Modulation of apoptotic pathways in B cells by Epstein-Barr virus

by

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Abstract

Epstein-Barr virus (EBV) is a gammaherpesvirus associated with several human malignancies including Burkitt's lymphoma (BL). Latent EBV infection protects BL cell lines from apoptosis induced by genotoxic drugs including cisplatin, roscovitine and nocodazole. This protection is dependent on the co-expression of EBNA3A and EBNA3C, which downregulate the pro-apoptotic Bim, but is independent of the tumour suppressor p53 (Anderton *et al.*, Oncogene (2008) 27, 421–33). However, using the calcium ionophore ionomycin as the apoptosis-inducing agent, another pathway by which BL cells undergo apoptosis has been identified. This is independent of both Bim and p53, but can also be blocked by latent EBV.

By assessing the response of a panel BL cells expressing restricted sets of viral latent genes to ionomycin, it was shown that EBNA1, EBNA2, LMP2A and LMP2B, full-length EBNA-LP, EBNA3A, EBNA3B, EBNA3C and EBERs are not required for ionomycin-resistance although LMP1 can provide partial protection. Recombinant and revertant EBV-BACs were constructed to investigate the contribution of the EBNA3 locus and the BHRF1 locus to ionomycin-resistance. It was determined that the EBNA3 locus is not involved in rescue from ionomycin. However, loss of the BHRF1 locus partially abrogates protection against ionomycin-induced apoptosis. The pro-apoptotic BH3-only protein Noxa is consistently induced at the level of protein and mRNA in BL cells sensitive to ionomycin irrespective of p53 status. Suppression of Noxa by shRNA-expressing lentiviruses attenuates ionomycin-induced apoptosis, indicating that EBV confers resistance at least in part by inhibiting the accumulation of Noxa through a yet to be identified mechanism. This Noxa-associated apoptotic pathway is also activated by the protein kinase inhibitor staurosporine.

Further investigation using the DNA topoisomerase II inhibitor etoposide indicates that in addition EBNA3A and EBNA3C, EBV can also confer resistance against genotoxic agents through the BHRF1 locus in a Bim-independent manner.

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Declaration

All the work presented in this thesis is the result of my own work (except where indicated) and in no way forms part of any other thesis. This work was carried out while I was a student in the Department of Virology, Imperial College London under the supervision of Prof Martin Allday and Dr Rob White.

Jade Yee

List of Abbreviations

Amp	Ampicilin
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
Bam W	*Bam*HI W repeats
BART	*Bam*HI-A rightward transcript
BCR	B-cell receptor
BH	Bcl-2 homology
Bim_{EL}	Extra long Bim isoform
Bim_{L}	Long Bim isoform
Bim_{S}	Short Bim isoform
BL	Burkitt's Lymphoma
bp	Base pair
BSA	Bovine Serum Albumin
CD40L	CD40 ligand
Cm	Chloramphenicol
CtBP	C-terminal binding protein
CTL	Cytotoxic T lymphocyte
ddH_20	Double distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EBER	EBV-encoded RNA
EBNA	EBV-associated Nuclear Antigen
EBV	Epstein-Barr virus
E.coli	*Escherichia coli*
ECL	Enhanced chemiluminescence

EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium Bromide
EtOH	Ethanol
ETP	Etoposide
FCS	Foetal Calf Serum
g	Constant of gravity
GC	Germinal centre
GFP	Green fluorescent protein
Gp	Glycoprotein
HDAC	Histone deacetylase
HL	Hodgkin's lymphoma
hrs	Hours
Hyg	Hygromycin B
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Ionomycin
IR	Internal repeat
Kan	Kanamycin
kDa	KiloDaltons
KPB	Potassium Phosphate Buffer
LB	Luria-bertani broth
LCL	Lymphoblastoid Cell Line
LMP	Latent membrane protein
LN_2	Liquid nitrogen
LP	Leader protein
mRNA	Messenger RNA
min	Minutes

NP40	Nonidet P-40
NPC	Nasopharyngeal carcinoma
O/N	Overnight
OD	Optical density
oriLyt	Origin of lytic replication for EBV
oriP	Origin of replication for EBV
PAGE	Poly-Acrylamide Gel Electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline containing 0.5% v/v Tween 20
PCR	Polymerase Chain Reaction
PMSF	Phenyl methyl sulphonyl fluoride
Puro	Puromycin
RBP-Jκ	Recombinant binding protein J kappa
Rev	Revertant
RIPA	Radioimmunoprecipitation
RGU	Raji green units
RNA	Ribonucleic acid
RNase A	Ribonuclease A
RPMI	Roswell Park Memorial Institute medium
RTQ-PCR	Real-time quantitative PCR
SB	Superbroth
SDS	Sodium Dodecyl Sulphate
shRNA	Short-hairpin RNA
STET	Sucrose Triton EDTA Tris
STS	Staurosporine
SV40	Simian virus 40
TBE	Tris-Borate-EDTA

TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	Terminal repeat
Tween 20	Polyoxyethylenesorbitanmonolaurate
UV	Ultraviolet
v/v	Volume by volume
WB	Western blotting
WT	Wild-type
w/v	Weight by volume

Chapter 1 Introduction

1.1 Epstein-Barr Virus

Epstein-Barr virus (EBV) is a human γ-herpesvirus originally identified from electron micrographs of cell lines established from Burkitt's lymphoma (BL) biopsies (Epstein *et al.*, 1964). The genome of the EBV laboratory prototype B95.8 was first sequenced by Baer and colleagues (Baer *et al.*, 1984) and is composed of linear, double-stranded DNA approximately 172kb in length (Figure 1.1A). There is a 12kb deletion from the B95.8 genome which removes three open reading frames (ORFs) present in common clinical isolates (Raab-Traub *et al.*, 1980; Skare *et al.*, 1982) as shown in Figure 1.1B. Since the EBV genome was sequenced from a *Bam*HI fragment library, viral genes, promoters and ORFs are often referenced to the corresponding *Bam*HI fragment (Baer *et al.*, 1984). The EBV genome is bounded on both termini by a variable number of serially arranged 0.5kb direct terminal repeats (TR) which are required for the processing and packaging of the virion DNA into the viral capsid (Zimmermann and Hammerschmidt, 1995). There are two distinct genetic strains of EBV, Type I and Type II, which were identified by sequence variations found in a subset of genes associated with latent infection (Adldinger *et al.*, 1985). However, the biological significance and role in disease pathogenesis of this divergence remains unclear.

In addition to Burkitt's lymphoma, EBV is also associated with several human malignancies of lymphoid and epithelial origin including Hodgkin's disease (HD), post-transplant lymphoproliferative disease, nasopharyngeal carcinoma (NPC) and gastric carcinoma. The precise role of EBV in the pathogenesis of these malignancies is still unclear (Young and Rickinson, 2004). *In vitro*, EBV preferentially infects primary B lymphocytes and has the unique ability to transform quiescent B lymphocytes into permanently proliferating, latently infected lymphoblastoid cell lines (LCLs) (Henle *et al.*, 1967). The ability of the virus to transform B cells *in vitro* is a reflection of its oncogenic potential *in vivo*. Primary epithelial cells are relatively resistant to EBV infection *in vitro* (Sixbey *et al.*, 1983).

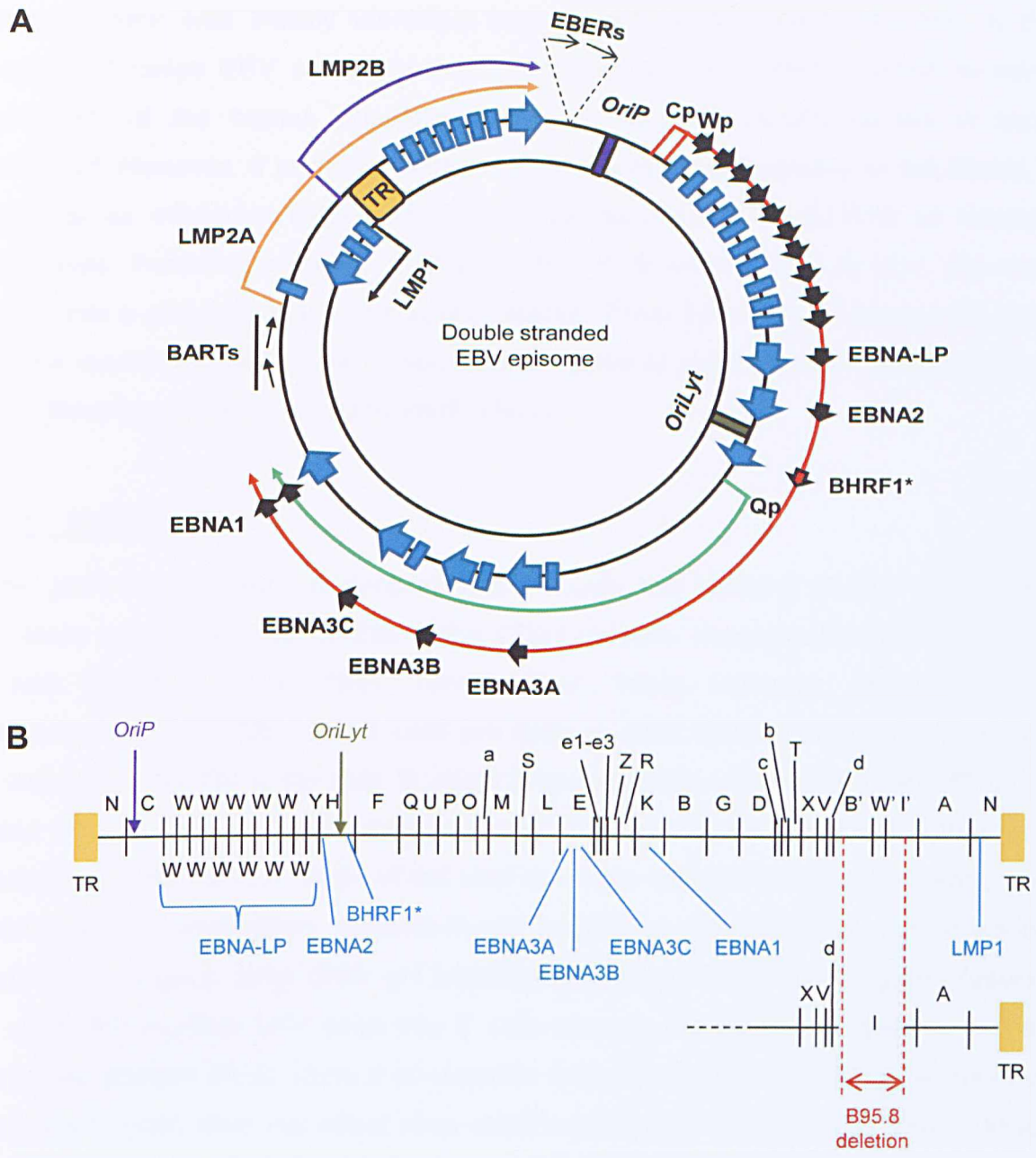


Figure 1-1 EBV genome during latency. **(A)** Schematic representation of the EBV genome in episomal form illustrating the location and transcription of latency-associated genes. Transcription of the EBV nuclear antigens (EBNAs) are initiated from the Wp, Cp or Qp promoters where individual mRNAs are generated from differential splicing of a long primary transcript. The location of *oriP*, the latent origin of replication, and *oriLyt*, origin of lytic replication, are also shown. *BHRF1, the viral Bcl-2 homologue, is expressed during latency in Wp-restricted cell lines. **(B)** *Bam*HI restriction map of the EBV genome showing the deletion carried in the prototype B95.8 strain of EBV. *Bam*HI fragments are named according to size, with A being the largest. The location of open reading frames for latent proteins are also shown. LMP2A and LMP2B transcripts span the terminal repeat (TR) region so these genes are only transcribed when the genome is circularised. Figure is not drawn precisely to scale.

Although EBV was initially identified from a tumour-associated cell line, in the majority of cases EBV establishes an asymptomatic, persistent infection in more than 95% of the human population. Primary infection usually occurs in early childhood. However, if primary infection occurs during adolescence or adulthood, it presents as infectious mononucleosis or glandular fever in 30-50% of infected individuals. Following primary infection of human B lymphocytes *in vivo*, the virus enters into a phase of lifelong persisting latency. From this state of latency, the virus can be reactivated giving rise to successive cycles of replication and re-infection of cells (Bornkamm and Hammerschmidt, 2001).

1.1.1 EBV Infection

EBV prefentially infects B lymphocytes through the binding of the major viral envelope glycoprotein gp350/220 to the CD21 receptor expressed on the surface of B cells (Nemerow *et al.*, 1987; Tanner *et al.*, 1988). However, other modes of interaction between EBV and B cells are likely to exist since a virus mutant lacking functional gp350/220 is still able to infect B lymphocytes, albeit at a lower efficiency (Janz *et al.*, 2000). Following attachment of the virus to the target cell, other viral proteins are required for fusion of the viral envelope with the cellular membrane and subsequent internalisation. Heterotrimeric complexes consisting of the envelope glycoproteins gp42, gp85 (EBV gH homologue) and gp25 (gL homologue) (Yaswen *et al.*, 1993) mediate EBV entry into B cells through the binding of gp42 to human leukocyte antigen (HLA) class II co-receptor molecules (Li *et al.*, 1997). Monoclonal antibody to gp85 does not affect virus attachment but blocks virus-cell fusion (Miller and Hutt-Fletcher, 1988). Similarly, recombinant EBV mutants which do not express gp42 cannot efficiently infect B lymphocytes (Wang and Hutt-Fletcher, 1998).

The presence of the EBV genome in NPC tumour cells (zur Hausen *et al.*, 1970; Wolf *et al.*, 1973) suggests that EBV can also infect epithelial cells *in vivo*. However, epithelial cells are relatively resistant to EBV infection *in vitro* (Sixbey *et al.*, 1984). The majority of epithelial cells susceptible to EBV infection do not express the EBV receptor, CD21, suggesting that CD21 is not important for infection of epithelial cells (Imai *et al.*, 1998). The loss of functional gp42 does not affect the ability of EBV

mutants to infect epithelial cells, confirming that gp42 is not critical for epithelial cells infection (Wang *et al.*, 1998). Recombinant EBVs which do not express gp85 is unable to adsorb to epithelial cells (Molesworth *et al.*, 2000) indicating there is a role for gp85/gp25 dimeric complexes in the interaction of EBV with an epithelial cell receptor or co-receptor, which has not yet been identified (Oda *et al.*, 2000b).

Infectivity of epithelial cells can be improved by co-culture with virus producing B cells (Imai *et al.*, 1998) suggesting that *in vivo* infection of the nasopharyngeal epithelium is dependent on initial virus replication in the lymphoid system. However, recent work by Shannon-Lowe and colleagues has shown that the rate of epithelial cell infection increases significantly when co-cultured with the virus and EBV-negative B cells (Shannon-Lowe *et al.*, 2006). The authors demonstrate that after initial binding of EBV to primary B cells, the virus can transfer efficiently to CD21-negative epithelial cells through the formation of an intracellular synapse. It was also shown that gp350-CD21 interaction and the viral glycoproteins, gp85 and gp110, are essential for this transfer interaction. The authors suggest that through binding to CD21, EBV has developed a mechanism to simultaneously access both lymphoid and epithelial systems.

Very little is known about EBV capsid dissolution, genome transport to the cell nucleus or DNA circularisation. EBV capsid transport to the nucleus is likely to be mediated by the cytoskeleton by analogy to other DNA viruses that replicate in the nucleus. Almost all EBV-infected cells carry extrachromosomal copies of the circular EBV episome (Adams and Lindahl, 1975).

1.1.2 EBV latency and persistence

EBV can be pathogenic as demonstrated by its association with various malignancies. However, EBV, like all herpesviruses, also has the ability to lie dormant or latent and persist within a host cell. In the vast majority of cases, persistent infection of humans is asymptomatic. These contradictory statements may be explained by the ability of EBV to establish different latency programmes (latency III, II, I and 0) in which subsets of viral latent genes are expressed, allowing the virus

to switch from a proliferative to a resting, immunologically silent phenotype (Thorley-Lawson, 2001).

1.1.2.1 Viral latency in vitro

In vitro infection of quiescent B cells by EBV results in the establishment of latent infection (reviewed in Kieff and Rickinson, 2001), where virus replication is generally minimal or undetectable. In EBV-immortalised B cells or LCLs, every cell carries multiple extrachromosomal copies of the viral episome and constitutively express a limited set of viral proteins, the latent proteins consisting of six EBV nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C and -LP, and three latent membrane proteins (LMPs) 1, 2A and 2B. In addition to the latent proteins, transcripts from the *Bam*HI A fragment of the viral genome (BARTs) have been detected in LCLs. Two species of non-polyadenylated (and therefore non-coding) RNAs are also expressed - EBER1 and EBER2. This pattern of EBV latent gene expression is referred to as the growth or latency III programme. These viral gene products maintain the latent infection and stimulate resting B cells to enter the cell cycle and continuously proliferate in culture. Studies conducted using virus deletion mutants and systems expressing individual EBV latent genes have shown that both EBNA2 and EBNA3C are an absolute requirement for the transformation process, and have highlighted crucial roles for EBNA1, EBNA3A and LMP1 (Bornkamm and Hammerschmidt, 2001; Dirmeier *et al.*, 2003; Hertle *et al.*, 2009).

The morphology of resting B cells changes dramatically upon infection by EBV. Transformed cells become large and irregular in shape due to the increased cytoplasmic volume required to support high rates of RNA and protein synthesis. LCLs also develop short villipodia projecting from the cell surface and strongly adhere to each other to form large clumps (Rowe, 1999). Infection by EBV causes the upregulation of the B-cell activation markers CD23, CD30, CD39 and CD70, the cell-adhesion molecules lymphocyte-function-associated antigen 1 (LFA1) and 3 (LFA3), and intercellular cell-adhesion molecule 1 (ICAM1) (Rowe *et al.*, 1987; Kieff and Rickinson, 2001). The phenotypic changes resemble those seen when resting B cells are transiently induced to become B-blasts and proliferate upon antigenic or mitogenic stimulation.

In vivo, the latency III programme serves to rapidly establish a pool of infected B cells. However, there is a reprogramming of EBV gene expression *in vivo* that limits viral gene expression to enable infected cells to persist despite cellular immune response to EBV proteins. This is discussed further in Section 1.1.2.3.

1.1.2.2 Regulation of viral latent gene expression

Following infection of B cells by EBV, the EBV genome circularises within 16-20 hours post-infection and is dependent on the activation of infected cells to the G1 stage of the cell cycle (Hurley and Thorley-Lawson, 1988). Inhibition of protein synthesis prevented circularisation of the viral genome (Hurley and Thorley-Lawson, 1988) but not viral gene transcription (Sinclair and Farrell, 1995). This suggests that transcription early after infection is from the linear rather than circular genome. The first viral mRNAs expressed after infection are transcribed from the promoter in the *Bam*HI W repeat region (Wp) by the host cell RNA polymerase II (Woisetschlaeger *et al.*, 1989). EBNA2 and EBNA-LP are the first viral proteins expressed, followed by EBNA1 (Allday *et al.*, 1989; Rooney *et al.*, 1989). Upon expression of EBNA1 and EBNA2, transcription switches from Wp to the C promoter (Cp) giving rise to transcripts encoding for EBNA-LP, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA1 (Woisetschlaeger *et al.*, 1991).

EBNA2 has been well-characterised as a transcriptional activator, which through its interaction with the DNA-binding protein recombinant binding protein-Jκ (RBP-Jκ; also known as Cp binding factor 1 or CBF1), can associate with cellular and viral promoters including Cp (Grossman *et al.*, 1994) (see Section 1.1.2.4). EBNA-LP acts as a co-activator of EBNA2-mediated transcription (Harada and Kieff, 1997) (see Section 1.1.2.4). EBNA2, together with EBNA-LP, can activate Cp through an upstream promoter element (Yoo *et al.*, 1997). EBNA1 is essential for the maintenance of the viral episome through its binding to the latent origin of replication, *oriP* (Rawlins *et al.*, 1985). *OriP* also functions as an EBNA1- and EBNA2-depedent enhancer of Cp (Reisman and Sugden, 1986). In contrast, EBNA3A, EBNA3B and EBNA3C function as transcriptional repressors of Cp. The EBNA3s bind to the same site on RBP-Jκ as EBNA2 (Johannsen *et al.*, 1996), thus acting as negative regulators of EBNA2-mediated transcription. Transcription of the latent membrane

proteins is mainly dependent on EBNA2. LMP1 and LMP2B are transcribed from an EBNA2-responsive bidirectional promoter (Laux *et al.*, 1994b; Johannsen *et al.*, 1995). EBNA2 has also been shown to be necessary for the transactivation of the LMP2A promoter (Meitinger *et al.*, 1994). Again, the EBNA3s can repress transactivation of these promoters by EBNA2 (Le Roux *et al.*, 1994; Marshall and Sample, 1995). LMP2A and LMP2B transcripts span the terminal repeat (TR) region so these genes are only transcribed when the genome is circularised (see Figure 1.1A&B). The switch to Cp-mediated transcription gives the virus a means to regulate expression of all the EBNAs as well as the LMPs, whose promoters are controlled by members of the EBNA family.

The existence of more restricted forms of latent infection became evident from the study of EBV-positive malignancies. Latency II is frequently observed in EBV-positive NPC tumours and the Reed-Sternberg cells of HD where expression of latent genes is restricted to EBNA1, the LMPs, EBERs and BARTs (Herbst *et al.*, 1991; Brooks *et al.*, 1992). During latency II, EBNA1 is transcribed from the *Bam*HI Q promoter (Qp) and Cp/Wp is silenced (Tao *et al.*, 1998). The majority of EBV-positive BL tumours exhibit type I latency where expression of latent genes is limited to EBNA1, EBERs and BARTs. Cp/Wp and LMP promoters are silenced and EBNA1 is transcribed from Qp (Schaefer *et al.*, 1991).

1.1.2.3 Viral latency and persistence in vivo

In vitro infection of resting B cells by EBV results in their activation and unregulated proliferation to form LCLs. However, as more than 95% of the human population are asymptomatically infected, uncontrolled proliferation is inhibited following EBV infection of B cells *in vivo*. In healthy carriers, the virus persists *in vivo* in a transcriptionally quiescent state within memory B cells which circulate in the peripheral blood (Miyashita *et al.*, 1997; Babcock *et al.*, 1999). Thorley-Lawson and colleagues have proposed a model in which EBV utilises its growth programme (latency III) to activate B cells and subsequently exploit the normal differentiation pathways of B cells to access the site of long-term EBV persistence, the memory B cell (Thorley-Lawson and Babcock, 1999). To achieve this, EBV uses specific latent genes to mimic the signals necessary to drive the differentiation of normal antigen

activated B cells (see Figure 1.2). The different forms of latent infection observed in EBV-associated malignancies may represent latency programmes which have evolved to accommodate persistence of the virus (see Table 1.1) (Thorley-Lawson, 2001). Gradually, during this process, there is a downregulation of viral gene expression that enables infected B cells to evade a developing cytotoxic T cell (CTL) response.

EBV is thought to spread through saliva exchange with an infected individual. The virus then enters into tonsillar crypts (Tang *et al.*, 1995) and penetrates the mucosal epithelium of the tonsils. The initial site of infection *in vivo* is thought to be resting naive B lymphocytes which reside below this epithelial layer (Thorley-Lawson and Babcock, 1999). EBV infection of naive B cells drives them to become proliferating B blasts through the expression of the growth programme or latency III. This resembles the outcome of B cell infection by EBV *in vitro*. At this stage, these EBV-infected B lymphocytes resemble normal B cells that have been activated by antigen (Wang *et al.*, 1990).

