ -thioglycerol

Filter sterilised using a 0.3 micron filter, aliquoted and stored at  $-20^{\circ}\text{C}$

**Sodium pyruvate**

100 mM stock solution in 1 X PBS (Gibco BRL)    Stores at  $4^{\circ}\text{C}$

**HEPES**

1 M stock pH 7.5 (Gibco BRL)    Stored at room temperature

**Geneticin G418 (stock solution 50 mg/ml) for tetracycline inducible cell lines**

0.1 g                        Geneticin

2 ml                        RPMI 1640

Filter sterilised using a 0.2 micron filter, aliquoted and stored at  $-20^{\circ}\text{C}$     20  $\mu\text{l}$  of the stock solution was added per ml of media to give a final concentration of 1 mg/ml

**Hygromycin B (stock solution 50 mg/ml supplied)**

Ten micro litres of the stock solution was added per ml of media to give a final concentration of 500  $\mu\text{g}$  per ml    Stored at  $4^{\circ}\text{C}$

**Tetracycline (stock 5 mg/ml)**

5 mg                      Tetracycline  
1 ml                      100% Ethanol

Stored at -20°C, 1 µl of tetracycline was added to 5 ml of media to give a final concentration of 1 µg per ml

**Geneticin G418 (stock solution 600 mg/ml) for transfected epithelial cell lines C33A Neo and LMP1**

0.6 g                      Geneticin  
1 ml                      1 M HEPES pH 7.5

Filter sterilised using a 0.2 micron filter, aliquoted and stored at -20°C. One µl of the stock solution was added per ml of media to give a final concentration of 600 µg per ml

**Okadaic acid (stock solution)**

0.4 mM in 10% DMSO  
Stored in aliquots at -20°C

**Sodium orthovanadate (stock solution)**

1M in dH<sub>2</sub>O. Boiled until translucent and pH readjusted to 10.0  
Stored in aliquots at -20°C

**PMA (phorbol 12-myristate 13-acetate) (stock solution)**

0.1 mg/ml in ethanol  
Stored in aliquots at -20°C

**Ionomycin (stock solution)**

2mM in DMSO  
Stored in aliquots at -20°C

## **Solutions for modified DEAE-Dextran Transfection Protocol**

### **T.E.**

10 mM                      Tris (pH 7.4)

1 mM                      EDTA

Prepared fresh on the day of use using autoclaved Tris and EDTA. It is important to ensure that the pH of the Tris is at 7.4 room temperature prior to use.

### **TBS**

25 mM                      Tris (pH 7.4)

137 mM                    NaCl

5 mM                      KCl

0.7 mM                    CaCl<sub>2</sub>

0.5 mM                    MgCl<sub>2</sub>

0.6 mM                    Na<sub>2</sub>HPO<sub>4</sub>

Prepared from autoclaved stocks, aliquoted and filtered before use. Again the pH of the Tris is critical.

### **DEAE Dextran**

1 mg/ml in TBS, prepared fresh and filter sterilised

### **β-galactosidase assay**

#### **100 x Mg solution**

0.1 M MgCl<sub>2</sub>

4.5 M 2-mercaptoethanol

Stored at -20°C

#### **1 x ONPG substrate (o-nitrophenyl β-D-galactopyranoside)**

4 mg/ml in 0.1 M sodium phosphate buffer pH 7.5

Stored at -20°C

## **SOLUTIONS FOR PROTEIN ISOLATION**

### **Suspension buffer**

0.1 M	NaCl
0.01 M	Tris-Cl (pH 7.6)
0.001 M	EDTA (pH 8.0)
1 µg/ml	leupeptin
100 µg/ml	PMSF
Stored at 4°C	

### **2X SDS gel loading buffer**

100 mM	Tris-cl
10%	2-mercaptoethanol
4% (w/v)	SDS
0.2% (w/v)	Bromophenol blue
20% (v/v)	Glycerol

Two times loading buffer was prepared without 2-mercaptoethanol and stored at room temperature. 2-mercaptoethanol was added just prior to use from a 14.4 M (100%) stock.