Normally, in order for antigen-activated B cells to differentiate into memory B cells, cells must undergo a germinal centre (GC) reaction. The germinal centre consists of rapidly dividing, antigen-specific B cells (centroblasts) which undergo Ig class-switching and somatic hypermutation of the immunoglobulin genes. After several divisions, cells undergo growth arrest and the resulting centrocytes are positively selected for high-affinity variants for antigen binding to exit into the memory B cell pool. Signals from the B cell antigen receptor (BCR) and T-helper cells are essential for survival in this competitive environment. All other cells die by apoptosis (McHeyzer-Williams *et al.*, 2001). EBV-infected B blasts are thought to migrate into the follicle of a lymph node, where they may form germinal centres and differentiate to become memory cells, in a similar way as antigen-activated B cells. The transcription programme switches from the growth programme to a more restricted form of transcription programme called the default programme or latency II in EBV-infected germinal centre cells (Babcock *et al.*, 2000). It is uncertain how this change occurs but it is likely to involve signals originating in the follicles. Only EBNA1, the

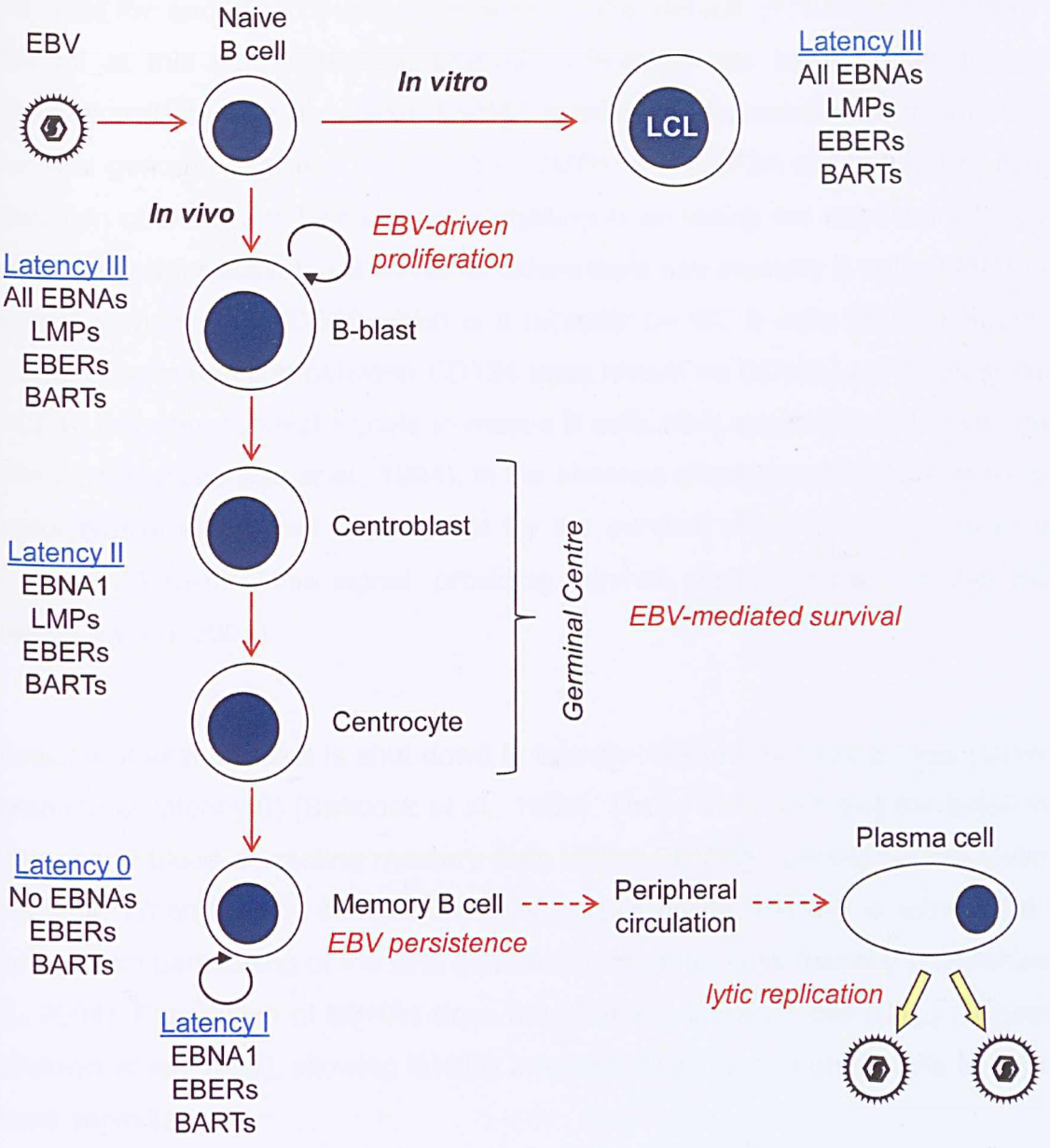


Figure 1-2 EBV persistence *in vivo*. Infection by EBV drives naive B cells to become proliferating B blasts through expression of latency-associated viral genes (latency III or the 'growth programme'). *In vitro*, EBV-transformed cells halt at the B blast or lymphoblastoid cell line (LCL) stage. *In vivo*, EBV is thought to utilise normal B cell development and differentiation pathways to gain access to its site of persistence, the memory B cell. Survival signals vital for normal B cell differentiation are mimicked through expression of a subset of viral latent genes – latency II or the 'default' programme'. EBV-infected cells leave the germinal centre and enter the memory B cell pool by shutting down expression of all viral latent proteins – latency 0 or the 'latency' programme'. When latently infected memory B cells divide, the express latency I. Memory B cells can recirculate through the peripheral blood system until stimulated to differentiate into plasma cells. This event triggers the activation of the EBV lytic cycle and production of progeny virus. This schematic is based on a model proposed by Thorley-Lawson (2001).

LMPs, EBERs and BARTs are expressed in the default programme. EBNA2 is turned off at this stage because EBNA2 expression has been shown to block differentiation (Polack *et al.*, 1996). EBNA1 expression is essential for maintenance of the viral genome (Rawlins *et al.*, 1985). LMP1 and LMP2A are thought to mimic the function of BCR and T-helper cell signalling in providing the necessary survival signals required for activated B blasts to differentiate into memory B cells. LMP1 is a functional homologue of CD40 which is a receptor on GC B cells (Zimber-Strobl *et al.*, 1996). The interaction between CD154 (also known as CD40L) on T-helper cells and CD40 provides survival signals to rescue B cells from apoptosis and drives their proliferation (Banchereau *et al.*, 1994). In the absence of antigen, the BCR delivers a non-proliferative signal that is essential for the survival of all B cells (Lam *et al.*, 1997). LMP2A mimics this signal, providing survival signals in place of the BCR (Thorley-Lawson, 2001).

Expression of viral proteins is shut down in latently infected memory B cells (latency programme or latency 0) (Babcock *et al.*, 1999). These cells then exit the tonsil into the peripheral blood as resting memory cells where EBV can persist for the lifetime of the host. When latently infected memory B cells divide, EBNA1 is expressed to ensure correct partitioning of the viral genome to daughter cells (latency I) (Hochberg *et al.*, 2004). Expression of EBNA1 does not elicit a cytotoxic T cell (CTL) response (Levitskaya *et al.*, 1995), allowing latently infected, dividing memory B cells to evade immune surveillance.

Latent virus in memory B cells can be reactivated to undergo lytic replication and release infectious virus into saliva for new infection of naive B cells. The mechanism involved in inducting reactivation of the virus is still unknown. Virus replication is thought to be associated with terminal differentiation of memory cells into plasma cells (Anagnostopoulos *et al.*, 1995; Laichalk and Thorley-Lawson, 2005). The plasma cell differentiation factor X-box-binding protein 1 (XBP-1) has been shown to mediate reactivation of EBV by activating transcription of the immediate-early gene promoters *BZLF1* and *BRLF1* (Bhende *et al.*, 2007; Sun and Thorley-Lawson, 2007).

This model was proposed based on evidence gathered from studies where distinct human B cell subsets are isolated by flow cytometry and analysed for the presence and frequency of EBV-infected cells as well as expression of viral genes by PCR. Although this model is supported by many studies, there are some issues which need to be resolved (Kuppers, 2003). Firstly, there is no evidence that naive B cells are the only primary target for EBV infection or that the expression of the growth programme is required to establish latency *in vivo*. Furthermore, *in vitro* naive and memory B cells show the same susceptibility to EBV infection so it is possible that memory B cells may also be infected by EBV *in vivo* (Ehlin-Henriksson *et al.*, 2003). Secondly, while cell-sorting experiments have indicated that the frequency of EBV-positive B cells among GC, memory and naive B cells is equal (Babcock *et al.*, 2000), immunohistochemical and *in situ* hybridisation studies of tonsillar tissues have shown that the EBV-positive B cells are rarely detected in the GC (Araujo *et al.*, 1999; Meru *et al.*, 2001). Finally, the expression of LMP1 in human B-cell lymphomas and *in vitro* studies display an inverse correlation with the expression of Bcl-6, a transcriptional repressor that controls GC differentiation in B cells (Cattoretti *et al.*, 1995; Carbone *et al.*, 1997). This suggests that proposed expression of LMP1 in GC B cells may interfere with differentiation.

A recent study has provided evidence directly linking EBV-infected B cells *in vivo* and the GC (Roughan and Thorley-Lawson, 2009). It was demonstrated that EBV-positive B cells in the tonsils retain expression of functional and phenotypic markers of GC cells, including Bcl-6 and the chemokine receptors CXCR4 and CXCR5. In addition, these cells are physically located in the GC and express the default latency programme. Thus, the expression of LMP1 in these cells does not appear to affect GC functionality *in vivo*.

Latency Programme	EBV Gene Expression	EBNA Promoter Usage	Occurence
0 (Latency)	None EBERs BARTs		Memory B cells in peripheral blood*
I	EBNA1 EBERs BARTs	Qp	Burkitt's lymphoma† Dividing memory B cells*
II (Default)	EBNA1 LMP1 LMP2A EBERs BARTs	Qp	Hodgkin's lymphoma† Nasopharyngeal carcinoma† Centroblasts and centrocytes*
III (Growth)	EBNA1 EBNA2 EBNA3A EBNA3B EBNA3C EBNA-LP LMP1 LMP2A&2B EBERs BARTs	Wp, Cp	EBV-immortalised lymphoblastoid cell lines (LCLs) Post-transplant lymphoproliferative disease† Activated B-blasts*

Table 1.1 EBV Latency Programmes. EBV latent gene expression and promoter usage associated with latency programmes. *Normal EBV life cycle and †EBV-associated lymphomas. Adapted from Thorley-Lawson, 2001.

1.1.2.4 Viral latent genes

EBNA1

EBNA1 is the only EBV nuclear antigen expressed in all dividing EBV-infected cells. The EBNA1 protein is separated into N- and C-terminus domains by a glycine-

alanine (Gly-Ala) repeat sequence which functions to stabilise the mature protein by preventing proteasomal breakdown (Levitskaya *et al.*, 1995).

EBNA1 is responsible for the maintenance and replication of the viral episome through sequence-specific binding of its C-terminal DNA-binding and dimerisation domain to the viral origin of replication, *oriP* (Rawlins *et al.*, 1985; Lee *et al.*, 1999). *OriP* consists of 20 EBNA1 binding sites arranged in an array of multiple tandem repeats (FR - family of repeats) and four sites arranged in dyad symmetry (DS element) (Yates *et al.*, 1984; Rawlins *et al.*, 1985). The binding of EBNA1 to the DS element enables DNA replication from this element (Gahn and Schildkraut, 1989; Yates *et al.*, 2000), while binding to the FR element is required for the partitioning of viral episomes (Lupton and Levine, 1985).

EBNA1 can also contribute to the transcriptional regulation of its own expression and that of the other EBNAs. It can drive transcription from the common EBNA promoter Cp (Reisman and Sugden, 1986; Sugden and Warren, 1989) and the LMP1 promoter (Gahn and Sugden, 1995) via binding to the FR element of *oriP* (Puglielli *et al.*, 1996). The EBNA1-exclusive promoter Qp (see Figure 1.1A) contains two EBNA1 binding sites allowing EBNA1 to negatively regulate its own expression (Sample *et al.*, 1992; Sung *et al.*, 1994).

There is speculation that EBNA1 may act as a transcription factor modulating the expression of cellular genes potentially important for oncogenesis including recombinase activating genes Rag1 and Rag2 (Srinivas and Sixbey, 1995), Bcl-xL (Tsimbouri *et al.*, 2002) and CD25 (Kube *et al.*, 1999). However, the role of EBNA1 as a transcription factor for cellular genes is still debatable. Kang and colleagues have suggested that EBNA1 exclusively activates promoters in the episomal viral DNA and does not operate on viral promoters integrated into the host genome or on cellular promoters (Kang *et al.*, 2001).

Gene knockout studies reveal that EBNA1 does not have a crucial role in *in vitro* transformation beyond the maintenance of the viral genome (Humme *et al.*, 2003).

The role of EBNA1 in oncogenesis is still unclear as demonstrated in two independent studies conducted to assess the tumorigenic potential of EBNA1 by generating mice transgenic for EBNA1. While Wilson and colleagues observed the development of B cell lymphomas in one model (Wilson *et al.*, 1996), Kang and colleagues found no evidence to suggest that EBNA1 can promote B-cell lymphomagenesis (Kang *et al.*, 2005).

EBNA2

EBNA2 was the first viral protein shown to be essential for transformation of B lymphocytes *in vitro*. The P3HR1 virus, a strain of EBV which carries a deletion of the gene encoding EBNA2 and the unique Y1Y2 exons of the gene encoding EBNA-LP (Figure 1.3), is transformation-incompetent (Miller *et al.*, 1974). Reinsertion of the EBNA2 gene in P3HR1 restored transforming ability (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989). EBV genomes from other BL-derived cell lines such as Daudi, Sal-BL and Oku-BL (see Figure 1.3) were subsequently found to carry deletions similar to that found in P3HR1 (Jones *et al.*, 1984; Kelly *et al.*, 2002). The deletions found in these cell lines also encompass regions of the BHLF1 gene and *oriLyt*.

Analysis of EBNA2 from numerous virus isolates provided evidence that there are two distinct genetic strains of EBV, type 1 and type 2. The sequence similarity between type 1 and type 2 EBNA2 is only approximately 55% while there is little variation in most other EBV genes (Bornkamm *et al.*, 1982; Adldinger *et al.*, 1985). The difference in transforming ability of EBV type 1 and type 2 strains (type 1 more efficient than type 2) was attributed to the differences in the EBNA2 genes (Rickinson *et al.*, 1987; Tomkinson and Kieff, 1992a).

EBNA2 functions as a crucial transactivator of many cellular and viral genes. EBNA2 does not bind DNA directly but instead interacts with cellular transcription factors such as the DNA binding protein, recombination binding protein 1 for Jκ or RBP-Jκ (also known as Cp binding factor 1 or CBF1) (Grossman *et al.*, 1994; Zimber-

Strobl *et al.*, 1994) and the *ets* family protein Spi-1/PU.1 (Laux *et al.*, 1994b) to activate transcription.

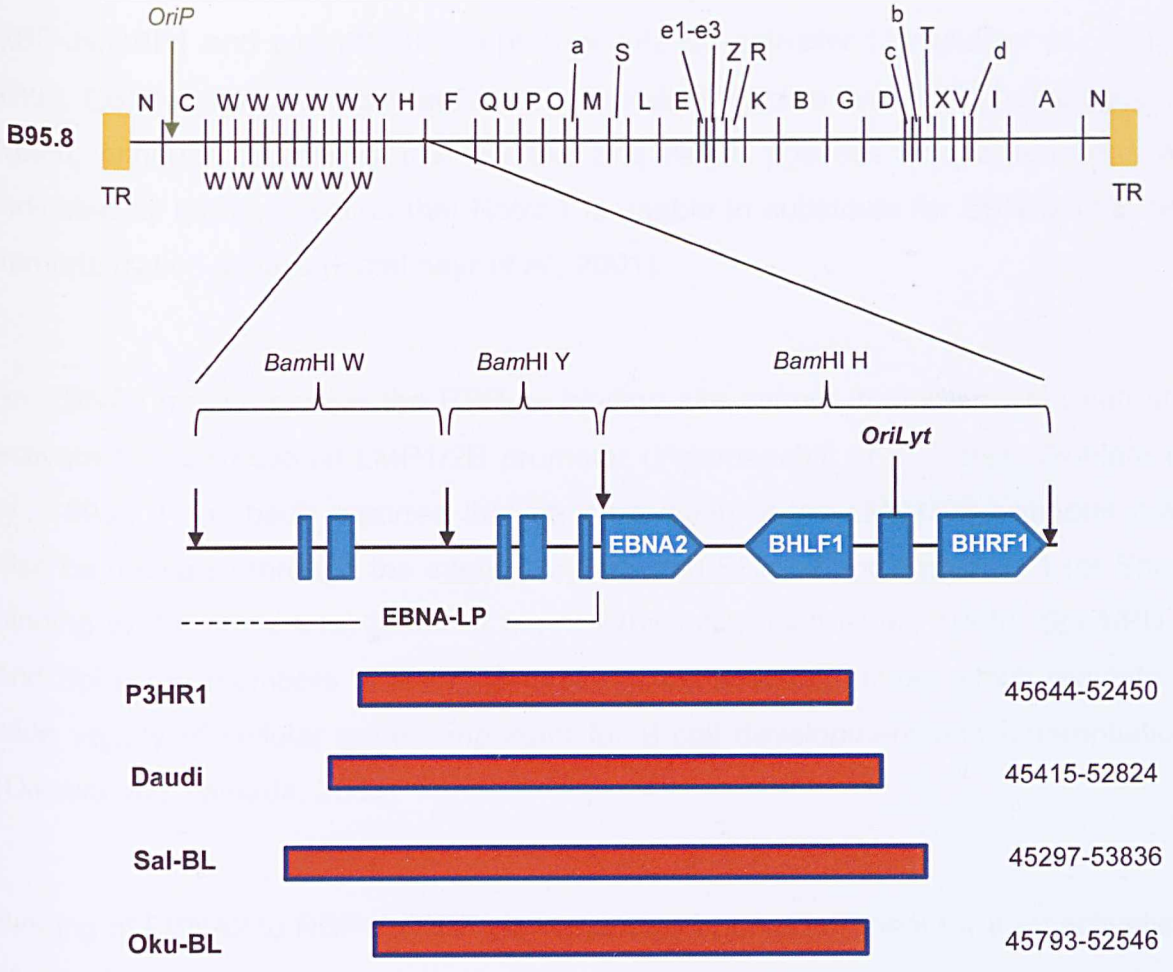


Figure 1-3 EBNA2 deletion found in the EBV genome of Wp-restricted BL cell lines is similar to P3HR1 deletion. Location of the EBNA2 gene deletion in Wp-restricted BL cell lines, Sal-BL and Oku-BL, shown in a *Bam*HI restriction map of the B95.8 EBV genome. Deletion coordinates (numbered according to the B95.8 sequence) are shown relative to the deletion found in the P3HR1 and Daudi EBV strains. Most deletions shown also encompass regions of the BHLF1 gene and *oriLyt*. Adapted from Kelly *et al.*, 2002. Figure is not drawn precisely to scale.

RBP-Jκ/CBF1 is a ubiquitously expressed protein and a component of the Notch signalling pathway. RBP-Jκ/CBF1 normally inhibits transcription by recruiting a co-repressor complex to the promoter. Binding sites for RBP-Jκ/CBF1 have been identified in all viral promoters responsive to EBNA2: Cp, LMP1/2B and LMP2A (Ling

et al., 1993; Zimber-Strobl *et al.*, 1993; Laux *et al.*, 1994b). Interaction of EBNA2 with RBP-Jκ/CBF1 displaces the co-repressor complex, and instead recruits transcriptional co-activators (Hsieh *et al.*, 1996; Sakai *et al.*, 1998). Upon ligand-mediated activation of Notch, cleaved Notch migrates to the nucleus and binds to RBP-Jκ/CBF1 and converts this repressor into an activator (Jarriault *et al.*, 1995). Thus, EBNA2 may be regarded as a constitutively active functional homologue of Notch, although it is likely that EBNA2 and Notch possess unique functions as indicated by the observation that Notch1 is unable to substitute for EBNA2 in B-cell immortalization assays (Hofelmayr *et al.*, 2001).

An EBNA2 mutant lacking the RBP-Jκ binding site cannot immortalise but can still activate the bidirectional LMP1/2B promoter (Yalamanchili *et al.*, 1994; Sjoblom *et al.*, 1995). It has been reported that transactivation of the LMP1/2B promoter may also be mediated through the interaction between EBNA2 and Spi-1/PU.1 (or Spi-B binding to the same site) (Laux *et al.*, 1994b; Johannsen *et al.*, 1995). Spi-1/PU.1 and Spi-B are members of the ETS family of transcription factors which regulate a wide variety of cellular genes important for B-cell development and differentiation (Oikawa and Yamada, 2003).

Binding of EBNA2 to RBP-Jκ/CBF1 is necessary but not sufficient for transactivation of the LMP2A promoter (Meitinger *et al.*, 1994). Other transcription factors involved in the activation of the LMP2A promoter have not yet been identified. The transactivation domain of EBNA2 has also been shown to interact with the transcription machinery (Tong *et al.*, 1995), histone acetyltransferases p300, CBP and PCAF (Wang *et al.*, 2000) and the chromatin remodelling protein hSNF5/Ini1 (Wu *et al.*, 1996).

The EBNA3 Family

EBNA3A, 3B and 3C are members of a family of nuclear proteins encoded by tandemly arranged genes, and transcribed from a single common promoter, Cp (see Figure 1.1A). The encoded EBNA3 proteins are likely to have distinct functions, despite modest homology (see Figure 1.4). Studies using EBV recombinant viruses

have shown that EBNA3C is essential for B cell immortalisation *in vitro* but EBNA3B is dispensable (Tomkinson and Kieff, 1992b; Tomkinson *et al.*, 1993). A recent study has demonstrated that while EBNA3A is not an absolute requirement for B cell immortalisation *in vitro*, it is crucial for the efficient outgrowth of LCLs (Hertle *et al.*, 2009). As previously observed with EBNA2, there are differences in nucleotide and predicted amino acid sequences between EBV type I and type II EBNA3 genes (Sample *et al.*, 1990). However, unlike EBNA2, substitution of any of type I EBNA3s in a type II background and vice versa does not alter the transformation efficiency of the virus (Tomkinson and Kieff, 1992a).

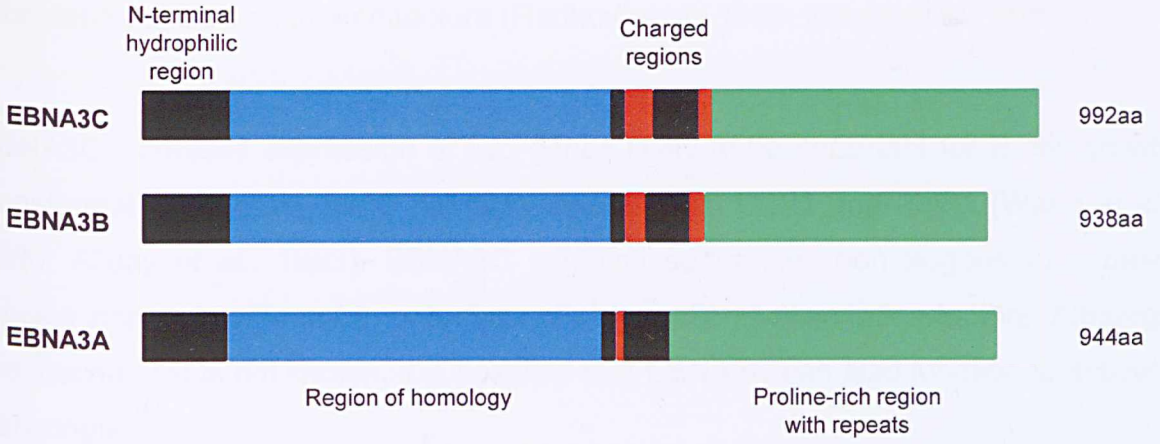


Figure 1-4 Structural and sequence homology between the EBNA3 family of proteins. The EBNA3 proteins share a common 90-95 amino acids (aa) hydrophilic N-terminal region. The 220-230aa region of homology (shown in blue) shows less than 28% amino acid identity. The EBNA3 proteins also contain a region of short sequences rich in positively- (shown in black) and negatively-charged (shown in red) amino acids. The C-terminal half is a proline-rich region containing repeated polypeptide domains (shown in green). Adapted from Le Roux *et al.*, 1994.

Despite these differences, all three EBNA3 proteins have been found to associate with the cellular DNA binding protein RBP-Jκ/CBF-1, and this interaction is mediated through a homologous domain in their N-terminal regions (Le Roux *et al.*, 1994; Robertson *et al.*, 1996; Waltzer *et al.*, 1996). EBNA2 also binds to RBP-Jκ and the binding of EBNA2 and EBNA3 proteins are mutually exclusive (Johannsen *et al.*, 1996). As such, it has been demonstrated that EBNA3A and EBNA3C can inhibit transcriptional activation of some EBNA2-responsive genes, including its own

promoter Cp, by preventing EBNA2 from binding to RBP-Jκ (Radkov *et al.*, 1997; Cludts and Farrell, 1998).

The ability of EBNA3A and EBNA3C to function as transcriptional repressors, is in part due to their ability to bind the co-repressor C-terminal binding protein (CtBP) (Touitou *et al.*, 2001; Hickabottom *et al.*, 2002). The interaction EBNA3A and EBNA3C with CtBP was also shown to be important in the ability of both EBNA3A and EBNA3C to immortalise and transform primary rat embryo fibroblasts in cooperation with activated ras (Parker *et al.*, 1996). EBNA3C has been shown to bind to histone deacetylases (HDAC) and may repress transcription through the modulation of chromatin architecture (Radkov *et al.*, 1999; Knight *et al.*, 2003).

EBNA3C increases expression of two genes likely to be important for B cell growth transformation by EBV, the B cell activation antigen CD21 and LMP1 (Wang *et al.*, 1990; Allday *et al.*, 1993). EBNA3C contains sequences homologous to a basic leucine zipper motif found in one class of mammalian transcription factors. Although the mechanism is not known, it is possible that EBNA3C can also function to activate transcription.

When overexpressed, EBNA3C can disrupt cell cycle check points at G1/S and at mitosis. This deregulation of the cell cycle could be associated with the ability of EBNA3C to interact with cyclin A and to enhance the activity of cyclin A/cyclin dependent kinase complexes (Knight and Robertson, 2004). Very little is known about EBNA3B, but like EBNA3A and EBNA3C, it can bind RBP-Jκ and act as a repressor of transcription (Paul Young - personal communication).

EBNA-LP

EBV nuclear antigen leader protein or EBNA-LP is composed of a N-terminal domain encoded by repeating W1 and W2 exons of the major internal repeat (IR1 or *Bam*HI W repeat region) and a C-terminal domain derived from two exons Y1 and Y2 located in the long unique region (Bodescot *et al.*, 1984; Speck *et al.*, 1986) (Figure

1.1B). EBNA-LP is highly variable in size due to variations in the number of W repeat exons in different virus isolates (Dillner *et al.*, 1985; Finke *et al.*, 1987). EBNA-LP and EBNA2 are the first viral genes transcribed upon infection of primary B lymphocytes with EBV (Allday *et al.*, 1989; Rooney *et al.*, 1989). Although EBNA-LP is not absolutely essential for immortalisation of primary B lymphocytes *in vitro,* it is critical for efficient outgrowth of transformed cells (Hammerschmidt and Sugden, 1989; Mannick *et al.*, 1991).

EBNA-LP is a co-activator of EBNA2-mediated transcription (Harada and Kieff, 1997) although it appears to specifically augment EBNA2-mediated transcription from the viral promoters, Cp and LMP1/2B promoter (but not LMP2A), rather than all EBNA2 targets (Peng *et al.*, 2005). Co-activation of transcription by EBNA-LP in may be mediated by its association promyelocytic leukaemia (PML) nuclear body-associated protein Sp100. This interaction displaces Sp100 and heterochromatin protein 1α (HP1α) from PML nuclear bodies (Ling *et al.*, 2005). The displacement of both Sp100 and HP1α from PML nuclear bodies by EBNA-LP is thought to prevent their transcriptional repressor function (Lehming *et al.*, 1998; Seeler *et al.*, 1998).

The finding that phosphorylation of EBNA-LP is dependent on the cell cycle stage has indicated that EBNA-LP may play a role in cell cycle regulation (Kitay and Rowe, 1996). It has also been reported that EBNA-LP, together with EBNA2, can induce cyclin D2 and cell cycle activation in primary human B cells pre-activated by stimulation of CD21 wth gp350 (Sinclair *et al.*, 1994). EBNA-LP was reported to co-localise with retinoblastoma (Rb) proteins in the nucleus and bind *in vitro* to p53 and Rb (Szekely *et al.*, 1993). However, there is no evidence for these interactions in LCLs or that the function of p53 and Rb is modified due to EBNA-LP binding (Inman and Farrell, 1995; Allday *et al.*, 1995a). The expression of EBNA-LP in transgenic mice had no apparent oncogenic consequences (Huen *et al.*, 1993).

LMP1

The LMP1 gene is located close to the right-hand terminus of the linear viral genome and is transcribed leftwards (see Figure 1.1A). LMP1 protein is comprised of a small

cytoplasm-orientated N-terminal domain, followed by six transmembrane domains separated from each other by short turns and a larger cytoplasmic domain at the C terminus. LMP1 is essential for the efficient immortalisation of primary B cells by EBV (Kaye *et al.*, 1993). Two regions of the C-terminal domain known as C-terminal activation regions (CTAR)-1 and -2 (also known as C-terminal transformation effector sites (TES)-1 and -2) have been identified as being essential for efficient B-cell growth transformation (Izumi *et al.*, 1997a; Izumi and Kieff, 1997b). LMP1 is able to activate the nuclear factor κB (NFκB) pathway through the association of tumour necrosis factor receptor (TNFR)-associated factors (TRAFs) to CTAR1 and CTAR2 (indirectly via TNFR-associated death domain protein or TRADD) (Hammarskjold and Simurda, 1992; Huen *et al.*, 1995; Izumi and Kieff, 1997b). Following infection of B lymphocytes by EBV, the LMP1 CTAR1 site can interact with TRAFs to mediate a low level activation of NFκB that drives initial proliferation of infected cells. The majority of NFκB activation is mediated via binding of TRADD to the LMP1 CTAR2 site and is required for the continued proliferation of EBV-infected B cells in culture (Izumi and Kieff, 1997b).

The signalling transduction activities of LMP1 also stem from its ability to interact with TRAFs. The activation of NFκB through sequential activation of NF-κB-inducing kinase (NIK), inhibitors of κB kinase (IKK-α and IKK-β), and inhibitor of κB (IκB-α) is mediated by interaction with TRAF2 (Sylla *et al.*, 1998). The c-jun N-terminal kinases (JNK) and p38 mitogen-activated protein kinases are also activated by LMP1 (Eliopoulos *et al.*, 1999; Kieser *et al.*, 1999). A third activation domain CTAR3 has been identified through its interaction with Janus-activated kinase (JAK)3, which results in activation of the signal transducer and activation of transcription (STAT) factors (Gires *et al.*, 1999). LMP1 modulates the expression of cellular genes such as adhesion molecules associated with antigen activation of B lymphocytes, anti-apoptotic proteins (such as Bcl-2 and A20) and various cytokines through these signalling pathways.

LMP1 acts like a constitutively active receptor of the TNFR family and activates several signalling pathways in a ligand-independent manner through its N-terminal

and transmembrane domains (Floettmann and Rowe, 1997; Gires *et al.*, 1997). LMP1 is functionally similar to CD40, another member of the TNFR family, and can partially substitute for CD40 *in vivo* (Eliopoulos *et al.*, 1996; Zimber-Strobl *et al.*, 1996). However, there are distinct differences as well. In a study conducted using LMP1 transgenic mice in CD40-knockout or wild-type backgrounds revealed that constitutive expression of LMP1 mimics CD40 signals to induce extrafollicular B cell differentiation but, unlike CD40, blocks germinal centre formation (Uchida *et al.*, 1999).

LMP2A & 2B

LMP2A and LMP2B are integral plasma membrane proteins which share their 12 transmembrane domains separated by short reverse turns and a short C-terminus domain. LMP2A carries an additional 119 amino acid N-terminal domain with eight tyrosine residues encoded by a separate first exon. The genes encoding LMP2A and LMP2B are located in the between *Bam*HI N fragment and span the terminal repeat region (see Figure 1.1A&B). Circularisation of the viral genome through the terminal repeats is therefore necessary for the transcription of LMP2A and LMP2B (Laux *et al.*, 1988; Sample *et al.*, 1989). LMP2A and LMP2B are dispensable for *in vitro* transformation of B cells by EBV (Longnecker *et al.*, 1993a; Longnecker *et al.*, 1993b).

LMP2A is associated with the Src family of tyrosine kinases. Two tyrosines located at positions 74 and 85 (Tyr74 and Tyr85) in the N-terminal domain of LMP2A form an immunoreceptor tyrosine activation motif (ITAM), and a tyrosine at position 112 (Tyr112) forms a consensus Src binding site. Once phosphorylated, the ITAM recruits Src family kinases and Syk tyrosine kinase and negatively regulates their activities (Burkhardt *et al.*, 1992; Beaufils *et al.*, 1993). LMP2A is also a substrate of the Src kinases. Tyr112 binds to Lyn and mediates the constitutive phosphorylation of the other tyrosine residues in LMP2A (Longnecker, 2000). This results in the inhibition of B cell receptor (BCR) signaling by sequestering these protein tyrosine kinases (Fruehling *et al.*, 1996) and by blocking translocation of the BCR into lipid rafts (Dykstra *et al.*, 2001). As BCR signalling has been shown to induce lytic replication in latently infected cells, so the blockade of BCR signalling by LMP2A

might be important for maintaining latency (Miller *et al.*, 1994b). Mutation of Tyr112 abolishes the ability of LMP2A to inhibit BCR signalling (Fruehling *et al.*, 1998).

Given that it lacks the N-terminal domain, LMP2B is unable to interact with Lyn and Syk. It has been suggested that LMP2B negatively regulates LMP2A by forming aggregates with it (Bornkamm and Hammerschmidt, 2001). LMP2A signalling also appears to be regulated through its association with Nedd4 ubiquitin ligases, which modulate levels of LMP2A and its associated proteins through ubiquitin-mediated protein degradation (Ikeda *et al.*, 2000; Winberg *et al.*, 2000).

In vivo expression of LMP2A in the B cells of transgenic mice provides a developmental and survival signal in the absence of BCR signalling (Caldwell *et al.*, 1998). It has also been demonstrated that LMP2A can rescue differentiation of B cells in a background where a functional BCR is absent (Caldwell *et al.*, 2000). Thus, *in vivo* LMP2A mimics a constitutively active BCR that provides survival signals important for long term persistence of latently infected B cells.

EBERs

The EBERs are two small non-polyadenylated, and therefore non-coding, RNAs which are abundantly expressed in latently infected cells (Rymo, 1979). The genes encoding EBER1 and EBER2 lie adjacent to each other immediately upstream of oriP (see Figure 1.1A). EBER1 and EBER2 are transcribed by cellular RNA polymerase III to yield transcripts of 167 and 172 nucleotides long respectively (Rosa *et al.*, 1981). Although the sequence similarity between EBER1 and EBER2 is only 54%, the EBERs have similar secondary structures – extensive base-paired structures containing several stem loops (Rosa *et al.*, 1981). The EBERs are also structurally highly conserved across different EBV strains (Arrand *et al.*, 1989). The EBERs localise to the nucleus (Fok *et al.*, 2006) and form ribonucleoprotein complexes with the La protein (Lerner *et al.*, 1981) and the ribosomal protein L22 (predominantly EBER1) (Toczyski and Steitz, 1993; Toczyski *et al.*, 1994). The EBERs are not essential for the immortalisation of primary B lymphocytes by EBV or for the replication of the virus (Swaminathan *et al.*, 1991) . However, it has been

demonstrated that EBER2, but not EBER1, contributes to the efficient growth of EBV-transformed B cells (Wu *et al.*, 2007). This is probably due to the induction of IL-6 by EBER2, but not EBER1.

EBERs can bind to and inhibit activation of the interferon-inducible protein PKR (Clarke *et al.*, 1991; Sharp *et al.*, 1993). It has also been demonstrated that EBERs can protect BL cells against IFN-α–induced apoptosis (Nanbo *et al.*, 2002) and epithelial cells against Fas-mediated apoptosis (Nanbo *et al.*, 2005) through inhibition of PKR. However, there have been several reports that the EBERs do not modulate interferon responses in EBV-transformed B cells and that PKR is not a direct target of the EBERs *in vivo* (Swaminathan *et al.*, 1992; Ruf *et al.*, 2005).

The EBERs might contribute to the pathogenesis of various EBV-associated malignancies. For example, EBERs can induce expression of IL-10, which is thought to act as an autocrine growth factor in BL cells, through RIG-I-mediated IRF3 signalling (Kitagawa *et al.*, 2000; Samanta *et al.*, 2008). Similarly, the EBERs have also been implicated in the induction of insulin-like growth factor 1 (IGF-1) in EBV-positive gastric carcinoma cells (Iwakiri *et al.*, 2003).

BARTs

The BARTs are a group of differentially spliced RNAs encoded by the *Bam*HI-A region of the EBV genome and were originally identified in NPC tumour cells, where they are abundantly expressed (Hitt *et al.*, 1989). BART transcripts were later shown to be detectable in peripheral blood of healthy EBV carriers (Chen *et al.*, 1999) and expressed at lower levels in other EBV-associated malignancies including gastric carcinoma (Sugiura *et al.*, 1996) and Burkitt's lymphoma (Smith *et al.*, 2000).

The BARTs are expressed in all three forms of EBV latency *in vitro* (Brooks *et al.*, 1993) but are not essential for immortalisation of primary B cells *in vitro* (Robertson *et al.*, 1994; Kempkes *et al.*, 1995). Approximately 12kb of the 20kb DNA sequence

encoding the BART transcripts is deleted in the transformation-competent, prototype laboratory strain B95.8 (Baer *et al.*, 1984; Parker *et al.*, 1990).

Several putative open reading frames (ORFs) have been identified within the BART transcripts (Smith *et al.*, 2000). However, proteins encoded by these ORFs remain undetectable within EBV-positive BL and NPC cells (van Beek *et al.*, 2003; Al-Mozaini *et al.*, 2009). The putative BART ORFs are thought to encode the proteins: BARF0 (Fries *et al.*, 1997), RK-BARF0 (a longer differentially spliced form of BARF0) (Kienzle *et al.*, 1999), RPMS1 and A73 (Smith *et al.*, 2000).

It has been reported that artificially expressed RK-BARF0 and RPMS1 can interact with components of the Notch signalling pathway. RK-BARF0 has been shown to interact with the Notch4 ligand binding domain, resulting in the translocation of unprocessed Notch4 to the nucleus (Kusano and Raab-Traub, 2001). It has been suggested that this interaction is responsible for the induction of LMP1 by RK-BARF0 in EBNA2-deleted P3HR1 BL cells. RPMS1 has been shown to interact with RBP-Jκ/CBF-1 and its co-repressor, CBF-1 interacting co-repressor (CIR) (Smith *et al.*, 2000; Zhang *et al.*, 2001). This interaction stabilizes the RBP-Jκ/CBF-1-CIR repressor complex on the DNA and can lead to the inhibition of EBNA2-mediated transactivation of a promoter. The functions of these proteins in the regulation of LMP1 are analogous to that of EBNA2 (RK-BARF0) and the EBNA3s (RPMS1). These proteins may therefore be important in Latency II where EBNA2 and the EBNA3s are absent.

A73 was reported to interact with the cytoplasmic receptor for activated protein kinase 1 (RACK1) (Smith *et al.*, 2000) and can potentially influence RACK1 regulation of protein kinase C (PKC) and *Src* tyrosine kinase signalling.

EBV-encoded microRNAs

EBV encodes microRNAs in two primary transcripts: BHRF1, which also encodes the viral Bcl-2 homologue, and BART (Skalsky and Cullen, 2010). The BHRF1 miRNAs

are encoded within the intronic region of the BHRF1 mRNA. miR-BHRF1-1 is positioned in the 5'UTR while miR-BHRF1-2 and miR-BHRF1-3 is located in the 3'UTR of the BHRF1 mRNA. 22 miRNAs are located within three clusters in introns of BART transcripts. Most of the BART miRNAs are deleted in the transformation-competent B95.8 EBV strain (see Figure 1.5) leaving only miR-BART1, 2, 3, 4 and 15 still present in B95.8. This indicates that the majority of BART miRNAs are not required for growth transformation of B cells *in vitro*. However, BART miRNAs could still be important for transformation of epithelial cells or B cells *in vivo*.

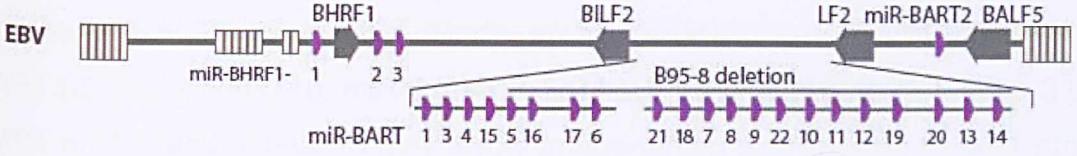


Figure 1-5 EBV-encoded microRNAs. Diagram illustrating the location of viral microRNAs encoded in the BHRF1 and BART transcripts. The BART gene deletion found in the EBV B95.8 strain is also shown. Adapted from Skalsky and Cullen, 2010.

Analysis of miRNA expression in EBV-infected B cells has revealed that the BHRF1 miRNAs are expressed at high levels in latency III B cells. The BHRF1 miRNAs are undetectable or present at very low levels in latency I B cells and latency II epithelial cells (Cai *et al.*, 2006). A recent study using stem-loop, quantitative real-time PCR to measure expression levels of viral miRNAs showed that miBHRF1-3 was detectable at similar steady-state levels in Oku-BL, which displays a Wp-restricted latency (see Section 1.1.4), and latency III BL-derived cells. Expression of viral miRNAs in Latency I BL cells was estimated to be approximately <20% of the levels observed in latency III BLs (Pratt *et al.*, 2009).

BART miRNAs were found to be expressed at high levels in latently infected epithelial cells but at lower, albeit detectable levels in B cells (Cai *et al.*, 2006). The steady-state levels of BART miRNAs were not dependent on the number of viral genomes per cell (Pratt *et al.*, 2009). The expression of individual BART miRNAs

within a single cell can differ dramatically even though it is like that they arise from one primary transcript (Edwards *et al.*, 2008; Pratt *et al.*, 2009). Although the levels of BART miRNAs differ among infected cells, the abundance of invidual BART miRNAs relative to each other were fairly consistent.

Some viral targets of EBV-encoded miRNAs have been identified. For example, miR-BART2 is transcribed antisense to the viral DNA polymerase BALF5 and can target BALF5 mRNA for degradation (Pfeffer *et al.*, 2004). Since BALF5 is a lytic gene and induction of the lytic cycle reduces miR-BART2 levels, it has been suggested that miR-BART2 regulates the switch from latent to lytic replication by preventing premature BALF5 expression (Barth *et al.*, 2008). Three BART miRNAs (miR-BART1-5p, miR-BART16, miR-BART17-5p) have been shown to target the 3'UTR of LMP1 mRNA and appear to play a role in fine-tuning expression of LMP1 protein in EBV-infected NPC cells (Lo *et al.*, 2007). Another study conducted using NPC cells reported that LMP2A expression can be regulated by miR-BART22 (Lung *et al.*, 2009).

EBV-encoded miRNAS also target cellular genes involved in cell proliferation, cell survival and antiviral defence pathways. The p53-upregulated modulator of apoptosis or Puma is targeted by miR-BART5 in NPC cells (Choy *et al.*, 2008). Inhibition of miR-BART5 led to an increase in apoptosis mediated by Puma in EBV-infected epithelial cells. Downregulation of the interferon-inducible T-cell attracting CXC-chemokine ligand 11 (CXCL11) by miR-BHRF1-3 may serve as an immunomodulatory mechanism in EBV-positive lymphomas (Xia *et al.*, 2008). The major histocompatibility complex class I-related chain B (MICB) is targeted by miR-BART2 (Nachmani *et al.*, 2009). MICB, which is induced by viral infection, is a ligand for natural killer (NK) and CD8+ T cells. Suppression of MICB by miR-BART2 leads to a reduction in NK cell-mediated killing of infected cells.

1.1.3 Lytic replication

Lytic replication of EBV permits the virus to spread within a population of individuals and from cell to cell. During lytic replication, viral genomes are amplified to

thousands of copies per cell for assembly into viral particles (Hammerschmidt and Sugden, 1988). The mechanisms which trigger the switch from latent infection to lytic replication *in vivo* is still not completely understood but expression of viral genes associated with latency and lytic replication are generally considered mutually exclusive (Kieff and Rickinson, 2001). EBV, like other herpesviruses, encodes a set of lytic replication enzymes and factors involved in nucleotide biosynthesis, RNA processing, viral DNA replication, and capsid and viral envelope synthesis.

The switch from EBV latent infection to lytic replication is governed by the immediate-early genes *BZLF1* and *BRLF1* which encode the transcriptional activator proteins (Countryman *et al.*, 1987; Hardwick *et al.*, 1988). Both BZLF1 and BRFL1 are essential for the switch to lytic replication (Feederle *et al.*, 2000). BZLF1 is capable of auto-regulating its own promoter, Zp, and that of BRFL1, Rp (Flemington and Speck, 1990). Most early lytic genes that are expressed before the onset of viral DNA replication are dependent on the BZLF1 or BRLF1 protein transcription factors for their expression (Kieff and Rickinson, 2001).

The origin of viral DNA replication that mediates replication during the lytic cycle is termed *oriLyt*. In most EBV strains, *oriLyt* is present twice in the EBV genome and is located approximately opposite each other on a circular viral episome (Hammerschmidt and Sugden, 1988). Viral strains which only carry one copy of *oriLyt*, such as B95.8, appear to replicate equally well. BZLF1 protein binds to and activates a promoter that serves as an integral component of *oriLyt* (Schepers *et al.*, 1996). DNA replication mediated by *oriLyt* is also dependent on six viral lytic replication proteins: BALF5 (DNA polymerase) (Hammerschmidt and Sugden, 1988), BMRF1 (polymerase proccesivity factor), BALF2 (single-stranded DNA-binding protein), BSLF1 (primase), BBLF4 (helicase) and BBLF2/3 (helicase-primase-associated protein) (Fixman *et al.*, 1992).

BZLF1 protein can also interact with the transcription factor NF-κB, the tumour suppressor p53 and virion proteins. Interaction with NF-kB inhibits the transactivating effects of BZLF1 (Gutsch *et al.*, 1994). Similarly, association with p53 appears to

transcriptionally inactivate both proteins (Zhang *et al.*, 1994). This could be important in preventing p53-induced apoptosis in response to lytic EBV DNA replication. Later in lytic infection, BZLF1 becomes associated with virion structural proteins, although BZLF1 has not been detected in virions (Katz *et al.*, 1992). It has been postulated that these interactions could downregulate BZLF1 activity late in infection so as to minimise the toxic effects of high-level BZLF1 expression.

A viral Bcl-2 homologue, BHRF1, is expressed early after lytic induction (Pearson *et al.*, 1987). BHRF1 protein localises to the nuclear membrane, mitochondria and endoplasmic reticulum (Austin *et al.*, 1988) and plays an important role in preventing apoptotic cell death during lytic infection (Henderson *et al.*, 1993). BALF1, which is expressed as an early lytic transcript, also has significant homology to Bcl-2 and is thought to possess similar anti-apoptotic activity (Marshall *et al.*, 1999). However, recent reports have suggested that BALF1 may function as an antagonist to BHRF1 (Bellows *et al.*, 2002). Some studies suggest that BHRF1 and BALF1 are not strictly lytic proteins but may also be expressed during latent infection (Austin *et al.*, 1988; Cabras *et al.*, 2005). This will be discussed further in Section 1.1.4 and Section 1.3.

The lytic cycle can be induced in cell lines latently infected with EBV by a range of stimuli including protein kinase C (PKC) agonists such as phorbol esters (for example 12-O-tetradecanoylphorbol-13-acetate or TPA), histone deacetylase (HDAC) inhibitors such as n-butyrate, cross-linking of BCR by anti-immunoglobulin (anti-Ig), calcium ionophores, and transforming growth factor-beta (TGF-β) (Takada and Ono, 1989; di Renzo *et al.*, 1994; Miller *et al.*, 1994a). The ability of these agents to induce the lytic cycle is dependent on the cell background in which the virus is latent (Gradoville *et al.*, 2002). For example, an LCL established by *in vitro* infection with B95.8 was activated into the lytic cycle by TPA but not by n-butyrate. In contrast, a subclone of the EBV-positive BL cell line P3J-HR1K, Zp and Rp were activated by HDAC inhibitors but not by TPA.