### **Protease Inhibitors (stock solutions)**

2 mg/ml	Leupeptin in water
0.1 M	PMSF (phenylmethylsulfonyl fluoride) in isopropanol
Stored at -20°C (PMSF was stored away from light)	

## **SOLUTIONS FOR SDS PAGE/WESTERN BLOTTING**

1 M	Tris-Cl pH 6.8
1.5 M	Tris-Cl pH 8.8
10% (w/v)	SDS
10% (w/v)	Ammonium persulphate (APS)

Acrylagel  
Bis-acrylagel  
TEMED

**10X Tris glycine running buffer (500 ml)**

15 138 g	Tris
71 125 g	Glycine
5 0 g	SDS

Made up to 500 ml with distilled water

**Destain**

100 ml	Acetic acid
400 ml	Methanol
500 ml	Distilled water

**Coomassie blue stain**

1 g	Coomassie blue R
200 ml	Destain

**Transfer Buffer (10X stock solution)**

30 3g	Tris
144 2g	Glycine

Adjusted to pH 8.3, made up to 1 L with distilled water, stored at room temperature

**Transfer Buffer (1X working Solution)**

100 ml	10X Stock Solution
200 ml	Methanol
700 ml	Distilled H <sub>2</sub> O

Stored at 4°C

**TBS (1X)**

6.1 g	Tris
8.8 g	NaCl

Made up to 1 L with distilled water and adjusted to pH 7.5 with HCl  
Autoclaved and stored at room temperature

**TBST (0.1%, v/v)**

1 L	TBS (as above)
1 ml	Tween 20

**Blotto**

1X TBS (as above)  
0.05% (v/v) Tween 20  
5% (w/v) non-fat dry milk (Marvel)  
0.02% NaN<sub>3</sub>

**Sodium azide (10%) (w/v)**

1 g	NaN <sub>3</sub>
10 ml	Distilled water

**REAGENTS FOR RNA ANALYSIS**

**RNA sample buffer**

50 % (v/v)	Deionized formamide
8.3 % (v/v)	Formaldehyde
0.027 M	MOPS pH 7.0
6.7 mM	Sodium acetate

**RNA loading buffer (containing ethidium bromide)**

50% (v/v)	High grade glycerol
1 mM	EDTA (pH 8.0)
0.4 % (w/v)	Bromophenol blue

0.1 µg/ml                      Ethidium bromide

Aliquoted and stored at -20°C

### **5 X MOPS Buffer (Formaldehyde gel running buffer)**

0.1 M MOPS (pH 7.0)

40 mM Sodium Acetate

5 mM EDTA (pH 8.0)

20.6g of 3-(*N*-morpholino) propanesulfonic acid (MOPS) was dissolved in 800 ml of DEPC treated 50 mM sodium acetate. The pH of the solution was adjusted to 7.0 using 2 M NaOH. 10 ml of DEPC-treated 0.5 M EDTA (pH 8.0) was added and the volume of the solution was adjusted to 1L using DEPC-treated H<sub>2</sub>O. The 5X solution was filter sterilised through a 0.2 micron filter prior to use.

### **Formaldehyde gel**

1 part Formaldehyde

3.5 parts agarose in DEPC H<sub>2</sub>O

0.68 g                      Agarose

35ml                      DEPC H<sub>2</sub>O

The agarose solution was boiled until fully dissolved and allowed to cool to 60°C. The following were then added:

11 ml                      5X MOPS buffer

10 ml                      Formaldehyde

56 ml                      Final volume

The gel was cast in a fume hood and allowed to set for approximately 45 min. The gel was electrophoresed in 1X formaldehyde gel running buffer.

### **RNA loading buffer**

50% (v/v)                      High grade glycerol

1 mM                      EDTA (pH 8.0)

0.25% (w/v)                      Bromophenol blue

0.25% (w/v) Xylene cyanol FF  
 DEPC-treated overnight, autoclaved and stored at room temperature

### 20XSSC

175.3g NaCl  
 88.2g NaCitrate  
 pH to 7.0 using a few drops of 10M NaOH and made up to 1 litre using up H<sub>2</sub>O DEPC  
 treated and autoclaved Stored at room temperature

### Hybridization Buffer

50% (v/v) Deionized formamide  
 6X SSC  
 1% (w/v) SDS  
 0.1% (v/v) Tween 20  
 100µg/ml tRNA  
 Prepared fresh prior to use stored at room temperature