1.1.4 Burkitt's Lymphoma

Burkitt's lymphoma (BL) is a malignancy of B lymphocytes which was first identified in 1958 as a tumour of the jaw occurring in children in regions of equatorial Africa, where it is the most common childhood cancer (Crawford, 2001). EBV was first discovered by Epstein and colleagues in 1964 in B lymphocytes cultured from an African (endemic) BL. Endemic BL (eBL) which is almost entirely (>95%) EBV-associated is restricted to areas of equatorial Africa and Papua New Guinea. Malaria is hyperendemic in areas with high incidence of eBL suggesting that malaria infection is an important co-factor in the pathogenesis of eBL. Sporadic or non-African cases of BL present worldwide, mainly in Western countries, at a much lower frequency. Sporadic BL (sBL) has a weaker EBV association with the virus present in about 15% of cases in North America and Europe. The association with EBV increases in patients infected with the human immunodeficiency virus (HIV) where 40-50% of BL tumours are EBV-positive (Crawford, 2001). Both type I and type II EBV strains have been found in association with eBL in Africa and Papua New Guinea but type I strains are dominant in EBV-positive sBL in Europe. This reflects the relative prevalence of these two virus strains within the general population in these areas (Rickinson and Kieff, 2001).

Irrespective of their origin and EBV association, BL is characterised by the chromosomal translocation juxtaposing the *c-myc* oncogene on the long arm of chromosome 8 onto either the immunoglobulin heavy chain (*IgH*) locus on chromosome 14 or the kappa (*IgK*) and lambda (*IgL*) light chain loci on chromosome 2 or 22 respectively (Lindstrom and Wiman, 2002). In 80% of cases, the t(8;14) translocation occurs with the *IgH* gene. The remainder involve either *IgK* t(2;8) or *IgL* t(8;22). These translocations bring the *c-myc* oncogene under the transcriptional control of an immunoglobulin locus leading to deregulate, constitutive expression of *c-myc* (Klein, 1983). The oncogene *c-myc* encodes a transcription factor and plays a critical role in regulating cell proliferation, growth, apoptosis, and differentiation in a cell type dependent manner (Pelengaris *et al.*, 2002). The overexpression of *c-myc* in B cells results in the continuous proliferation of cells and inhibition of differentiation. Constitutive expression of *c-myc* can enhance cell cycle progression through induction of cyclin D and cyclin E (Santoni-Rugiu *et al.*, 2000) or by

disrupting cell cycle control through downregulation of cell cycle inhibitors such as p21WAF/CIP1 and p27KIP1 (Gartel *et al.*, 2001; Yang *et al.*, 2001).

Deregulated expression of *c-myc* can sensitize cells to apoptosis in addition to increasing proliferation (Evan *et al.*, 1992; Hoffman and Liebermann, 2008). As such, there is a strong selective pressure to overcome apoptosis and ensure tumour progression. It has been shown that *c-myc*-induced apoptosis in B cells is primarily induced through the indirect activation of the tumour suppressor p53. *c-myc* stimulates, via E2F transcription factors, the expression of $p14^{ARF}$ ($p19^{ARF}$ in mice) which is an antagonist for the negative regulator of p53, MDM2 (Zindy *et al.*, 1998; Eischen *et al.*, 1999). It has been reported that many BL biopsies and BL-derived cell lines carry point mutations in the tumour suppressor gene p53 (Farrell *et al.*, 1991; Bhatia *et al.*, 1992) or carry defects in the $p14^{ARF}$-MDM2-p53 pathway (Lindstrom *et al.*, 2001). These genetic changes contribute to the inhibition of *c-myc*-induced apoptosis and the promotion of cellular proliferation.

Point mutations in codon 57 (Pro57Ser) and 58 (Thr58Ala) of *c-myc* have also been identified in BL cells. These mutants are able to stimulate proliferation and activate $p14^{ARF}$/p53 but are defective at promoting apoptosis. This is explained by the failure of these mutants to induce the expression of the pro-apoptotic protein Bim when overexpressed, unlike wild-type *c-myc* (Hemann *et al.*, 2005). Bim is a member of the Bcl-2 family (see Section 1.2) and is an important regulator of apoptosis in B lymphocytes. The loss of even a single allele of Bim accelerates lymphomagenesis in *Eμ-myc* transgenic mice indicating that Bim is a haploinsufficient tumour suppressor. Tumours arising from *Eμ-myc* mice with two functional alleles of Bim often carry mutations which inactivate the $p19^{ARF}$-MDM2-p53 pathway. In Bim -/- mice however, lymphomagenesis occurs at an increased rate and selection pressure for the functional inactivation of the p53 pathway appears to be removed (Egle *et al.*, 2004). As such, it has been suggested that the promotion of *c-myc*-induced lymphomagenesis is dependent on the inactivation of either the $p14/p19^{ARF}$-MDM2-p53 pathway or Bim (via mutation of *c-myc* alleles) (Allday, 2009).

BL cells display a cell surface profile ($CD10^+/CD77^+$) consistent with germinal centre (GC) B cells (Rickinson and Kieff, 2001). In addition, the consistent presence of the GC-associated protein Bcl-6 (Carbone *et al.*, 1997) and the detection of the Ig gene hypermutation (Klein *et al.*, 1995) support suggestions that BL is a tumour of GC B cells.

The mechanisms by which EBV contributes to the pathogenesis of BL are still not well understood. The vast majority of EBV-positive BL tumours display the highly restricted latency I infection where none of the growth-promoting latent genes are expressed (Rowe *et al.*, 1987). It has been suggested that EBV plays an initiating role in which it establishes a pool of transformed B cells which are at risk of *c-myc* translocation or by enhancing the survival and proliferation of a translocation-positive clone. This is followed by a switch to latency I as accumulated cellular changes which allow cells to grow independently of viral growth-promoting genes such as EBNA2 and LMP1. This scenario was modelled *in vitro* by using LCLs established using recombinant EBV expressing a conditional estrogen receptor-EBNA2 fusion protein such that EBNA2 function (and consequently LMP1 expression) is estrogen-dependent (Polack *et al.*, 1996). When these cells were transfected with a *c-myc* expression plasmid, constitutive expression of *c-myc* allowed the cells to grow independently of estrogen. The cell surface profiles of cells which grew in the absence of estrogen had also switched from a lymphoblastoid-like to latency I BL-like phenotype, where CD10 and CD38 were upregulated while activation markers and adhesion molecules were shut off. A later study using the same model has highlighted the apparent differences between the EBNA2-driven and *c-myc*-driven growth programmes in B cells which indicate that evolution to BL can only occur if the EBV latency III programme is suppressed (Pajic *et al.*, 2001). The selection pressure against the full set of viral latent genes is demonstrated in a subset of BL tumours in which the EBNA2 gene has been deleted from the EBV genome. In a study conducted by Kelly and colleagues, approximately 15% of eBL biopsies were shown to harbor viral genomes containing a deletion of the EBNA2 gene and the Y_1Y_2 unique exons of EBNA-LP similar to the deletion found in the P3HR1 EBV strain (see Figure 1.3) (Kelly *et al.*, 2002). These tumour cells displayed a novel form of EBV latency that is characterized by usage of Wp instead of Qp (termed 'Wp-

restricted latency') leading to the expression EBNA1, EBNA3A, EBNA3B, EBNA3C, a truncated form of EBNA-LP, EBERs and BARTs. Expression of the viral Bcl-2 homologue BHRF1 is also detectable at very low levels in these cells (Kelly *et al.*, 2009). Some of the tumour cells additionally carried a wild-type EBV genome, but none carried the wild-type genome alone. However, only the Wp-restricted form of latency was detected.

EBV may also contribute to the pathogenesis of BL through the latency I genes themselves. The reported oncogenicity of EBNA1 in transgenic mice remains controversial as previously described in Section 1.1.3 (Wilson *et al.*, 1996; Kang *et al.*, 2005). Furthermore, the contribution of EBNA1 to *in vitro* B cell transformation by EBV appears to be limited to maintenance of the viral genome (Humme *et al.*, 2003). However, recent experiments in which EBNA1 function in EBV-positive BL cell lines was blocked by a dominant negative EBNA1 have demonstrated that EBNA1 does promote survival (Kennedy *et al.*, 2003). While it has been shown that the EBERs can induce expression of IL-10, which is thought to act as an autocrine growth factor in BL cells (Kitagawa *et al.*, 2000), the role of the EBERs as potential oncogenes is still debatable. The EBERs have also been reported to upregulate the anti-apoptotic protein Bcl-2 to promote survival in EBV-negative BL cells (Komano *et al.*, 1999). Thus, it is possible that while EBV may play an initiating role in the pathogenesis of BL, the virus continues to contribute to the growth phenotype of malignant cells by prolonging their survival through the action of latency I genes.

A recent model for the pathogenesis of EBV-positive BL proposed by Thorley-Lawson and Allday suggests that EBNA3A and EBNA3C co-operate to induce epigenetic repression of the pro-apoptotic factor Bim in infected B-blasts. Following a switch from the growth programme to the default programme (see Figure 1.2), cells then differentiate into GC cells with a repressed *Bim* gene. Translocation of *c-myc* is thought to occur at the GC stage. Repression of Bim in GC cells inhibits apoptosis resulting from deregulated *c-myc* leading to uncontrolled proliferation. Infected GC cells then follow the normal B cell differentiation pathway and leaves to become a memory cell. This is accompanied by the transition from the EBV default programme to the more restricted latency I programme. Proliferation promoted by

overexpression of *c-myc* prevents latently infected cells from becoming a resting memory cell. These cells then grow as an EBV-positive BL tumour that expresses the latency I programme (Thorley-Lawson and Allday, 2008).

Malaria and HIV infection are important risk factors for the development of BL. The exact role of malaria and HIV in the pathogenesis of BL is still unclear. Immunosuppression by malaria and HIV leads to a decrease in the levels of EBV-specific cytotoxic T cells (CTLs). CTL-mediated control over the outgrowth of EBV-infected B cells is impaired during acute malaria infection (Moss *et al.*, 1983). EBV loads in the peripheral blood of individuals infected with acute malaria are five times higher than in healthy individuals. It has recently been reported that the malarial parasite *Plasmodium falciparum* can directly activate B cells and promote their survival. This is facilitated by the binding of a cysteine-rich interdomain region 1α (C1DR1a) of *P falciparum* erythrocyte membrane protein 1 (PfEMP1) to surface Ig (Chene *et al.*, 2007).

1.2 Apoptosis

Apoptosis or programmed cell death removes damaged, infected and superfluous cells and is important for normal development, immune function and tissue homeostasis. Indeed, deregulation of apoptosis can lead to a variety of human pathologies including cancer, autoimmune and neurodegenerative disorders (Cory and Adams, 2002). Apoptosis in mammalian cells is mediated by a family of intracellular cysteine proteases known as caspases. These caspases are initially synthesized as inactive procaspase precursors. There are two pathways by which caspase activation is triggered: the extrinsic and intrinsic apoptotic pathways.

1.2.1 The extrinsic apoptotic pathway

The extrinsic pathway is induced by the engagement death receptors, which are specialized cell-surface receptors such as Fas/CD95, tumor necrosis factor-alpha (TNF-α) receptor 1, and two receptors, DR4 and DR5, by the TNF-α related apoptosis-inducing ligand (TRAIL) (Krammer, 2000). This pathway activates the

initiator caspases-8 and -10 through adaptor proteins that include Fas-associated death domain protein (FADD). The activation of caspase-8 and -10 in turn activates downstream caspases such as caspases-3, -6 and -7 (Budihardjo *et al.*, 1999). This culminates in the cleavage of several hundred cellular proteins leading to morphological changes associated with apoptosis, including DNA degradation, chromatin condensation, and membrane blebbing (Danial and Korsmeyer, 2004). In some cell types, the downstream caspase cascade cannot be propagated directly following activation of caspase-8 and -10 and has to be amplified via the mitochondria (Scaffidi *et al.*, 1998). In these cells, caspase-8 cleaves the pro-apoptotic Bcl-2 protein Bid and truncated Bid can then activate the intrinsic apoptotic pathway.

1.2.2 The intrinsic apoptotic pathway

The intrinsic or mitochondrial apoptotic pathway (Green, 2005) is triggered by various extracellular and intracellular stresses including DNA damage, growth-factor withdrawal and UV radiation. The intrinsic apoptotic pathway is regulated by the Bcl-2 family of pro-survival and pro-apoptotic proteins (see Section 1.2.3). It begins with the permeabilisation of the outer mitochondrial membrane by pro-apoptotic members of the Bcl-2 family (Kowaltowski *et al.*, 2002), leading to the release of proteins, including the electron carrier protein cytochrome c, into the cytosol.

Cytochrome c then binds and activates the apoptotic peptidase activating factor (Apaf-1), resulting in its recruitment of caspase-9 to form the apoptosome. The initiator caspase-9 is thereby activated, and this triggers the downstream activation of caspases-3, -6 and -7 (Budihardjo *et al.*, 1999). These effector caspases then cleave key substrates in the cell that result in the degradation of DNA and proteins (Danial and Korsmeyer, 2004). One of the substrates cleaved by effector caspases, especially caspase-3, is poly (ADP-ribose) polymerase (PARP). Cleavage of the 113kDa full-length PARP generates 89- and 24-kDa polypeptides (Nicholson *et al.*, 1995). PARP cleavage is a universal phenomenon observed during programmed cell death induced by a variety of apoptotic stimuli and as such, is widely considered as one of the most valuable indicators of apoptosis.

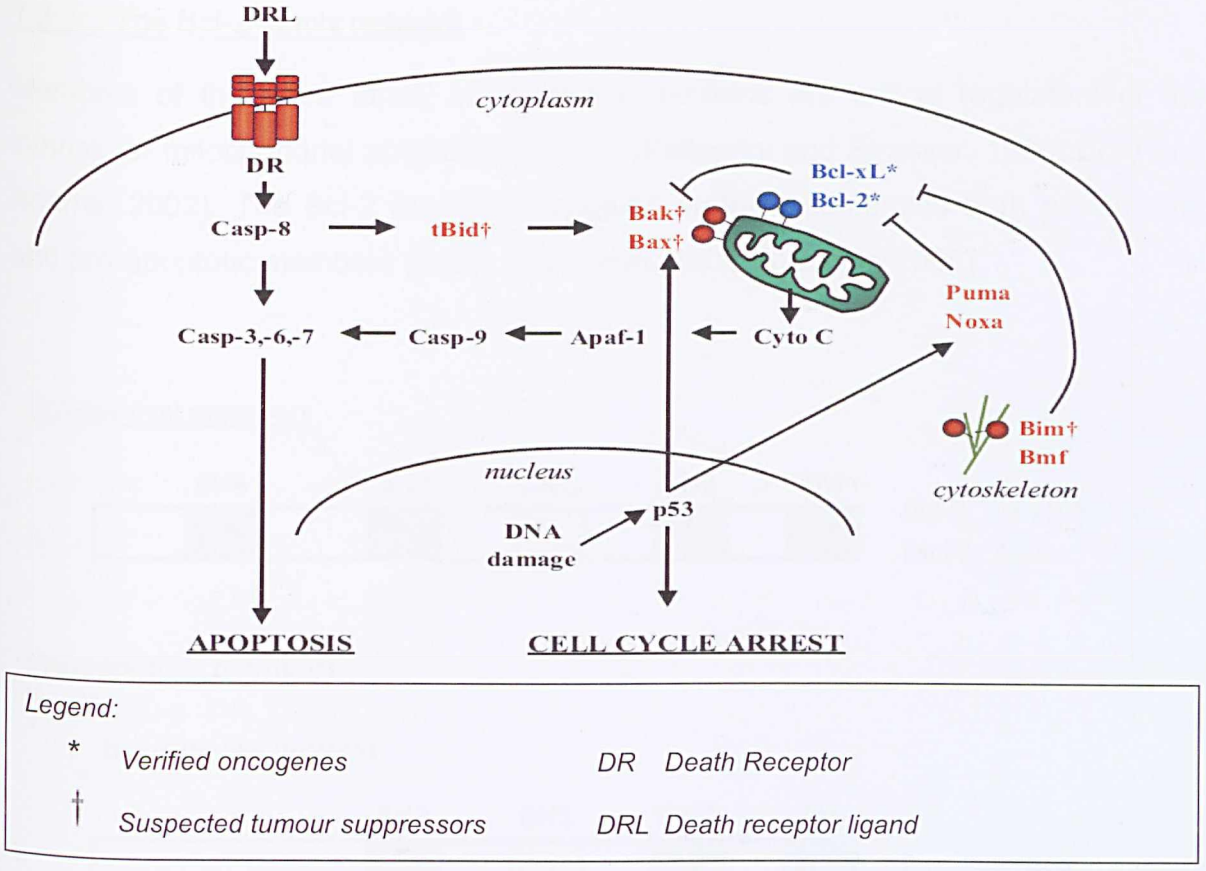


Figure 1-6 Apoptotic pathways. The extrinsic apoptotic pathway is induced by the engagement of the death receptor on the cell surface by death receptor ligands. This leads to the activation of the initiator caspase-8, subsequent activation of effector caspases-3, -6 and -7 and ultimately cell death. In some cell types, the pro-apoptotic BH3-only protein Bid is cleaved by caspase-8 and truncated Bid then activates the intrinsic apoptotic pathway. The intrinsic apoptotic pathway is mediated by the Bcl-2 family of proteins and can be induced by a range of apoptotic stimuli including DNA damage. Binding of BH3-only proteins such as Bim and Puma to pro-survival proteins (eg. Bcl-2 and Bcl-xL) releases pro-apoptotic Bax/Bak-like proteins. This mediates the release of cytochrome c from the mitochondria and leads to the activation of Apaf-1. The activation of caspase-9 by Apaf-1 triggers a caspase cascade which leads to cell death. Adapted from Kirkin *et al.*, 2004.

The release of cytochrome c into the cytosol is a pivotal event in this apoptotic pathway as once it has occurred, the cell is committed to cell death and the downstream cascade of caspase activation is irreversible (Green and Reed, 1998; Jiang and Wang, 2004).

1.2.3 The Bcl-2 family network

Members of the Bcl-2 family of interacting proteins are critical regulators of the intrinsic or mitochondrial apoptotic pathway (Pellegrini and Strasser, 1999; Cory and Adams, 2002). The Bcl-2 family of apoptotic proteins comprises both pro-survival and pro-apoptotic members (Droin and Green, 2004; Strasser, 2005).

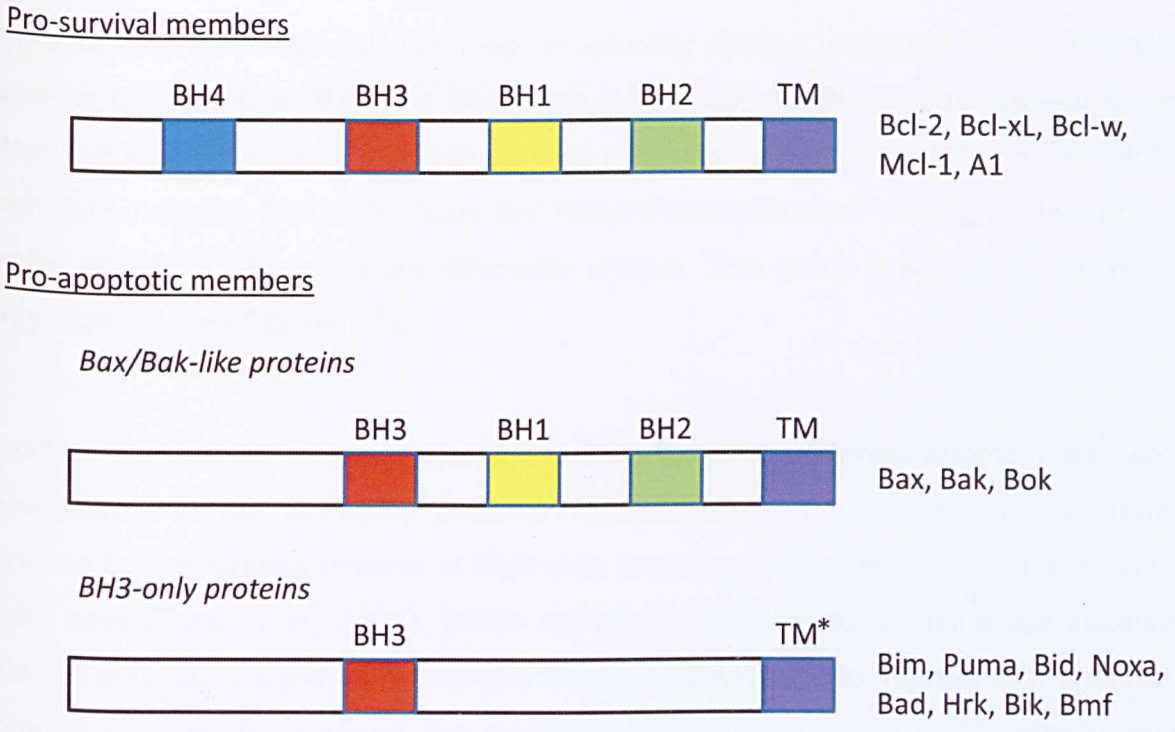


Figure 1-7 The Bcl-2 family of apoptotic proteins. The pro-survival family members - Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1 - each possess up to four distinct Bcl-2-homology (BH) domains. The pro-apoptotic members of the Bcl-2 family can be subdivided into two groups: the Bax/Bak-like proteins — Bax, Bak and Bok - which contain two or three distinct BH domains; and the BH3-only proteins –Bim, Puma, Bid, Noxa, Bad, Hrk, Bik and Bmf - which share with each other and other members of the Bcl-2 family only the short BH3 domain. *The transmembrane domain (TM) is only found in some of the BH3-only proteins –Bim, Hrk and Bid (Strasser, 2005; Giam *et al.*, 2008).

Pro-survival members, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1, share up to four regions of structural homology known as the Bcl-2 Homology (BH) domains (see Figure 1.7). Evidence for the existence of a BH4 domain in Mcl-1 and A1 remains controversial. The BH1, BH2 and BH3 domains interact to form a hydrophobic

groove on the surface of the protein (Willis *et al.*, 2003). This groove accommodates the BH3 domain of its pro-apoptotic binding partners and is essential for the pro-survival function of these proteins. Studies have shown that the overexpression of pro-survival Bcl-2 proteins prevents the release of cytochrome c from the mitochondria and subsequent caspase activation (Kluck *et al.*, 1997; Yang *et al.*, 1997). However, the mechanism by which this occurs is still unclear.

Pro-apoptotic members fall into two structurally distinct subgroups. Bax/Bak-like proteins such as Bax, Bak and Bok have 3 BH regions, BH1-3, and appear to be structurally very similar to their pro-survival relatives. In contrast, other pro-apoptotic members including Bim, Bid, Puma and Noxa share with each other only the short 9 amino acid BH3 region but are otherwise unique. This group is known as the BH3-only proteins (see Figure 1.7).

Bax/Bak-like proteins are essential in the activation of the intrinsic apoptotic pathway downstream of the BH3-only proteins. Genetic studies have shown that death induced by the overexpression of BH3-only proteins is completely blocked in $Bax^{-/-}$ $Bak^{-/-}$ cells (Zong *et al.*, 2001). When activated, Bax/Bak-like proteins are inserted into the outer mitochondrial membrane where they are able to interact with channel-forming proteins to increase the permeability of the membrane (Tsujimoto and Shimizu, 2000) and allow for the release of cytochrome c into the cytosol. The formation of these channels involves change in conformation and oligomerisation of Bax and Bak (Eskes *et al.*, 2000; Wei *et al.*, 2000).

BH3-only proteins bind with high affinity to at least one of the pro-survival Bcl-2 family members. The α-helical BH3 domain of BH3-only proteins insert into the hydrophobic cleft formed by the BH1-3 domains on the surface of pro-survival Bcl-2 family members described above (Sattler *et al.*, 1997; Liu *et al.*, 2003). Only Bim and Puma engage all pro-survival proteins. Others such as Bad (which binds to Bcl-2, Bcl-xL, and Bcl-w) and Noxa (which binds to Mcl-1 and A1) interact with distinct subsets and exhibit complementary killing (Chen *et al.*, 2005). Individual BH3-only proteins are thought to transduce different and specific death signals. For example,

Bim is important for apoptosis of lymphocytes following cytokine deprivation (Bouillet *et al.*, 1999), Bmf is required for cell death triggered by anoikis (Puthalakath *et al.*, 2001), and Noxa and Puma appear to be mediators of p53-induced apoptosis (Oda *et al.*, 2000a; Nakano and Vousden, 2001).

It has been observed that Bid can bind to and directly activate Bax/Bak-like proteins to induce release of cytochrome c (Wei *et al.*, 2000). Recent studies have shown that Bim and Puma may also interact directly with Bax/Bak-like proteins, induce oligomerisation of these proteins and subsequent release of cytochrome c (Letai *et al.*, 2002; Kuwana *et al.*, 2005).

The mechanism by which Bcl-2 family members interact to promote permeabilisation of the outer mitochondria membrane and the subsequent release of cytochrome c is still debatable. Two models, the 'direct' and 'indirect' models of activation of Bax/Bak-like proteins, have been proposed (see Figure 1.8) (van Delft and Huang, 2006). In the 'direct' model, Bid, Bim and Puma are described as 'activators' based on their ability to interact directly with Bax/Bak-like proteins. 'Sensitizers' such as Noxa and Bad are unable to directly activate Bax and Bak, and function by displacing 'activators' bound to pro-survival Bcl-2-like proteins. The 'activators' are then free to interact with Bax/Bak-like proteins. The 'indirect' activation model proposes that Bax/Bak-like proteins are inhibited by pro-survival proteins. BH3-only proteins can activate apoptosis by displacing Bax/Bak-like proteins bound to pro-survival proteins, allowing Bax/Bak-like proteins to oligomerise and permeabilise the mitochondria.

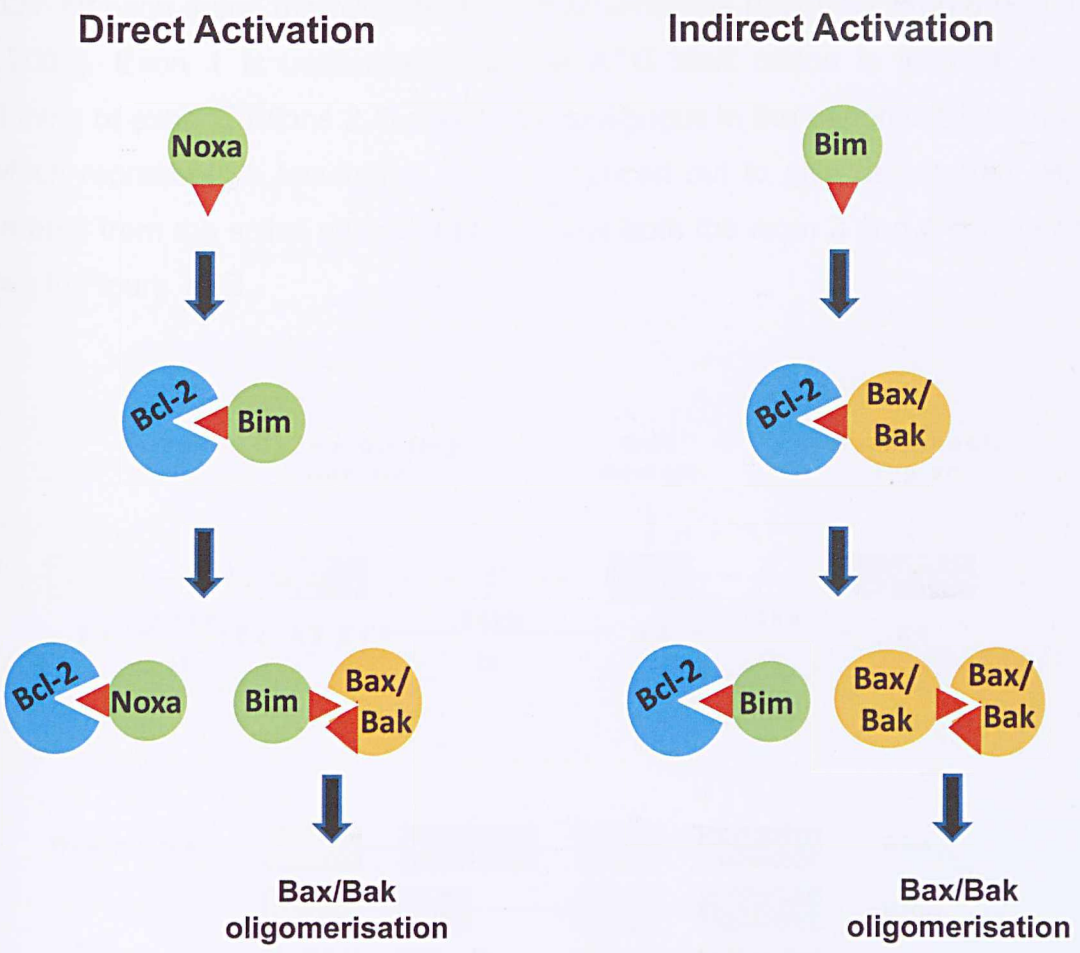


Figure 1-8 Models of Bax/Bak activation by BH3-only proteins. In the direct activation model, 'sensitizer' BH3-only proteins (eg. Noxa and Bad) induce apoptosis by displacing 'activator' BH3-only proteins (eg. Bim and Puma) bound to pro-survival Bcl-2-like proteins, allowing the 'activator' BH3-only proteins to directly trigger Bax/Bak activation. In the indirect activation model, pro-survival Bcl-2-like proteins inhibit Bax/Bak-like proteins. BH3-only proteins trigger apoptosis by neutralising pro-survival proteins, releasing Bax/Bak-like proteins and allowing Bax/Bak activation to occur spontaneously in the absence of pro-survival activity. Adapted from van Delft and Huang (2006).

1.2.3.1 Bim

Bim or Bcl-2 interacting mediator of death is a pro-apoptotic BH3-only member of the Bcl-2 family of proteins. There are 3 major isoforms of Bim as produced by alternative mRNA splicing: extra long (Bim_{EL}), long (Bim_L) and short (Bim_S) (O'Connor *et al.*, 1998). Human *Bim* has six exons (Figure 1.9A): the BH3 domain, critical for the pro-apoptotic function of Bim, is encoded by exon 5, a dynein binding

domain by exon 4 and the hydrophobic transmembrane region by exon 6 (Bouillet *et al.*, 2001). Exon 1 is untranslated as the ATG start codon is located near the beginning of exon 2. Exons 2, 3 and 4 are contiguous in the genomic DNA and exon 3, which represents a facultative intron, is spliced out to give rise to Bim_L. Bim_S is generated from the splice variant that removes both the exon 3 and 4 sequences as shown in Figure 1.9B.

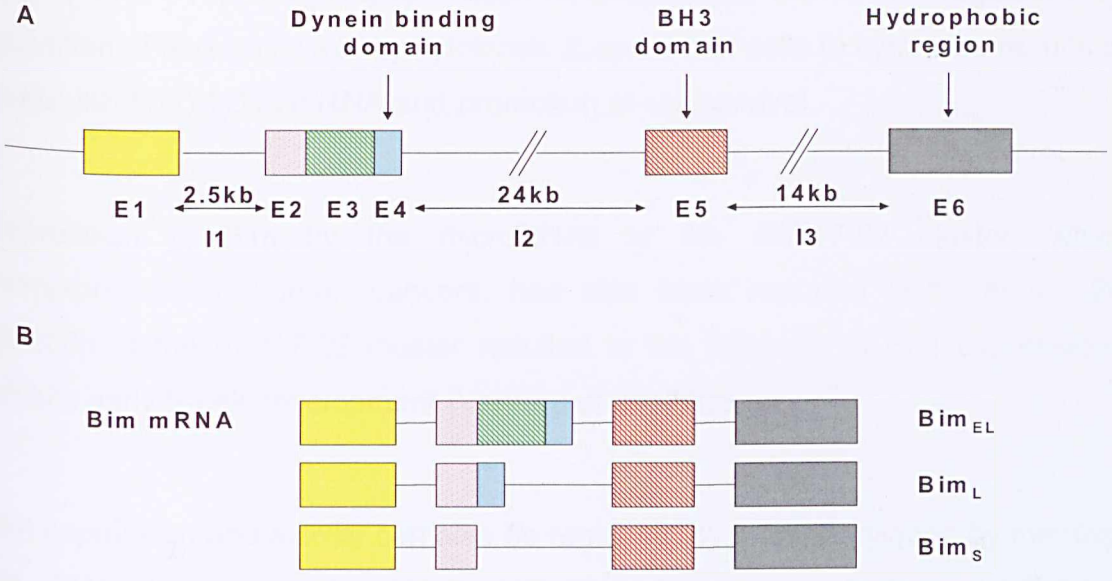


Figure 1-9 Human *Bim* (A) Organisation of human Bim gene **(B)** Bim mRNA splice variants that encode the extra long, long and short forms of the Bim protein. The most predominantly expressed isoform in most tissues is Bim_{EL} while only very low levels of Bim_S are usually detected. While Bim_S is usually expressed at very low levels, it is thought to be the most potent of the three isoforms. In healthy cells, Bim_{EL} and Bim_L are sequestered to the microtubular dynein motor complex through interaction with the dynein light chain (DLC). Bim_S lacks the dynein binding domain and therefore cannot be sequestered to the cytoskeleton (Puthalakath *et al.*, 1999). This appears to be the reason Bim_S is the most potent killer of the three isoforms.

Studies show that Bim expression can be regulated both at the level of transcription and post-transcription. Bim is transcriptionally upregulated by the forkhead transcription factor FOXO3a (or FKHR-L1) in response to cytokine deprivation in leukocytes (Dijkers *et al.*, 2000) and by E2F1 when this transcription factor is overexpressed (Hershko and Ginsberg, 2004). Upregulation of Bim through CHOP-

C/EBPalpha-mediated transcription is observed in breast carcinoma cells subjected to endoplasmic reticulum stress (Puthalakath *et al.*, 2007).

At the post-transcriptional level, Matsui and colleagues observed that heat-shock cognate protein 70 (Hsc70) can regulate Bim mRNA stability by binding to AU-rich elements in the 3'UTR and enhances its stability upon cytokine withdrawal (Matsui *et al.*, 2007). The binding efficiency of Hsc70 is modulated by co-chaperones such as Bag-4, CHIP, Hip, and Hsp40. These co-chaperones are in turn regulated by the activation of Ras pathways by cytokines. Exposure of cells to cytokines results in the destabilization of Bim mRNA and promotion of cell survival.

Repression of Bim by the microRNAs of the miR17-92 cluster, which is overexpressed in human cancers, has also been reported (Xiao *et al.*, 2008). Deletion of the miR17-92 cluster resulted in the increase of Bim expression and inhibits early B-cell development (Ventura *et al.*, 2008).

Bim expression and activity can also be regulated by phosphorylation by members of the MAP kinase family. Upon activation by growth factors, ERK-1/2 can phosphorylate Bim_{EL} at Ser-69 leading to ubiquitylation of the protein and subsequent proteasomal degradation (Ley *et al.*, 2003; Luciano *et al.*, 2003). Exposure of kidney cells to UV radiation revealed JNK-mediated phosphorylation of Thr-56 in Bim_L (Lei and Davis, 2003). As this phosphorylation site is within the DLC binding domain, it is postulated that JNK-mediated phosphorylation leads to the release of Bim_L from the dynein motor complex and an increase in the apoptotic potential of Bim_L. So far, there have been no reports of the regulation of Bim_S through phosphorylation.

Gene targeting experiments have indicated that Bim plays a central role in the control of lymphocyte homeostasis. Bim-deficient mice have abnormally high levels of T cells, B cells and macrophages, and at 4-8 months, accumulate up to a 200-fold increase the number of immunoglobulin-secreting plasma cells (Bouillet *et al.*, 1999). Lymphocytes from *Bim* $^{-/-}$ mice were shown to be resistant to certain apoptotic stimuli

such as cytokine deprivation but have almost normal sensitivity to Bcl-2-inhibitable apoptotic stimuli including DNA damage or treatment with phorbol ester.

There is also evidence that Bim functions as a tumour suppressor. Deletion of just one allele of *Bim* in *Eμ-myc* transgenic mice resulted in an increased rate of *c-myc*-induced tumourigenesis (Egle *et al.*, 2004).

1.2.3.2 Noxa

Noxa is a BH3-only pro-apoptotic member of the Bcl-2 family which was first identified as a phorbol 12-myristate 13-acetate(PMA; also known as TPA)-responsive gene in human peripheral blood mononuclear cells, Jurkat T-cell lymphoma cells, and human embryonic lung cells (Hijikata *et al.*, 1990) and given the name PMA-induced protein 1(PMAIP1). PMAIP1 was later rediscovered as a p53-responsive gene in wild-type and IRF-1/p53 double deficient mouse embryonic fibroblasts exposed to γ-irradiation and renamed Noxa (Latin for damage). A p53 response element was also identified in the Noxa promoter (Oda *et al.*, 2000a).

Many early studies have shown Noxa to be a critical mediator of apoptosis induced by p53. Generally, the involvement of Noxa in p53-dependent cell death is quite variable and appears to be cell-type and stimulus specific. Studies also suggest that p53 promotes apoptosis mainly through transcriptional activation of Noxa (and Puma) rather than through other proposed mechanisms (Vousden and Lu, 2002). However, recently several reports have described upregulation of Noxa in a p53-independent manner. Induction of Noxa in response to some apoptotic stimuli, such as hypoxia and E1A overexpression, has been observed in certain p53-deficient cells (Kim *et al.*, 2004; Flinterman *et al.*, 2005). p73 (specifically the transactivating isoform), a homologue of p53, has been postulated to be the possible mediator of Noxa induction in cells which lack p53 or have functionally inactive, mutated p53, as is the case with majority of human cancers. A recent study has demonstrated that p73 is activated by genotoxic agents in the presence of NF-κB and consequently, induces expression of Noxa in cells carrying mutant p53 (Martin *et al.*, 2009).

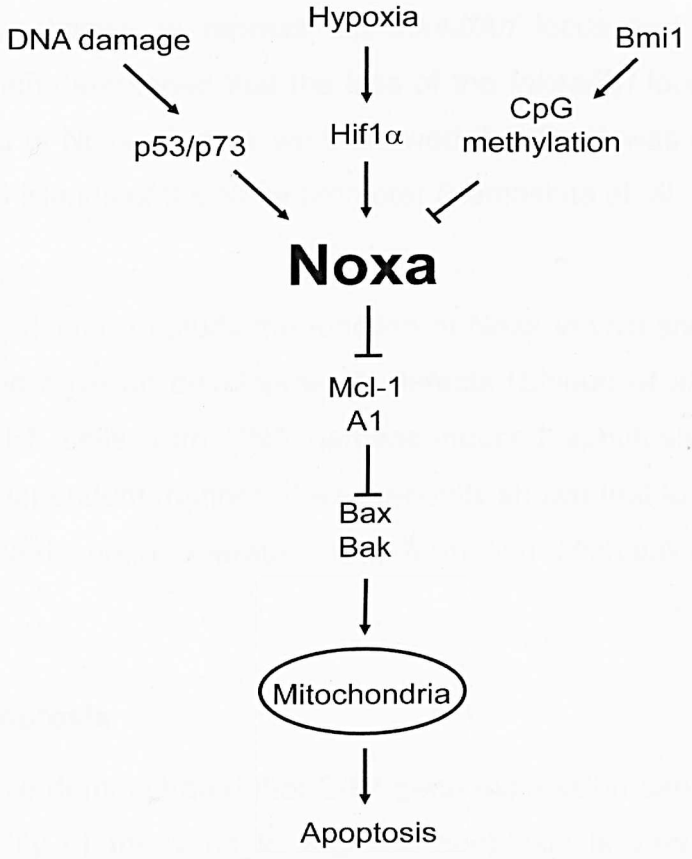


Figure 1-10 Noxa expression can be induced by multiple p53-dependent and –independent signals. Noxa expression is upregulated in response to apoptotic stimuli such as DNA damage (via p53 or p73) and hypoxia (via Hif1α). Noxa is also regulated by Bmi1 (via CpG methylation) in memory T cells. Noxa performs its pro-apoptotic function by neutralising the pro-survival Mcl-1/A1, thereby allowing activation of Bax/Bak-like proteins, permeabilisation of the mitochondria and finally apoptosis.

Modulators of Noxa expression other than p53 and its relatives have since been identified. Under hypoxic conditions, Noxa was found to be upregulated in various normal and malignant cells via direct activation of the Noxa promoter by hypoxia-inducible factor 1 alpha (HIF-1α) (Kim *et al.*, 2004). The transcription factor E2F1 has also been implicated in directly regulating Noxa expression (Hershko and Ginsberg, 2004).

Yamashita and colleagues observed a repression of Noxa gene expression in memory T cells by the polycomb group (PcG) gene Bmi1 (Yamashita *et al.*, 2008).

Loss of Bmi1 resulted in the upregulation of Noxa and an increase in cell death. Since Bmi1 is also known to repress the *Ink4a/Arf* locus and subsequent p53 signaling, it was then determined that the loss of the *Ink4a/Arf* locus or p53 did not affect the induction of Noxa. Further work showed that Bmi1 was required for DNA methylation at CpG islands of the Noxa promoter (Yamashita et. al., 2008).

Genetic studies conducted to study the function of Noxa *in vivo* showed that Noxa$^{-/-}$ mice are viable and have no developmental defects (Shibue *et al.*, 2003). Loss of Noxa protected MEF cells from DNA damage-induced apoptosis in both a p53-dependent and -independent manner. It was recently shown that loss of Noxa in *Eμ-myc* transgenic mice did not accelerate tumour formation (Michalak *et al.*, 2009).

1.3 EBV and Apoptosis

Several studies have demonstrated that EBV gene expression can be strongly anti-apoptotic. The ability of the virus to regulate apoptosis is very important since apoptosis is a major antiviral response used by multicellular organisms for the removal of infected cells. As such, EBV has evolved various mechanisms to ensure the survival of infected cells long enough for the virus to establish persistent latent infection and replicate. Furthermore, B lymphocytes, which are the primary target for EBV infection, are very prone to apoptosis when they become activated due to positive selection for those with high-affinity binding to antigen. During B cell development, multiple regulators of apoptosis act to remove defective B cells at different stages of their development and maintain the homeostasis of surviving B cells (Defrance *et al.*, 2002). Inhibition of apoptosis by EBV also makes a major contribution to the malignant transformation of infected cells.

Studies investigating EBV and apoptosis have identified several viral latent gene products with anti-apoptotic activity. LMP1 was originally shown to protect against apoptosis induced by serum starvation (Henderson *et al.*, 1991). Subsequently, it was demonstrated that LMP1, in some circumstances, is responsible for the upregulation of pro-survival Bcl-2 (Henderson *et al.*, 1991) and Mcl-1 (Wang *et al.*,

1996) proteins. LMP1 has also been shown to induce expression of A20 which is a zinc finger protein that protects from TNF-α induced apoptosis and could in this way interfere with p-53 mediated apoptosis (Laherty *et al.*, 1992).

Inhibition of EBNA1, using a dominant negative EBNA1, was shown to induce apoptosis in BL cells (Kennedy *et al.*, 2003). EBNA2 protects B cells against Nur77-mediated cell death (Lee *et al.*, 2004). LMP2A inhibits apoptosis induced by transforming growth factor-β1 probably by altering TGF-β signalling rather than affecting the apoptosis machinery (Fukuda and Longnecker, 2004). Expression of the viral RNA species, EBERs, can restore resistance to apoptosis and upregulate pro-survival Bcl-2 when introduced into EBV-negative BL cells (Komano *et al.*, 1999).

Studies in this lab and others have demonstrated the resistance of EBV-positive BL cells lines to apoptosis induced by various cytotoxic agents, including those causing DNA damage or those disrupting the mitotic spindle, relative to their EBV-negative counterparts (Wade and Allday, 2000; Leao *et al.*, 2007). This resistance to apoptosis was shown to be dependent on the co-expression of EBNA3A and EBNA3C since these latent proteins functionally interact to downregulate expression of the pro-apoptotic BH3-only protein Bim (Anderton *et al.*, 2008). Subsequently, it was shown that repression of Bim by EBV initially involves the repressive epigenetic histone modification H3K27-Me3 (trimethylation of histone H3 lysine 27) followed by DNA methylation at CpG islands throughout the Bim promoter (Paschos *et al.*, 2009). These findings imply that EBV can reprogram B cells to enhance the survival of infected cells and promote tumourigenesis.

Recently, a truncated mutant form of EBNA-LP has been shown to confer resistance against the caspase-dependent apoptosis induced by verotoxin 1 and the protein kinase inhibitor staurosporine (Garibal *et al.*, 2007), most probably through the functional inactivation of protein phosphatase 2A (PP2A). PP2A is a major serine-threonine phosphatase involved in several cellular signal transduction pathways and has been reported to be involved in apoptosis as it can activate pro-apoptotic and inhibit anti-apoptotic proteins of the Bcl-2 family (Van Hoof and Goris, 2003).

EBV encodes two Bcl-2 homologues, BHRF1 and BALF1, which are expressed in the early lytic phase of the virus replication cycle. BHRF1 suppresses apoptosis induced by cytokine depletion (Henderson *et al.*, 1993; Foghsgaard and Jaattela, 1997), γ-irradiation and certain cytotoxic drugs (McCarthy *et al.*, 1996). BHRF1, like most other viral Bcl-2 homologues, lack the BH4 domain (Marshall *et al.*, 1999) and has been reported to localise to the mitochondria (Cross *et al.*, 2008). BHRF1 is thought to prevent apoptosis through its inhibitory interaction with the pro-apoptotic protein Bak as well as the BH3-only proteins Bid, Bim and Puma (Cross *et al.*, 2008; Flanagan and Letai, 2008). The role of BALF1 in regulation of apoptosis is still debatable. Although it has been reported that BALF1 exerts anti-apoptotic effects through its association with the pro-apoptotic proteins Bax and Bak (Marshall *et al.*, 1999), other reports have suggested that this second Bcl-2 homologue actually inhibits the anti-apoptotic activity of BHRF1 (Bellows *et al.*, 2002). Unlike BHRF1, BALF1 is reported to possess a BH4 domain which is thought to be important for the anti-apoptotic activity of Bcl-2 proteins. It has therefore been hypothesised that the lack of conservation of the BH4 domain in BHRF1 suggests that BALF1 may have a function distinct from that of BHRF1 (Marshall *et al.*, 1999).

It was reported that BHRF1 and BALF1 prevent primary B lymphocytes from undergoing premature apoptosis to allow productive infection with EBV. The expression of these viral Bcl-2 homologues becomes undetectable once a latent infection is established (Altmann and Hammerschmidt, 2005). However, there appears to be evidence suggesting that BHRF1 is expressed at low levels during latency in Wp-restricted BL cells (see Section 1.1.4) and can promote survival of cells exposed to anti-IgM and the calcium ionophore ionomycin (Kelly *et al.*, 2009).

The EBV-encoded miR-BART5 has been shown to repress expression of the pro-apoptotic BH3-only member Puma in NPC cells and protect NPC cells against apoptosis induced by the genotoxic agents etoposide and adriamycin (Choy *et al.*, 2008). It has recently been reported that the BHRF1 miRNAs can inhibit apoptosis and promote cell cycle progression and proliferation early after B cell infection (Seto *et al.*, 2010).