## REAGENTS FOR RIBONUCLEASE PROTECTION ASSAY

### Five percent denaturing polyacrylamide gel

A five percent denaturing polyacrylamide gel was prepared for resolution of labelled riboprobes and analysis of protected RNA fragments. The following formula was employed to determine the amount of acrylamide and bis-acrylamide required

V<sub>a</sub> = volume of acrylamide  
 V<sub>b</sub> = volume of bis-acrylamide  
 V<sub>t</sub> = total volume of gel mix (150 ml)  
 C = % crosslinking  
 A = % gel

$$V_a = \frac{A V_t}{30} \qquad V_b = \frac{A C V_t}{200}$$

$$V_a = 5 \cdot 150 / 30 = 25 \text{ ml}$$

$$V_b = 5 \cdot 5 \cdot 2 \cdot 150 / 200 = 19.5 \text{ ml}$$

### **5 % Denaturing PAGE**

63 g	Urea
25 ml	Acrylamide
19.5 ml	Bisacrylamide
15 ml	10X TBE

Made up to 150 ml with up H<sub>2</sub>O

Nine hundred µl of 10% (w/v) APS and 150 µl TEMED were added, and mixed briefly, before pouring

### **10X TBE (per 500 ml)**

54 g	Tris base
27.5 g	Boric acid
20 ml	0.5 M EDTA (pH 8.0)

One X concentration was used for polyacrylamide gel preparation

### **10% (w/v) Ammonium persulphate**

0.1 g APS/ml ultra pure H<sub>2</sub>O

### **Elution Buffer**

0.5M ammonium acetate

10.0 mM Magnesium acetate

1mM EDTA pH 8.0

0.1% SDS

made up in DEPC-treated upH<sub>2</sub>O

### **Developer (5 L)**

1.50 L H<sub>2</sub>O

1 25 L Developer

2 25 L H<sub>2</sub>O

Stirred for 2 min

**Fixer (5 125 L)**

3 625 L H<sub>2</sub>O

1 250 L A fixer

0 250 L B fixer

Stirred for 2 min

**REAGENTS FOR EMSA**

**Nuclear Protein Extraction buffers**

Buffer A

10mM Hepes pH7.9

1.5 mM MgCl<sub>2</sub>

10mM KCl

0.5mM PMSF

0.5mM DTT

Buffer C

20mM Hepes pH 7.9

420 mM NaCl

1.5mM MgCl<sub>2</sub>

0.2mM EDTA

25% glycerol

0.5 mM PMSF

0.5mM DTT

#### Buffer D

10mM Hepes pH7.9

50mM KCl

0.2mM EDTA

20% glycerol

0.5mM PMSF

0.5mM DTT

Buffer A, C and D were stored at 4°C for no more than 1 week. PMSF and DTT were added to the buffers just before use.

#### **Poly (dI-dC).Poly (dI-dC) (stock)**

Dissolve 10 U in 393.5 µl ultrapure H<sub>2</sub>O

Store in aliquots at -20°C

#### **Binding buffer (10 x)**

Glycerol                      40%

EDTA                          10 mM

Tris pH 7.5                    100mM

NaCl                            1 M

BSA                             1 mg/ml

DTT                             50 mM

#### **Bandshift polyacrylamide gel**

The formula used to calculate the volume of Accugel [40% (29:1) Acrylamide:Bisacrylamide solution] used to prepare a gel of a given percentage is as follows:

$V_a$  = volume of accugel to be used (ml)

$V_t$  = Total volume of gel casting solution required (ml)

X = % gel desired

$$V_a = \frac{(V_t)(X)}{40}$$

For a 4% or 5% gel of 50 ml volume

<u>Solution (stock)</u>	<u>Volume (ml)</u>		
	4%	5%	8%
Accugel	5 0	6 2	10 0
10xTBE	5 0	5 0	5 0
Water (ultrapure)	39 9	38 7	34 9

Then, 5µl of 1M DTT was added to the gel mixture followed by 50mg of APS and 15µl of TEMED and the gel mixture swirled briefly and poured into the gel mould. The comb was then inserted and the gel allowed to set for at least 45 min.