EBV genes	Anti-apoptotic activity
LMP-1	Inhibit apoptosis induced by serum starvation (Henderson *et al.*, 1991), can upregulate of pro-survival Bcl-2 (Henderson *et al.*, 1991), Mcl-1 (Wang *et al.*, 1996) and A20 (Laherty *et al.*, 1992) proteins.
LMP2A	Inhibits apoptosis induced by transforming growth factor-β1 (Fukuda and Longnecker, 2004)
EBNA2	Protects B cells against Nur77-mediated cell death (Lee *et al.*, 2004)
EBNA1	Inhibition of EBNA1 induces apoptosis in BL cells (Kennedy *et al.*, 2003)
EBERs	Can restore resistance to apoptosis and upregulate Bcl-2 when introduced into EBV-negative BL cells (Komano *et al.*, 1999)
EBNA3A and EBNA3C	Protect against apoptosis induced by various genotoxic drugs, downregulate pro-apoptotic protein Bim (Anderton *et al.*, 2008)
EBNA-LP	Truncated mutant form of EBNA-LP protects against verotoxin 1- and staurosporine-induced apoptosis (Garibal *et al.*, 2007)
BHRF1	Protects against apoptosis induced by cytokine depletion (Henderson *et al.*, 1993; Foghsgaard and Jaattela, 1997), anti-IgM and ionomycin (Kelly *et al.*, 2009) γ-irradiation and cytotoxic drugs (McCarthy *et al.*, 1996). Inhibits apoptosis early after B cell infection (Altmann and Hammerschmidt, 2005)
BALF1	Can interact with the pro-apoptotic proteins Bax and Bak (Marshall *et al.*, 1999) but role of BALF1 still controversial. Inhibits apoptosis early after B cell infection (Altmann and Hammerschmidt, 2005)

EBV miRNAs	BHRF1 miRNAs inhibit apoptosis early after B cell infection (Seto *et al.*, 2010), miR-BART5 downregulates Puma (Choy *et al.*, 2008)

Table 1.2 Summary of EBV genes reported to possess anti-apoptotic activity.

1.4 Inducers of Apoptosis

The following inducers of apoptosis were used in this study to investigate the effect of EBV infection in the regulation of apoptosis in Burkitt's lymphoma-derived cells.

1.4.1 Ionomycin

Ionomycin is a calcium ionophore which induces an increase in the levels of intracellular Ca^{2+} in treated cells. Initially, it was assumed that ionomycin directly facilitated the transport of Ca^{2+} across the plasma membrane thereby bypassing natural entry pathways. However, it now appears that ionomycin stimulates the influx of Ca^{2+} by a store-regulated cation entry mechanism (Morgan and Jacob, 1994).

Increase of intracellular Ca^{2+} level induces apoptosis in thymocytes (Youn *et al.*, 1999), human B lymphocytes (Aagaard-Tillery and Jelinek, 1995) and various cancer cells. Following addition of calcium ionophores, Ca^{2+} released from stores within the endoplasmic reticulum is rapidly taken up by the mitochondria. The depletion of Ca^{2+} from the stores and overload of Ca^{2+} in the mitochondria would lead to stimulation of stress signals resulting in the activation of the intrinsic apoptotic pathway (Berridge *et al.*, 1999).

It has been shown that the addition of calcium ionophores such as ionomycin leads to a rapid activation of ubiquitous calpains (Molinari *et al.*, 1994). Calpains are a family of intracellular calcium-dependent cysteine endopeptidases which are structurally unrelated to caspases. Recent work has suggested that calpains trigger apoptosis by activating the intrinsic apoptotic pathway through the regulation of several Bcl-2 apoptotic proteins. Gil-Parrado and colleagues have shown that calpains can cleave Bcl-2, Bcl-xL and Bid *in vitro* (Gil-Parrado *et al.*, 2002). Other

authors have suggested that activation of calpains occurs via caspase-mediated cleavage of calpastatin, its endogenous and highly specific inhibitor, during initiation of apoptotic execution (Porn-Ares *et al.*, 1998). Ionomycin is also known to induce the viral lytic cycle in some B lymphocytes latently infected with EBV (Faggioni *et al.*, 1986).

1.4.2 Staurosporine

Staurosporine is a potent inhibitor of protein kinases, including the tyrosine protein kinase and protein kinase C (Tamaoki *et al.*, 1986). Protein kinases catalyse the transfer of ATP to a free hydroxyl group of serine, threonine or tyrosine residues belonging to specific proteins. Staurosporine binds with high affinity to protein kinases and prevents the binding of ATP to the molecule.

Staurosporine is widely used *in vitro* as an initiator of apoptosis in many types of cells, but the mechanism involved remains controversial. It is generally thought that staurosporine activates the intrinsic apoptotic pathway (Tafani *et al.*, 2001). Overexpression of the pro-survival Bcl-xL (but not Bcl-2) has been shown to protect cells from apoptosis induced by staurosporine (Yuste *et al.*, 2002). Although many studies have shown apoptosis induced by staurosporine to be caspase-dependent, there is some evidence that staurosporine may also induced apoptosis independently of caspase activation (Belmokhtar *et al.*, 2001; Xue *et al.*, 2003). In some cell types, treatment of cells with caspase inhibitors prior to staurosporine exposure failed to protect them from cell death (Yuste *et al.*, 2002).

1.4.3 Etoposide

Etoposide is an inhibitor of DNA topoisomerase II used as an antineoplastic agent against several types of tumours including leukemia, lymphoma, testicular and small cell lung cancers (van Maanen *et al.*, 1988). Topoisomerase II is an enzyme which reversibly unwinds DNA to facilitate DNA replication. Topoisomerase II attaches itself covalently to both strands of the DNA helix simultaneously, breaks one double helix reversibly to allow a second double helix to pass through the break, then reseals the

break and dissociates from the DNA. This reaction prevents DNA tangling problems that arise during DNA replication (Berger, 1998).

It is thought that etoposide prevents the re-ligation of DNA, generating single and double stranded breaks in intact cells and in isolated cell nuclei. Introduction of these DNA strand breaks leads to inhibition of DNA synthesis and induction of apoptosis. Etoposide is very active against cells in the late S and G2 phases of the cell cycle and may induce S phase and G2/M arrest in certain cancer cells (Nam *et al.*, 2010). The pro-apoptotic protein Puma is induced in a p53-dependent manner in response to etoposide (Villunger *et al.*, 2003). Etoposide may also induce apoptosis by upregulating expression of Fas ligand (Friesen *et al.*, 1996). The activation of the extrinsic or intrinsic apoptotic pathway appears to be dependent on cell type.

1.5 RNA interference gene silencing technology

RNA interference (RNAi) is now established as a method of silencing gene expression in a variety of organisms. RNAi was used in this study to knock down the expression of BH3-only proteins such as Noxa, Bim and Puma in BL-derived cells in order to investigate the role of these pro-apoptotic factors in the EBV-mediated resistance to ionomycin-induced apoptosis.

The introduction of long double-stranded RNA (dsRNA) molecules into cells interferes with the expression a particular gene which shares a homologous sequence with the dsRNAs. The use of RNAi for sequence-specific gene silencing was first demonstrated by injection of dsRNA into *Caenorhabditis elegans* (Fire *et al.*, 1998).

RNAi is mediated by small interfering RNAs (siRNAs) that are produced from the dsRNA through a cell-autonomous mechanism (McManus and Sharp, 2002; Dykxhoorn *et al.*, 2003; Medema, 2004) as depicted in Figure 1.11. siRNAs are 21-23 nucleotide (nt) dsRNA duplexes with symmetric 2-3nt 3' overhangs and 5'-phosphate and 3'-hydroxyl groups. The first step involves the cleavage of long

dsRNA molecules into siRNAs by the ribonuclease III enzyme known as Dicer in an ATP-dependent reaction. The siRNAs produced are then assembled into the RNA-induced silencing complex (RISC) which contains a helicase that unwinds the duplex siRNAs. The ATP-dependent unwinding of the siRNA duplex is required for the activation of the RISC. RISC uses the antisense strand of the unwound siRNA as a guide to identify the homologous messenger RNA (mRNA) and to promote its specific cleavage. The complementary mRNA is cleaved at a single site near the centre of the duplex region spanned by the siRNA (Elbashir *et al.*, 2001a). The nuclease responsible for the cleavage of the target mRNA, which is distinct from Dicer, has yet to be identified.

The introduction of long dsRNA (>30nt) into mammalian cells triggers a potent interferon response that leads to a general, sequence-nonspescific silencing of gene expression. Interferon induces mRNA degradation and thereby inhibits protein synthesis (Stark *et al.*, 1998). This complication was overcome by the use of chemically synthesised siRNA duplexes which resemble Dicer cleavage products. These siRNA duplexes are too short to trigger the interferon response and can be incorporated directly into RISC to target mRNA degradation (Caplen *et al.*, 2001; Elbashir *et al.*, 2001b) However, this approach can only produce transient gene silencing.

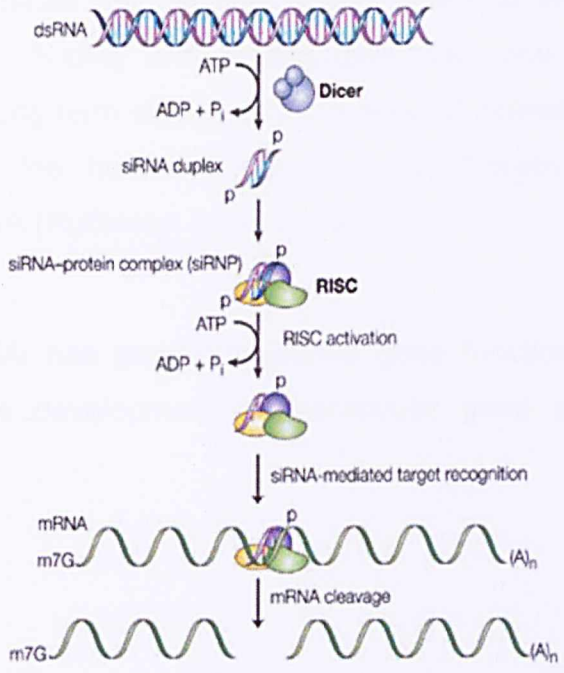


Figure 1-11 RNA interference pathway. Long dsRNA is cleaved by the endoribonuclease Dicer into siRNAs in an ATP-dependent reaction. The siRNAs are then incorporated into RISC and the complex becomes activated by the unwinding of the siRNA duplex. The unwinding of the siRNA duplex requires ATP. The unwound antisense strand guides RISC to the complementary mRNA and promotes its cleavage near the middle of the region bound by the siRNA. Taken from Dykxhoorn *et al.*, 2003.

The most widely used approach to silence gene expression over long periods of time is the use of vector-based short hairpin RNA (shRNA) systems (Hannon and Conklin, 2004). These vectors make use of RNA Polymerase III promoters, such as the U6 promoter (Miyagishi and Taira, 2002) and the H1 promoter (Brummelkamp *et al.*, 2002), to drive the stable expression of short hairpin RNA. These vectors are designed to contain an inverted repeat of 19-29 nucleotides encompassing the desired targeting sequence, separated by a short loop of 6-9 nucleotides. Upon synthesis of the nascent RNA, it will form a stem-loop structure that is processed by the Dicer molecule to functional siRNAs that can be taken up by RISC and direct sequence-specific mRNA degradation. This approach enables production of shRNAs within cells and makes possible the creation of continuous cell lines in which stable knockdown of a target gene is maintained by RNAi. In this study, lentiviral vectors

expressing shRNA were used to deliver shRNA into BL cells and generate stable cell lines in which target genes are knocked down. Lentiviral vectors are capable of infecting both actively dividing and resting cells. Lentiviral-mediated delivery of shRNA is suitable for long-term silencing of the gene of interest as the viral genome integrates stably into the host cell chromosome, thereby ensuring sustained expression of the shRNA (Rubinson *et al.*, 2003).

The application of RNAi has greatly facilitated gene function analysis and offers much potential for the development of therapeutic gene silencing (Dallas and Vlassov, 2006).

1.6 Aims of Thesis

Much work has been done showing that the presence of latent EBV in BL cells rescued them from apoptosis induced by a range of exogenous apoptotic stimuli including cytotoxic drugs, cytokine deprivation and γ-irradiation (see Section 1.3). Recent studies in this lab showed that the EBV-mediated protection against apoptosis induced by the cyclin dependent kinase inhibitor roscovitine, the DNA cross-linking agent cisplatin and the mitotic spindle poison nocodazole is dependent on the co-expression of EBV latent proteins EBNA3A and EBNA3C (Leao *et al.*, 2007; Anderton *et al.*, 2008). This correlates with the downregulation of the pro-apoptotic BH3-only protein Bim by EBNA3A and EBNA3C in BL cells. This protection also appears to be independent of the tumour suppressor p53, which is often mutated in BL cells. When similar experiments were repeated using BL2 cells, which carry wild-type p53 but do not express Bim, it was observed that latent EBV failed to protect BL2 cells against apoptosis induced by the genotoxic agents described above (Anderton *et al.*, 2008). This is because these agents induce the activation of p53 and an apoptotic pathway independent of Bim in BL2 cells.

As such, it was interesting to note that Kelly and colleagues have previously observed that EBV protects both BL31 and BL2 cells against apoptosis induced by the calcium ionophore ionomycin (Kelly *et al.*, 2005). We therefore hypothesised the

existence of a second pathway by which BL cells undergo apoptosis, which is both independent of both Bim and p53, but can also be blocked by EBV.

The aims of this thesis are to characterise the Bim- and p53-indepedent apoptotic pathway induced by ionomycin in BL cells and to investigate the role of EBV in the protection of BL cells against ionomycin-induced apoptosis. This was achieved by:

- Assessing the response of a panel of EBV-positive and -negative BL-derived cell lines to the calcium ionophore ionomycin.

- Identifying candidate viral gene(s) responsible for the repression of this apoptotic pathway by using EBV-positive BL cells expressing a limited subset of latent genes from naturally occurring and recombinant mutant EBVs.

- Constructing new recombinant EBVs containing deletions of further candidate gene(s) to investigate the role of each candidate gene in the EBV-mediated protection against ionomycin-induced apoptosis.

- Identifying cellular factors involved in the apoptotic pathway activated by ionomycin to elucidate the mechanism by which EBV suppresses it, and thereby provides protection against apoptosis.

- Assessing the response of our panel of BL cells to other apoptotic stimuli such as the protein kinase inhibitor staurosporine and the DNA topoisomerase II inhibitor etoposide to determine if the Bim- and p53-independent apoptotic pathway is unique to ionomycin.

Chapter 2 Materials and Methods

All chemical grade reagents were supplied by BDH chemicals, UK and were of AnalR grade purity unless otherwise stated. All autoradiography film (Hyperfilm™), Amplify™ and Enhanced Chemi-Luminescence (ECL)™ reagents were supplied by GE Healthcare, UK.

2.1 Solutions and Buffers

Unless otherwise stated, all solutions were prepared in ddH_2O.

6x Agarose gel loading buffer

20% (w/v) sucrose

0.1% (w/v) Bromophenol blue

10% (v/v) 10x TBE

Alkaline SDS

1% SDS

0.2M NaOH

Blocking Solution for Western Blots

5% (w/v) skim milk powder (Sigma, UK) was reconstituted in 1xPBS/0.05% Tween (Sigma, UK)

20x KPB (Potassium Phosphate Buffer)

0.34M KH_2PO_4

1.44M K_2HPO_4

Luria Broth (LB)

1% (w/v) Bactotryptone

0.5% (w/v) Yeast extract

0.5% (w/v) NaCl

LB Agar

1% (w/v) Bactotryptone

1% (w/v) Yeast extract

0.5% (w/v) NaCl

1.5% (w/v) Agar

NP40 lysis buffer

1% (v/v) NP40

50mM Tris pH8

150mM NaCl

1mM EDTA

10mM β-glycerophosphate

1mM DTT

10mM NaF

2mM Na_3VO_4

1mM PMSF

4% (v/v) Complete™ protease inhibitor cocktail solution (Roche)

Phosphate Buffered Saline (PBS), pH 7.2

12.5mM NaCl

2mM Na_2HPO_4

1mM NaH_2PO_4

100mM PMSF (phenylmethylsulfonyl fluoride)

Dissolved in isopropanol and stored at –20°C

Protease Inhibitor Cocktail Solution

1 tablet of Complete™ Protease Inhibitors Cocktail (Roche) dissolved in 2ml ddH_2O.

Stored at -20°C for up to 2 months

5x Radioimmunoprecipitation (RIPA) Lysis Buffer

0.75M NaCl

5% (v/v) NP40

2.5% (w/v) DOC

0.5% (w/v) SDS

0.25M Tris-HCl pH 8.0

RFI

30mM Potassium acetate

50mM $MnCl_2$

100mM KCl

10mM $CaCl_2$

15% (v/v) Glycerol

Adjusted to pH7.5

RFII

10mM NaMOPS

75mM $CaCl_2$

10mM KCl

15% (v/v) Glycerol

Adjusted to pH7.0

RNase A solution

2mg/ml dissolved in ddH2O. Stored at 4°C.

10x SDS Running Buffer for SDS-PAGE

250mM Tris

1.92M Glycine

1% (w/v) SDS

2x SDS Sample Buffer

100mM Tris-HCl (pH 6.8)

20% (v/v) Glycerol

1% (v/v) β-mercaptoethanol

1% (w/v) SDS

0.025% (w/v) Bromophenol blue

SOB

2% (w/v) Bactotyrptone

0.5% (w/v) Yeast extract

100mM NaCl

2.5Mm KCl

20Mm $MgSO_4$

STET

8% Sucrose

5% Triton X-100

50mM EDTA

50mM Tris-HCl pH8

Superbroth (SB)

1.2% (w/v) Bactotryptone

2.4% (w/v) Yeast extract

0.4% (v/v) Glycerol

5% (v/v) 20x KPB

10x TBE (Tris-Borate-EDTA)

89mM Tris

89mM Boric acid

10mM EDTA

TE

10mM Tris-HCl pH8

1mM EDTA pH8

Transfer Buffer for Western Blots

300ml 10xSDS Running Buffer

700ml EtOH

2L ddH_2O

Other reagents

Ionomycin and staurosporine were obtained from Sigma, UK. Etoposide was obtained from Calbiochem, UK. All were reconstituted in DMSO and stored protected from light at -20°C. Stock solutions were made at a concentration of 2mg/ml for ionomycin, 1mM for staurosporine and 1μg/ml for etoposide. Recombinant IFN-α IFN-γ were obtained from R&D Systems, UK. IFN-α and IFN-γ were reconstituted in PBS containing 0.1% bovine serum albumin (BSA) and stored at -70°C. Stock solutions were made at a concentration of 10μg/ml for IFN-α and 0.1mg/ml for IFN-γ.

Table 2.1 Antibiotics

Antibiotic	Stock	Working concentration
Ampicillin (Amp)	100mg/ml in 70% EtOH	0.1mg/ml
Chloramphenicol (Cm)	12.5mg/ml in 70% EtOH	12.5µg/ml
Hygromycin B (Hyg)	50mg/ml in PBS	100-200µg/ml
Kanamycin (Kan)	50mg/ml in ddH_2O	50µg/ml
Puromycin (Puro)	1mg/ml in ddH_20	1µg/ml
Tetracycline (Tet)	5mg/ml in 70% EtOH	10µg/ml

Table 2.2 Primary Antibodies

Antigen	Antibody	Species	Supplier	Application
Bad		Rabbit	Cell Signalling	WB 1:1000
Bcl-2	124	Mouse	Dako	WB 1:200
BHRF1	5B11	Mouse	Chemicon	WB 1:1000
Bid		Mouse	BD Transduction	WB 1:1000
Bik		Rabbit	Cell Signalling	WB 1:1000
Bim/BOD		Rabbit	Stressgen	WB 1:1000
Bip/GRP78		Rabbit	Cell Signalling	WB 1:1000
Bmf		Rabbit	Cell Signalling	WB 1:1000
BZLF1	BZ1 (Young *et al.*, 1991)	Mouse	Prof. Paul Farrell	WB 1:500
Early lytic antigens	EE serum from patient with chronic IM	Human serum	Prof. Paul Farrell	WB 1:10000
EBNA1		Human serum	Prof. Paul Farrell	WB 1:750
EBNA2	PE2	Mouse	DAKO	WB 1:500
EBNA3A		Sheep	Exalpha	WB 1:1000
EBNA3B		Sheep	Exalpha	WB 1:333

EBNA3C	A10 (Maunders *et al.*, 1994)	Mouse[1]	Dr. Martin Rowe	WB 1:10
EBNA-LP	JF186 (Finke *et al.*, 1987)	Mouse[1]	Dr. Lindsey Spender	WB 1:10
Hrk		Rabbit	Abcam	WB 1:200
LMP1	CS1-4	Mouse	DAKO	WB 1:500
Noxa	114C307	Mouse	Calbiochem	WB 1:500
p53	DO-1	Mouse[2]	Dr. Xin Lu	WB 1:1000
Par-4		Rabbit	Cell Signalling	WB 1:1000
PARP		Rabbit	Roche	WB 1:4000
Puma		Rabbit	Sigma	WB 1:1000
Tis11b	SB1/30	Rat	Dr. Martin Turner	WB 1:400
γ-tubulin	GTU-88	Mouse	Sigma	WB 1:2000

[1] Tissue culture supernatant [2] Ascites fluid

WB = western blotting

Table 2.3 Secondary Antibodies

Specificity	Species-Conjugate	Supplier	Application
Human Ig	Rabbit-peroxidase conjugated	DAKO	WB 1:2000
Mouse Ig	Sheep–peroxidase conjugated	GE Healthcare	WB 1:2000
Rabbit Ig	Goat-peroxidase conjugated	DAKO	WB 1:2000 (1:10,000 tertiary)
Rat Ig	Rabbit-peroxidase conjugated	DAKO	WB 1:2000
Sheep Ig	Rabbit-peroxidase conjugated	DAKO	WB 1:2000

All antibodies were diluted in 5% Milk/0.05% Tween-20/PBS.

2.2 Tissue culture

RPMI Medium 1640 containing L-glutamine was supplied by Invitrogen, UK. Media was supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) and penicillin/streptomycin (100units/ml). BL2 and BL31 cells were additionally supplemented with 1mM sodium pyruvate (Sigma) and 50μM α-thioglycerol (Sigma). Hygromycin B (Roche, UK) was added at a concentration of 150μg/ml to all HEK293 cells carrying a hygromycin-resistant BAC, and BL cells infected with recombinant hygromycin-resistant EBV were selected in media containing 200μg/ml Hyg and maintained in media containing 100μg/ml Hyg.

For passaging, suspension cells were diluted into fresh, warmed medium every 2 to 3 days (see Table 2.4). For experiments, cells were resuspended in fresh medium at a density of $3x10^5$ cells/ml 24 hours before manipulation. Adherent cells were washed once in PBS and then incubated with an appropriate volume of 0.05% trypsin/EDTA (Invitrogen) at 37°C until cells detached from the flasks. An equal volume of fresh medium was added to halt further trypsinisation and cells were split into fresh medium. All cells were cultured at 37°C in a humidified incubator with 10% CO_2.

Viable cell counts were performed by diluting cells 1:1 with 0.4% trypan blue (Sigma) and placing a small volume on a haemocytometer (Assistent, Germany). Living cells, which exclude trypan blue dye, were counted under a light microscope.

For long-term storage of cell stocks, 10^7 cells were pelleted and resuspended in 1ml freezing medium (20% FCS, 10% DMSO in RPMI). The cells were placed in cryochambers and transferred to liquid nitrogen (LN_2) following storage at -80°C overnight (O/N).

Table 2.4 Growth medium used for tissue culture

Cell Lines	Medium
BL41, BL41/B95.8, BL41/P3HR1, BL41/MTLM5, BL41/MTLM11, Oku-BL, Sal-BL, Mutu I, Mutu III, LCL-BF, LCL-CH, HEK293, DG75, BJAB	RPMI/10% FCS
BL2, BL31	RPMI/10%FCS + 1mmol sodium pyruvate + 50μmol α-thioglycerol
BL2 and BL31 established with recombinant knockout and revertant EBVs	RPMI/10% FCS + 1mmol sodium pyruvate + 50μmol α-thioglycerol + 100μg/ml Hygromycin

2.3 Protein Analysis

2.3.1 Harvesting Cells

Suspension cells

Cells, which were seeded at $3x10^5$ cells/ml overnight, were centrifuged at 700×*g* for 4 minutes at 4°C. The media was aspirated and the cell pellet washed with 5ml of cold PBS, before being centrifuged again at 700×*g* for 4 minutes at 4°C. The supernatant was removed again, and the pellet was resuspended in 1ml of cold PBS and transferred into an eppendorf. The cells were then centrifuged at 700×*g* for 4 minutes at 4°C and the supernatant removed.

Adherent cells

Cells were seeded in 10cm dishes overnight in 10ml of media at $1x10^6$ cells/ml. The media was transferred into a falcon tube and the cells washed with 2.5ml of cold

PBS. The wash solution was also transferred into the falcon tube and the cells were scrapped off the 10cm dish using a cell scraper and transferred into the same falcon tube. The 10cm dish was washed again with 2.5ml of cold PBS and the solution transferred into the falcon tube. The cells were then centrifuged at 700×*g* for 4 minutes at 4°C. The media was removed and the cell pellet was washed twice with cold PBS using the same method as for suspension cells above.

2.3.2 Preparation of Protein Extracts

After washing in PBS, cells were lysed in 50–100µl 1× RIPA buffer (containing 4% (v/v) Complete™ protease inhibitor cocktail solution and 1mM PMSF) and left to incubate for 10 minutes on ice. 1× RIPA lysis buffer was prepared as follows:

	Volume
5× RIPA	200µl
Protease Inhibitor Cocktail solution	40µl
100mM PMSF	10µl
dH_20	750µl

After centrifugation at 14000×*g* for 10 minutes at 4°C to remove cell debris, the protein concentration of the supernatant was measured by calibration against a known standard concentration of gamma globulin using the BioRad detergent-compatible protein assay (BioRad, UK). An appropriate volume of SDS sample buffer was added to 20-50µg of protein before heating to 100°C for 5 minutes, centrifuging at 14000×*g* for 1 minute and loading onto sodium dodecyl sulphate-polyacrylamide or SDS-PAGE gels.

2.3.3 SDS-PAGE and Western Blotting

For the detection of high molecular weight proteins (>90kDa), 7.5% polyacrylamide gels were used, for proteins of 40-90kDa 10% gels were used, for 20-40kDa proteins, 12.5% polyacrylamide gels were used and smaller proteins were separated on 15% polyacrylamide gels. Gels were prepared as described below.

Resolving gel

	7.5%	10%	12.5%	15%
30% Acrylamide/Bis-acrylamide solution (37.5:1)	1.98ml	2.64ml	3.30ml	3.96ml
1 M Tris-HCl (pH 8.8)	2.96ml	2.96ml	2.96ml	2.96ml
SDS (10% w/v)	79.2µl	79.2µl	79.2µl	79.2µl
ddH_2O	2.96 ml	2.3ml	1.64 ml	0.98 ml
APS (10% w/v)	26.4µl	26.4µl	26.4µl	26.4µl
TEMED	5.3µl	5.3µl	5.3µl	5.3µl

Stacking gel (5%)

30% Acrylamide/Bis-acrylamide solution (37.5:1)	828µl
1 M Tris-HCl (pH 6.8)	620µl
SDS (10% w/v)	49.6µl
ddH_2O	3.47ml
APS (10% w/v)	24.8µl
TEMED	5µl

6µl of the ladder used (Full-Range or High-Range Rainbow™ markers, Amersham) was combined with 6µl of SDS sample buffer and was boiled for 5 minutes before being loaded into a well. Gels were run at a constant voltage of 150V for approximately 70 minutes. Proteins were then transferred onto nitrocellulose membranes at 100V for 1 hour in transfer buffer using BioRad transfer apparatus. Membranes were incubated in blocking solution for 1 hour before addition of the primary antibody, which was left overnight at 4°C with agitation. For detection of primary antibodies, the appropriate secondary antibody was applied to the membrane for 1 hour at room temperature. Four washes of PBS-T were carried out between antibody layers and proteins were visualised using the ECL

chemiluminescence detection kit and exposure on Hyperfilm™ as recommended by the manufacturer (GE Healthcare).

2.4 DNA Analysis and Cloning Techniques

Similar techniques were used to analyse both bacterial artificial chromosomes (BACs) and standard high-copy number plasmids, but where methods did differ, this is indicated in the text.

2.4.1 Preparation of competent DH5α bacteria

2ml of an O/N culture of *E.coli* strain DH5α was diluted into 250ml of SOB solution and agitated at 37°C until the optical density reading of the solution at a wavelength of 550nm was 0.5 (determined by spectrophotometry) to signify that the bacteria were in an exponential phase of growth. The bacteria were pelleted by centrifugation at 4000×*g* for 5 min at 4°C. The pellet was resuspended in 50ml of ice-cold RFI and left on ice for 40 min. Bacteria were pelleted as before and this time the pellet resuspended in 10ml of ice-cold RFII before 0.25ml aliquots of bacteria were snap frozen on dry ice and then stored at -80°C for later use.

2.4.2 DNA Transformation

E.coli DH5α competent cells were allowed to defrost on ice. The DNA to be transformed was added to 50µl of *E.coli*, and the mix kept on ice for 30 minutes. The mixture was then heat shocked for 1 minute at 42°C. 1ml of LB was added, and the mixture placed in a shaking incubator for 1 hour at 37°C. Bacteria were pelleted and resuspended in 100µl LB and this transformation mix was then plated out on Agar plates containing antibiotics for overnight incubation at 37°C.

2.4.3 Small scale isolation of plasmid DNA: "Minipreps"

Single bacterial colonies were picked into 1.5ml superbroth (SB) or Luria broth (LB) containing antibiotics and grown overnight at 37°C in a shaking incubator. Samples were centrifuged at 2000×*g* for 10 minutes to pellet the bacteria. The supernatant

was removed by aspiration. 70µl STET was added to the pellet, which was resuspended by vortexing. 200µl of alkaline SDS was added while vortexing. While still vortexing 150µl of 7.5M ammonium acetate was added, before transferring the sample to ice. Samples were left on ice for 5 minutes, and then centrifuged at 14000×*g* for 20 minutes at 4°C in a pre-cooled rotor. The supernatant was then poured into a fresh eppendorf. 240µl isopropanol added and the DNA precipitated by vortexing or inversion. The sample was centrifuged at room temperature for 5 minutes at 9500×*g*. The supernatant was discarded and the pellet washed in 200µl of 70% ethanol and then centrifuged at 9500×*g* for a further 5 minutes. The supernatant was then aspirated and the pellet air-dried for approximately 10 minutes before the DNA was resuspended in 50µl TE supplemented with 10µg/ml RNaseA.

2.4.4 Large scale isolation of plasmid DNA: "Maxipreps"

Single bacterial colonies were picked into 250ml (high-copy plasmids) or 500ml (low-copy plasmids and BACs) of LB containing antibiotics and grown overnight at 37°C in a shaking incubator. Isolation of plasmid DNA was carried out the following day using the Plasmid Maxi Kit (Qiagen, Germany) as per the manufacturer's instructions. The DNA pellet was resuspended in an appropriate amount of TE at pH8. The plasmid DNA isolated was quantified by diluting 1:50 in dH_20 and the absorbance at 260nm was measured using a spectrophotometer. The concentration of each sample was estimated by multiplying the absorbance at 260nm by 2.5. The purity of the isolated plasmid DNA was determined by measuring the ratio of the absorbance of the diluted DNA (1:50) at 260nm over the absorbance at 280nm. The ratio of the DNA used fell in the range of 1.8-2.

2.4.5 Caesium chloride gradient DNA banding

10.55g of Caesium chloride (CsCl) was added to maxiprep plasmid DNA and made up to 10ml in TE. The solution was mixed and 200µl of ethidium bromide (10mg/ml) was added. The solution was transferred into 16mm x 76mm quick-seal centrifuge tubes (Beckmann, US) and centrifuged at 60,000×*g* for 16-20 hours (Beckmann 70Ti rotor) at 20°C. Bands were visualised with normal light and the lower band

containing plasmid DNA was removed. Butanol extraction was carried out six times using TE/butanol saturated solution to remove ethidium bromide. The plasmid DNA was then precipitated by adding 2 volumes of ethanol and 0.5 volumes of 7.5M ammonium acetate. The mixture was centrifuged at 13,000×*g* at 4°C for 15 minutes. The supernatant was discarded and the pellet was washed in 70% ethanol. The mixture was spun at 13,000×*g* for 5 minutes and the supernatant discarded, The DNA pellet was allowed to air-dry before the pellet was resuspended in 500µl TE.

2.4.6 Agarose gel electrophoresis

Agarose gels were made with 1% or 2% agarose dissolved in 1x TBE depending on the size of the DNA fragment of interest. Staining SYBRsafe (Invitrogen, UK) was added to the mixture. Samples were mixed with agarose gel loading buffer before loading and running in 1x TBE running buffer, at 80V for approximately 1 hour before visualization on a UV trans-illuminator. In all cases 1kb or 100bp DNA ladder (New England Biolabs, UK) was used as a marker and run alongside DNA samples.

2.4.7 Restriction Enzyme Digests

Preparative digests for cloning were carried out in 30µl total volume with the following reagents:

3µl plasmid DNA

3µl 10x restriction enzyme buffer

1.5µl 20x BSA

Make up to 30µl with ddH_2O

To this was added 0.5µl of appropriate restriction enzyme (New England Biolabs) and the digest was incubated for 3 hours at optimal temperature of the enzyme. When a double digest needed to be performed but could not be performed simultaneously due to incompatibility of buffers, two individual single digests were performed on the same sample, with a DNA precipitation step (see Section 2.4.8) in between to remove all traces of the buffer. The digests were heat inactivated to stop

the reaction. Diagnostic restriction digests were carried out in 15µl total volume using the same reagents and conditions but with 1µl of miniprep plasmid DNA or 5µl miniprep BAC DNA. Digested BAC DNA was analysed by pulsed-field gel electrophoresis (see Section 2.4.11).

2.4.8 DNA Precipitation

0.5 volumes of 7.5M ammonium acetate was mixed with 1 volume of DNA solution. 2.5 volumes of ethanol were added to this, mixed by inversion and put on ice for 5 minutes. The mixture was centrifuged at 13000×*g* at 4°C for 30 minutes. The supernatant was discarded and 200µl of 70% ethanol was added to wash the pellet. The mixture was spun again at 13000×*g* at 4°C for 5 minutes and the supernatant removed. The DNA pellet was allowed to air-dry and then resuspended as desired.

2.4.9 DNA Gel Extraction

Gel extraction was carried out following QIAquick Gel Extraction Kit protocol (Qiagen) using a microcentrifuge. All buffers were provided by Qiagen. The DNA band (approximately 200µg) was excised from the agarose gel and 600µl of Buffer QG was added. This was incubated at 50°C for approximately 10 minutes until the agarose was completely melted. 200µl of isopropanol was added and the solution mixed. 700µl of this solution was put into a 'quick spin' column (Qiagen) within a 2ml collection tube and centrifuged for 1 minute at 10,000×*g*. The flow-through was discarded and the remaining 700µl of the solution was added before centrifuging again for 1 minute at 10000×*g*. The flow-through was once again discarded and 750µl of Buffer PE added to the column and centrifuged for 1 minute at 10000×*g*. The flow-through was again discarded and the column centrifuged for another minute at 10000×*g*. The column was placed in an eppendorf and 30µl of Buffer EB was added to the centre of the column membrane, allowed to sit for 2-3 minutes before centrifuging for 1 minute at 10000×*g*.

2.4.10 DNA Ligation

DNA was combined in a T4 DNA ligase reaction at room temperature for 4 hours to yield resultant plasmids. Ligations were performed in 10µl volumes using roughly 3 times as much insert as vector:

Component	Amount
Vector DNA	0.5-1µl
Insert DNA	1-2µl
10 × T4 DNA ligase buffer	1µl
T4 DNA ligase	0.5µl
Total (make up volume with dH_20)	10µl

2.4.11 Pulsed-field gel electrophoresis

Pulsed-field gels were run in a Bio-Rad CHEF DR II system. Gels were made with 1% agarose dissolved in 0.5x TBE (no EtBr) and were run in 2L of 0.5x TBE running buffer. A 1:1 mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders (both from NEB, see Appendix A) were used as size markers to run alongside DNA samples. Both DNA samples and ladders were mixed with an appropriate volume of 6x agarose gel loading buffer and the λ–DNA based ladders were heated at 50°C for 5 min prior to loading. The settings for pulsed-field gel electrophoresis of EBV-BACs were as follows:

Initial Sweep Time	1 s
Final Sweep Time	10 s
Voltage	6 V/cm
Running time	14 hrs
Running temperature	14°C

When the run was complete, the gel was stained with EtBr at a concentration of 0.5µg/ml in 0.5x TBE for 1-2 hours before visualisation on a UV transilluminator.

2.5 Construction of recombinant EBV-BACs

A system of RecA-based markerless recombination (Lalioti and Heath, 2001; White *et al.*, 2003) was used to delete the EBNA3 locus, the BHRF1 open reading frame (ORF) and the BHRF1 locus from the pHB9 EBV-BAC. A similar process of recombination was then used to restore the deletions to create revertant EBV-BACs. All EBV-BACs contain chloramphenicol (Cm) and hygromycin (Hyg) resistance genes for selection in prokaryotic and eukaryotic cells respectively. EBV-BACs also carry a green fluorescent protein (GFP) expression cassette for visualisation in eukaryotic cells.

2.5.1 Generation of targeting constructs

2.5.1.1 *Targeting constructs to delete and reinsert EBNA3 locus*

Plasmids containing segments corresponding to regions of the EBNA 3 genes in the EBV genome (p4752.2 – EBNA3A exon1 and p4800.2 – EBNA3C end) were available in the lab. These constructs were made by amplifying regions of the EBV genome by PCR and cloning into pBluescriptII® plasmids. This PCR product pair of half-sites was subcloned from each of the plasmids and ligated together in pBluescriptII® to generate pJY05.1 [p4752.2 (*Psi*I/*Cla*I) + p4800.2 (*Cla*I/*Psi*I)], which contains an internal deletion of the EBNA3 locus flanked by fragments of the EBV genome. The pair of half-sites, now in succession within pJY05.1 were then excised and ligated into p4487.1 (pKOV-Kan-ΔCm^R containing a different polylinker; White *et al.*, 2003) to yield the targeting construct, pJY06.1 [pJY05.1 (*Bss*HII/*Sac*I) + p4487.1 (*Sac*I/*Bss*HII)]. The shuttle plasmid p4487.1 contains positive and negative selection markers: a kanamycin (Kan) resistance gene, a temperature-sensitive origin of replication with permissive temperature of 30-33°C, and the *SacBII* gene of *Bacillus amyloliquefaciens* (colonies containing this gene do not grow in medium containing sucrose) (Gay *et al.*, 1983; Gay *et al.*, 1985). Diagnostic restriction digests were

carried out at each stage to ensure successful cloning (data not shown). Restriction enzyme maps of p4487.1 and the pHB9 EBV-BAC can be found in Appendix B.

In order to construct a revertant BAC, a targeting construct was generated by cloning the entire EBNA3 locus from p4852.3 (a plasmid containing all three EBNA3 genes cloned out of the pHB9 EBV-BAC) into the p4487.1 shuttle plasmid via a cloning intermediate, pJY07.1 [p4852.3 (*Hind*III/*Nhe*I) + p4674.1 (*Xba*I/*Hind*III) - p4674.1 is pBluescriptII® modified to include a different polylinker]. The targeting construct pJY09.2 was the generated by ligation of the fragments pJY07.1 (*Not*I/*Sal*I) + p4887.1 (*Sal*I/*Psp*OMI).

2.5.1.2 *Targeting constructs to delete and reinsert the BHRF1 ORF and the BHRF1 locus*

The region surrounding and including the BHRF1 locus was cloned out of the pHB9 EBV-BAC into p4908.1 (pBluescriptII® modified to include a different polylinker) to generate p8199.5. The *Xho*I/*Sal*I fragment of p8199.5 was excised and the plasmid religated to generate pJY19.1. The *Bgl*II/*Fse*I fragment of pJY19.1 was excised, Klenow treated and religated to generate a deletion of the BHRF1 ORF (pJY22.1). The fragment containing an internal deletion of the BHRF1 ORF was cloned into the shuttle plasmid p4487.1 via a cloning intermediate pJY23.1 [pJY22.1 (*Psp*OMI/*Bst*BI) + p4908.1 (*Not*I/*Cla*I)]. The targeting construct pJY24.1 was generated by cloning the SacI/*Sal*I fragment of pJY23.1 into the multiple cloning site of p4487.1.

The *Psp*OMI/*Bsp*HI fragment of pJY19.1 was cloned into *Bsm*BI/*Bfu*AI pJY18.2 (pBluescriptII® modified to include a different polylinker) to generate pJY20.2. The *Pst*I/*Sma*I fragment of pJY20.2 was excised, Klenow treated and religated to generate a deletion of the BHRF1 locus (pJY21.1). In order to insert regions of homology flanking the BHRF1 locus, the *Bgl*II/*Psp*OMI fragment of p8199.5 was cloned into pJY21.1 (*Bfu*AI/*Bgl*II) to generate pJY26.10. The *Bsp*HI/*Bss*HII fragment of p8199.5 was then cloned into pJY26.10 (*Mlu*I/*Bsm*BI) to generate pJY27.1. The targeting construct, pJY28.1, was generated by cloning the *Bgl*II/*Not*I fragment of pJY27.1 into the *Psp*OMI/*Bam*HI sites of p4487.1.

In order to construct a revertant BHRF1 ORF BAC, a targeting construct was generated by cloning the BHRF1 ORF into the p4487.1 shuttle plasmid via a cloning intermediate, pJY29.1 [pJY19.1 (*Psp*OMI/*Bst*BI) + p4908.1 (*Not*I/*Cla*I)]. The targeting construct, pJY30.5, was the generated by ligation of the *Sal*I/*Sac*I fragment of pJY29.1 into the multiple cloning site of p4487.1.

The BHRF1 locus was cloned from p8199.5 (*Bgl*II/*Sfi*I and *Sfi*I/*Sac*II) and ligated into *Bgl*II/*Sac*II of pJY18.2 to generate pJY31.1. The targeting construct, pJY32.2, was generated by cloning the *Xho*I/*Sac*I fragment of pJY31.1 into *Sal*I/*Sac*I of p4487.1.

2.5.2 Preparation of competent DH10B bacteria containing EBV-BACs

E.coli DH10B cells harbouring wild-type (pHB9) or recombinant EBV-BACs were made competent for transformation by the following method. Single bacterial colonies were picked into 2ml selective LB and grown O/N in a shaking incubator at an appropriate temperature (43°C for cointegrants, 37°C for other EBV-BACs). The next day, 100µl of O/N culture was added to 10ml selective LB and allowed to grow for 3 hours in a shaking incubator at the appropriate temperature. Cells were placed on ice for 30 min before being centrifuged at 3000×*g* for 15 min at 4°C. The pellet was resuspended in 2ml ice-cold 100mM $CaCl_2$, left on ice for 30 min and centrifuged at 3000×*g* for 15 min at 4°C. The pellet was then resuspended in 200µl 100mM ice-cold $CaCl_2$ with 15% (v/v) glycerol. Aliquots of 50µl were stored at -80°C.

2.5.3 Recombination between targeting constructs and EBV-BACs: making cointegrants

Targeting constructs (5µl miniprep DNA) and a RecA-expressing plasmid (pDF25-Tet; 1µl miniprep DNA) were co-transformed into 50µl competent *E.coli* DH10B cells containing wild type or EBNA3 deletion EBV-BACs according to the method described in section 2.4.2. Following recovery for 1 hour at 30°C, bacteria were plated on LB agar containing Cm, Kan and tetracycline (Tet) and grown O/N at 30°C. Colonies were pooled in 1ml LB and 100µl of this plated onto LB agar containing Cm and Kan and grown O/N at 43°C. Colonies were then screened for the presence of

successful cointegrants by miniprep DNA isolation, diagnostic restriction digest and pulsed-field gel electrophoresis.

2.5.4 Resolving cointegrants

After *E.coli* containing cointegrants had been made competent (section 2.5.2), they were transformed with 2µl of pDF25-Tet miniprep DNA according to the method described in section 2.4.2. Following recovery for 1 hour at 30°C, bacteria were plated on LB agar containing Cm and Tet and grown O/N at 30°C. Colonies were pooled and grown in 1ml LB containing 5% sucrose, Tet and Cm at 30°C for 3 to 4 hours, after which 100µl was transferred to fresh 1ml LB containing 5% sucrose with the same selection and grown for another 4 hours at 30°C. From this culture, 100µl was transferred to 1ml LB containing 5% sucrose, Cm and Tet and grown O/N at 30°C. Approximately 5µl of this O/N culture was streaked onto a large (14.5 cm diameter) LB agar plate containing Cm and 10% sucrose and grown O/N at 43°C. The next day, colonies were replica plated onto LB agar plates containing Cm and Kan, and then to a control plate containing just Cm. These replica plates were incubated at 43°C O/N. A selection of colonies that grew on the Cm plate but not on the plate containing Cm and Kan were chosen for miniprep DNA isolation, restriction digest and pulsed-field gel electrophoresis. This screening procedure allowed cointegrants to be distinguished from BACs which had resolved to generate deletion and revertant EBV-BACs.

2.6 Production of infectious virus from recombinant EBV-BACs and generation of BL cell lines

Once recombinant EBV-BACs had been constructed, they were transfected into HEK293 cells and stable cell lines established. Infectious virus was then generated from 293 producer cell lines by induction of the lytic cycle.

2.6.1 Transfection of EBV-BACs into 293 cells (LID transfection system)

293 cells were seeded at $1x10^6$ cells/well of a 6-well plate 24 hours prior to transfection. The LID transfection system consists of Lipofectin® Reagent (Invitrogen), Integrin-Targeting Peptide 6 (Hart *et al.*, 1998) and DNA. For 1µg of plasmid DNA, 0.75µl of Lipofectin® Reagent (1mg/ml) was combined with 40µl Peptide 6 (0.1mg/ml) and left to stand for 5 min. This solution was added to 1µg of maxiprep-purified BAC DNA [made up to 100µl volume in OptiMEM (Invitrogen)] and left for 20 min. The medium was then removed from cells in the 6-well plate and carefully replaced with the entire LID transfection mixture which had been made up to 1ml with OptiMEM. Cells were incubated at 37°C for 4 to 6 hours after which the transfection mixture was replaced with 4ml of RPMI/10% FCS.

2.6.2 Isolation of stably transfected 293 cell clones

Two days after transfection of BAC DNA into 293 cells, the medium on the cells was changed for 4ml RPMI/10% FCS containing 150µg/ml Hyg for selection of successfully transfected cells and left overnight. The next day cells in each well of the 6-well plate were trypsinised and diluted with growth medium and 200µl aliquots transferred to a 10cm dish. Cells in both 100mm dishes and 96-well plates were grown in RPMI/10% FCS/150µg/ml Hyg and incubated at 37°C with 10% CO_2.

The growth of colonies of GFP-positive transfected cells was monitored by fluorescence microscopy. Once colonies in 10cm dishes were greater than 4-5mm in diameter, each colony was isolated with a ring and transferred individually to one well of a 24-well plate (ring-cloning).

Single cell clones of transfected 293 cells isolated by ring-cloning methods were expanded until cells were confluent in a $25cm^2$ tissue culture flask. At this stage there were sufficient cells to freeze down a stock in LN_2 and to seed a 6cm dish ready for episome rescue.

2.6.3 Episome rescue from transfected cells

Episomal (non-chromosomal, low molecular weight) DNA can be extracted from cells in a similar manner to the isolation of plasmid DNA from bacterial cells. Transfected 293 cell clones were grown to 90% confluency in a 60mm dish. The growth medium was removed and the cells washed in cold PBS before being scraped into 1.5ml PBS and transferred to an eppendorf tube. Cells were centrifuged at 700×*g* for 3 min and the pellet resuspended thoroughly in 60µl STET by gentle vortexing. Cells were lysed by the addition of 130µl alkaline SDS while vortexing briefly. This was then neutralised by the addition of 110µl 7.5M ammonium acetate. The mixture was incubated on ice for 5 min before centrifuging at 15000×*g* for 30 min at 4°C. The supernatant was transferred to a 1.5ml MaXtract High Density Gel tube (Qiagen), 200µl of phenol:chloroform:isoamyl alcohol (25:24:1; Sigma) added and the tube rapidly inverted for 30s to mix before centrifuging at 10000×*g* for 5 min. The upper aqueous layer was then transferred to a new MaXtract Gel tube and 200µl of chloroform was added. The tube was again inverted to mix and centrifuged for 5 mins at 10000×*g*. This was repeated once. The upper aqueous layer was transferred to a fresh eppendorf and the DNA precipitated by addition of 670µl of 100% EtOH and washed with 200µl of 70% EtOH. The pellet of episomal DNA was allowed to air-dry for 15 min before being resuspended in 50µl TE containing 10µg/ml RNaseA.

2.6.4 Screening of episomal DNA rescued from 293 cells

2µl of this low molecular weight DNA preparation was added to 20µl of Electromax® electrocompetent DH10B *E.coli* (Invitrogen) on ice. This was electroporated at 1.8kV, 25µFD and 200Ω using a Gene pulser® (Bio-Rad) before 400µl of SOC (Invitrogen) was added and the mixture placed in a shaking incubator at 37°C for 1 hour to recover. The entire culture volume was plated onto LB agar containing Cm and incubated O/N at 37°C. Colonies were chosen for miniprep DNA isolation and then screened by analytical restriction digest of 5µl DNA with *Eco*RI and *Age*I restriction enzymes. Restriction fragments were separated by pulsed-field electrophoresis (section 2.4.11) and compared to those produced by digestion of 2µl of the originally transfected EBV-BAC maxiprep DNA.

2.6.5 Generating virus from stably transfected 293 cells

293 producer cell lines harbouring recombinant EBV-BACs were induced to produce virus by transient transfection of BZLF1 (Countryman and Miller, 1985; Hammerschmidt and Sugden, 1988; Delecluse *et al.*, 1998), and BALF4 (Neuhierl *et al.*, 2002). 293 cells were seeded at a density of $5x10^5$ cells/well of a 6-well plate. The following day, each well of cells was transfected with 0.5μg BZLF1 and 0.5μg BALF4 (1μg in total) by the LID transfection method (see section 2.6.1). Following incubation of cells for 4 to 6 hours, the transfection mixture was replaced with RPMI/10% FCS. Three days after transfection, virus-containing supernatant was harvested and filtered through a 0.45μm filter. Infectivity of the supernatant was assayed by infection of Raji-BL cells. Ten-fold dilutions of viral supernatant harvested from 293 cells (1ml, 0.1ml and 0.01ml made up to 1ml volume using RPMI/10% FCS) were used to infect $1x10^5$ Raji cells in 0.5ml RPMI/10% FCS. After 2 days, 0.5ml of RPMI/10% FCS containing 20nM TPA (Sigma) and 5mM sodium n-butyrate (Sigma) was added to the cells. The next day, viral titre was estimated by counting the number of GFP-positive Raji cells in various dilutions of viral supernatant using fluorescence microscopy.

2.6.6 Infection of EBV-negative BL cells

The EBV-negative BL cell lines BL31 and BL2 were infected with recombinant and wild type EBV generated from EBV-BACs. 100μl of viral supernatant from a 293 producer cell line was added to $1x10^5$ BL cells in 1ml of growth medium. After 48 hours, cells were selected in Hyg at a concentration of 200μg/ml to produce EBV-converted BL lines. Cells were monitored periodically for GFP expression by fluorescence microscopy. Once cell lines were established, Hyg selection was reduced to 100μg/ml. Expression of EBV latent proteins was validated by western blotting of protein extracts.

2.7 Lentiviral-mediated shRNA

Lentiviruses carrying shRNA directed against Bim, Puma and Noxa were obtained from Sigma. A non-targeting shRNA lentiviral vector was also obtained from Sigma for use as a negative control. All lentiviral vectors carry a puromycin (Puro) resistance gene. EBV-negative BL31 and BL2 cells were seeded at $2x10^5$ cells/ml 24 hours before transduction. Cells were diluted to $1x10^5$ cells/ml and aliquoted (100µl) into wells of a 96-well plate. Lentiviral particles were added to cells at an MOI of 10 (approximately 10-30µl). Titres of lentiviruses obtained were in the range of 10^6-10^7TU/ml. After 24 hours, cells were selected in puromycin at a concentration of 1µg/ml. Once stable cells lines were established, cells were harvested for protein and RNA analyses to determine the levels of knockdown achieved by shRNA.

Table 2.5 Lentiviral shRNA vectors

Gene/TRC Clone Number	shRNA sequence	Target region
Bim 1 TRCN0000001051	CCGGATGGTTATCTTACGACTGTTACTCGA GTAACAGTCGTAAGATAACCATTTTTT	CDS
Bim 2 TRCN0000001053	CCGGACGAATGGTTATCTTACGACTCTCGA GAGTCGTAAGATAACCATTCGTTTTTT	CDS
Bim 3 TRCN0000001054	CCGGAGCCGAAGACCACCCACGAATCTCG AGATTCGTGGGTGGTCTTCGGCTTTTTT	CDS
Puma 1 TRCN0000033609	CCGGCGTGAAGAGCAAATGAGCCAACTCG AGTTGGCTCATTTGCTCTTCACGTTTTTG	3' UTR
Puma 2 TRCN0000033610	CCGGGTACAATCTCATCATGGGACTCTCGA GAGTCCCATGATGAGATTGTACTTTTTG	CDS
Puma 3 TRCN0000033611	CCGGCGACCTCAACGCACAGTACGACTCG AGTCGTACTGTGCGTTGAGGTCGTTTTTG	CDS

Puma 4 TRCN0000033612	CCGGGAGGGTCCTGTACAATCTCATCTCG AGATGAGATTGTACAGGACCCTCTTTTTG	CDS
Puma 5 TRCN0000033613	CCGGACGGTCCTCAGCCCTCGCTCTCTCG AGAGAGCGAGGGCTGAGGACCGTTTTTTG	CDS
Noxa 1 TRCN0000150555	CCGGGCATTGTTGTTGTTGCTGTTTCTCGA GAAACAGCAACAACAACAATGCTTTTTTG	3' UTR
Noxa 2 TRCN0000151311	CCGGGCTTCTGTTCAGATGATCTTTCTCGA GAAAGATCATCTGAACAGAAGCTTTTTTG	3' UTR
Noxa 3 TRCN0000153637	CCGGCTCAGGAGATTTGGAGACAAACTCG AGTTTGTCTCCAAATCTCCTGAGTTTTTTG	CDS
Noxa 4 TRCN0000155570	CCGGCTTCCGGCAGAAACTTCTGAACTCG AGTTCAGAAGTTTCTGCCGGAAGTTTTTTG	CDS
Noxa 5 TRCN0000155978	CCGGCAAACTGAACTTCCGGCAGAACTCG AGTTCTGCCGGAAGTTCAGTTTGTTTTTTG	CDS

2.8 RNA Analysis

2.8.1 Preparation of RNA Extracts

Cells were seeded at $3x10^5$ cells/ml the day prior to RNA isolation. Total RNA extraction was performed using RNeasy Mini kits (Qiagen) as per the manufacturer's instructions. The RNA pellet was resuspended in RNase free water and stored at -80°C. RNA extracts were quantified using a NanoDrop spectrophotometer.

2.8.2 Real-time Quantitative PCR (RTQ-PCR)

Real-time quantitative PCR was performed using the QuantiFAST SYBR Green RT-PCR kit (Qiagen) and ABI 7900HT real-time PCR machine (Applied Biosystems, US). Experiments were carried out with triplicate RNA samples using primers specific for Noxa and Bim_{EL}. Noxa and Bim_{EL} mRNA levels in each sample were normalised with respect to ubiquitin C (UBC) or delta-aminolevulinate synthase 1

(ALAS1) mRNA levels. The initial steps in the reaction include the reverse transcription of RNA to cDNA by reverse transcriptase (RT) at 50°C for 10 minutes and the denaturation of RT at 95°C for 5 minutes. Cycling conditions are 95°C for 10s (denaturation) and 60°C for 30s (combined annealing and extension). The reaction was carried out with a total volume of 25µl which consists of the following components:

	Volume
2x SYBRGreen RT Mix	12.5µl
RT Mix	0.25µl
RNA (0.1µg/µl)	1µl
Forward Primer (10µM)	2.5µl
Reverse Primer (10µM)	2.5µl
RNase free H_20	6.25µl
Total	25µl

Dissociation curve analysis was performed during each run to confirm the absence of non-specific products.

Table 2.6 Primer sequences

Bim_{EL} Fwd	GCTGTCTCGATCCTCCAGTG
Bim_{EL} Rev	GTTAAACTCGTCTCCAATACG
Noxa Fwd	GAACGCGCCAGTGAACCCAA
Noxa Rev	CTTTGTCTCCAATCCTCCGG
ALAS1 Fwd	GGATTCGAAACAGCCGAGTG
ALAS1 Rev	TGACATCATTGTGGCGGAAG
UBC Fwd	ATTTGGGTCGCGGTTCTTG
UBC Rev	TGCCTTGACATTCTCGATGGT

2.9 Luciferase Reporter Assays

DG75 and BJAB cells were transfected for reporter assays by electroporation with 2μg of SV40 β-gal and 2μg of each of the Noxa promoter reporter plasmids or pGL3 basic. Cells were split at a density of $2x10^5$ cells/ml 24 hrs prior to transfection. DNA was added to $0.4cm^3$ cuvettes (Bio-Rad) and the volume was made up to 50 μl with unsupplemented RPMI media. $1x10^7$ cells were harvested per transfection. These cells were pelleted at 1300×*g* for 4 mins at 4°C. The supernatant was retained as conditioned medium. The cell pellet was resuspended in 150μl of unsupplemented RPMI, added to the cuvette, and incubated on ice for 10 mins. Electroporation was performed at 250V, 960μF using a Gene Pulser (Bio-Rad). The cuvettes were then incubated at 37°C for 15 mins, after which the transfected cells were resuspended by pipette and added to 10ml of medium (7.5ml of conditioned medium and 2.5ml of fresh RPMI with 10% FCS). Transfected cells were incubated at 37°C for 24hrs before adding ionomycin at 1μg/ml.

After 6hrs of exposure to ionomycin, cells were collected by centrifugation and washed in PBS. Cells were then pelleted by centrifugation and the pellet was lysed in 100μl of reporter lysis buffer (Promega) for 15 mins at room temperature. Debris was removed by centrifugation at 12,000×*g* and the supernatant was transferred to a fresh Eppendorf tube. 20μl of supernatant per transfection was used in the luciferase assay or in the β-galactosidase assay. Luciferase activity was measured on an Autolumat luminometer using a luciferase detection reagent (Promega). To measure β-galactosidase activity, 20μl of cell lysate per transfection was added to one well of a 96-well tissue culture plate. 150μl of reaction mixture (1.25mm $CaCl_2$, 25μm chlorophenol red-β-galactopyranoside) was added to each well and plates were incubated at 37°C until the yellow color of the mixture began changing to red. Absorbance at 570 nm was taken as a measure of β-galactosidase activity. All luciferase activity values were normalized to β-galactosidase activity in the lysate.

2.10 Cell Viability Assays

Cell viability was measured using the CellTiter-Glo™ Luminescent Cell Viability Assay kit (Promega). Cells were seeded at a density of $3x10^5$ cells/ml the day before manipulation and aliquoted at 100µl per well in a 96-well tissue culture plate. Control wells containing media without cells were also prepared to obtain a value for background luminescence. Ionomycin, etoposide, staurosporine or DMSO (vehicle control) were added to experimental wells and incubated for the appropriate amount of time. CellTiter-Glo Reagent was prepared according to the manufacturer's instructions. The 96-well plate and its contents were left to equilibrate at room temperature for approximately 30 mins. An equal volume of 100µl CellTiter-Glo Reagent solution was then added to each assay well. The plate was left on an orbital shaker to mix contents and induce cell lysis for 2 mins. The plate was then allowed to incubate at room temperature for 10 mins to stabilise luminescent signal. Luminescence of each sample was recorded using an Autolumat luminometer.

Chapter 3 The effect of EBV on the response of B cells to the calcium ionophore ionomycin

Previous work has shown that BL cells are sensitive to a variety of pro-apoptotic stimuli including cytokine deprivation, genotoxic agents, calcium ionophores and anti-IgM. It has been shown that the latent infection of BL cells with EBV significantly increased their resistance to apoptosis (Gregory *et al.*, 1991; Kelly *et al.*, 2005; Kelly *et al.*, 2006; Leao *et al.*, 2007; Anderton *et al.*, 2008). Recent studies have also demonstrated that the protection conferred by EBV against apoptosis induced by agents that can directly or indirectly damage DNA, such as the cyclin dependent kinase inhibitor roscovitine, the DNA cross-linking agent cisplatin and the mitotic spindle poison nocodazole, is dependent on the co-expression of the EBV latent proteins EBNA3A and EBNA3C and the repression of the cellular pro-apoptotic protein Bim (which is downregulated by EBNA3A and EBNA3C) but is independent of the tumour suppressor p53 (Anderton *et al.*, 2008). It has also been shown, however, that EBV did not protect lymphoblastoid cell lines (LCLs) against apoptosis induced by the DNA damaging drugs described above, presumably because they have an intact p53-mediated apoptosis pathway (Allday *et al.*, 1995a; O'Nions and Allday, 2003).

Results from studies conducted using another known inducer of apoptosis in B cells, the calcium ionophore ionomycin (Aagaard-Tillery and Jelinek, 1995), indicated that there could be a second independent pathway by which BL cells undergo apoptosis, which can also be inihibited by EBV (Kelly *et al.*, 2005; Kelly *et al.*, 2006). We wanted to confirm and investigate further these observations by assessing the response of our panel of BL-derived cells and LCLs to the calcium ionophore ionomycin.

3.1 The response of EBV-negative and -positive BL cells to ionomycin

3.1.1 EBV latent gene expression confers resistance against ionomycin-induced apoptosis

In order to investigate the effects of ionomycin (IM) on BL cells, initially the EBV-negative BL41 cell line and two EBV-positive BL41 cell lines converted by *in vitro* infection using different virus strains, B95.8 and P3HR1, were treated with ionomycin at a concentration of 1μg/ml over a 48 hour time period. BL41 is null for WT p53 because it carries only a single, mutant allele (Allday *et al.*, 1995b). Cell survival was determined by counting viable cells which exclude trypan blue at 24 hour intervals throughout the course of the treatment. Cells were also harvested at the same time intervals for protein extraction and analysis.

Viable cell counts were plotted as a percentage of the starting population. The graph shown in Figure 3.1A was constructed using data from one experiment but is representative of data from three independent experiments. After treatment with ionomycin for 48 hours, less than 20% of BL41 cells remain viable. In contrast, BL41/B95.8 cells, which display an EBV latency III phenotype, appear to be completely resistant to ionomycin-induced apoptosis with the number of viable cells greater than the starting population after 48 hours. A similar response was observed in BL41/P3HR1 cells, which express a restricted set of latent proteins. The P3HR1 virus carries a deletion in the region of the EBV genome encoding EBNA2 and the two C-terminal exons encoding EBNA-LP. Hence, BL41/P3HR1 cells only express the latent proteins EBNA1, EBNA3A, EBNA3B, EBNA3C, a truncated form of EBNA-LP, and the EBERs and BARTs. The LMPs are not expressed because EBNA2 is required to transactivate their expression. The experiment described above was repeated and results from three independent experiments were pooled. The mean and standard deviation of viable cell counts after 48 hours of treatment with ionomycin were plotted as a histogram in Figure 3.1B.

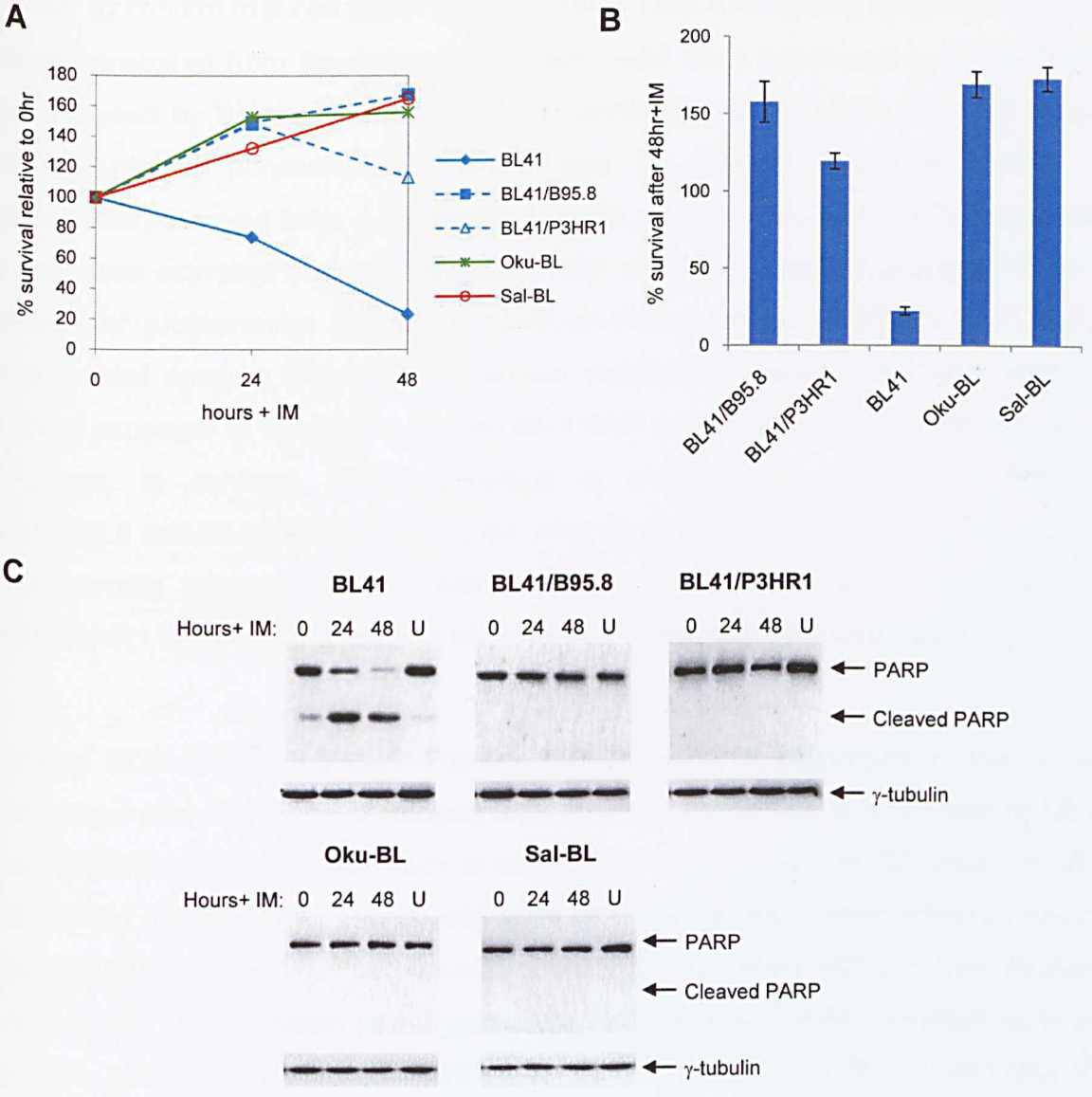


Figure 3-1 Resistance of EBV-positive BL cells to ionomycin-induced apoptosis. BL41 and its EBV-positive counterparts, BL41/B95.8 and BL41/P3HR1, as well as two recently isolated EBV-positive BL lines, Oku-BL and Sal-BL were treated with 1µg/ml ionomycin for up to 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Cell survival is expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments are shown. **(C)** Protein analysis was carried out by Western blotting using an antibody directed against PARP. γ-tubulin was used as a loading control. IM=ionomycin

In order to confirm that cell death was a result of cells undergoing apoptosis, protein extracts prepared from treated and untreated cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects both full length poly(ADP-ribose) polymerase or PARP and its cleaved fragments. PARP is proteolytically cleaved from a full length 113kDa protein to 89 and 24kDa fragments by caspases activated during apoptosis. PARP cleavage is widely accepted to be a hallmark of programmed cell death (Casciola-Rosen *et al.*, 1996). In BL41 cells, Western blot analysis (Figure 3.1C) shows significant cleavage of PARP after 24 hours of exposure to ionomycin. Full-length PARP is almost completely cleaved after 48 hours. In contrast, PARP cleavage is undetectable in the EBV-positive BL41/B95.8 and BL41/P3HR1 cells even after 48 hours of treatment with ionomycin. These results suggest that the restricted set of EBV latent genes expressed in BL41/P3HR1 is sufficient to protect BL cells from ionomycin-induced apoptosis.

Because BL41/B95.8 and BL41/P3HR1 cells have been passaged in culture for many years and may have developed anti-apoptotic properties independent of EBV, it was important to extend the study to include two recently isolated BL lines, Oku-BL and Sal-BL. These early passage cell lines were newly established from BL biopsy material (Kelly *et al.*, 2002) and found to carry EBV genomes with a similar deletion to that of the P3HR1 strain (see Figure 1.3). Oku-BL and Sal-BL therefore express the same subset of EBV genes as BL41/P3HR1. However unlike BL41, both Oku-BL and Sal-BL carry wild-type (WT) p53 (Anderton *et al.*, 2008).

Oku-BL and Sal-BL were treated with ionomycin at a concentration of 1μg/ml for up to 48 hours. Viable cells which exclude trypan blue were counted and cells were harvested for protein analysis at 24 hour intervals. Viable cells counts shown in Figures 3.1A and 3.1B indicate that both Oku-BL and Sal-BL are resistant to ionomycin-induced apoptosis. Furthermore, Western blotting analyses show that full-length PARP is unaffected in both cell lines even after 48 hours, indicating that Oku-BL and Sal-BL do not undergo apoptosis in response to ionomycin (Figure 3.1C). These data show that the ionomycin resistance phenotype is not specific to BL41 and are consistent with the restricted set of EBV viral proteins associated with the

P3HR1 strain being sufficient to protect BL cells against ionomycin-induced apoptosis.

To address this issue further, two EBV-negative BL-derived cell lines, BL31 and BL2, recently infected with a recombinant EBV from which the EBNA2 open reading frame (ORF) had been deleted to produce a P3HR1-like virus (E2KO, Kelly *et al.*, 2005) was also included in this study. BL31 and BL2 cell lines differ in two ways. While BL31, like most BL-derived cell lines, only expresses mutant p53, BL2 carries WT p53 (Anderton *et al.*, 2008). Also, BL31 cells express the pro-apoptotic BH3-only protein Bim but the protein is undetectable by Western blotting in BL2 cells. The EBV-positive converts, BL31+E2KO and BL2+E2KO, express all viral latent proteins with the exception of EBNA2 and the LMPs. This expression pattern is very similar to that of BL41/P3HR1, Oku-BL and Sal-BL except for expression of a full-length, rather than a truncated, EBNA-LP. The study of these recently established converts and their EBV-negative parental lines allowed us to address the question of whether EBV gene expression was directly responsible for the phenotype described above and to reinforce the hypothesis that a limited pattern of viral proteins was sufficient.

Although we confirmed that LMP1 is undetectable by Western blotting in BL2+E2KO, it appears that very low levels of the protein is expressed in BL31+E2KO (data not shown). In order to rule out any effects resulting from LMP1 expression in BL31+E2KO, a second recombinant virus harbouring a deletion of the EBNA2 ORF (not identical to Kelly *et al.*, 2005) was constructed by Ian Groves in the lab. BL31 cell lines were established using this new recombinant EBNA2-knockout (E2KO-IG) EBV. It was confirmed that these newly established cell lines do not express EBNA2 or the LMPs (Ian Groves – personal communication). A revertant virus in which the EBNA2 ORF deletion was reinserted was also constructed as a control for any second site mutations which may have occurred during the recombination process. BL31 cell lines were also established using this EBNA2-revertant virus (E2rev).

BL2 and its converted E2KO counterpart, BL2+E2KO, were treated with 1μg/ml ionomycin over a 48 hour time period. Once again, viable cells which exclude trypan

blue were counted at 24 hour intervals and cells harvested for protein analysis. Viable cell counts were expressed as a percentage of the starting population. The graph shown in Figure 3.2A was constructed using data from one experiment but is representative of data from three independent experiments. Results from three independent experiments were pooled and the mean and standard deviation of viable cell counts after 48 hours of treatment with ionomycin were plotted as a histogram in Figure 3.2B.

After 48 hours of exposure to ionomycin, less than 40% of BL2 cells remain viable as shown in Figures 3.2A&B. Stable infection of BL2 with recombinant E2KO virus protected these cells from ionomycin-induced apoptosis with the population of viable cells actually increasing after 48 hours. This is consistent with Western blot analyses which show evidence of PARP cleavage in EBV-negative BL2 cells 48 hours following treatment with ionomycin. In contrast, full-length PARP remains unaffected in BL2+E2KO even at the end of the time course (Figure 3.2C).

Similar experiments were conducted using parental BL31, its EBNA2-knockout and EBNA2-revertant counterparts. BL31 cells established using virus produced from the original wild-type (WT) EBV-BAC, from which these recombinant viruses were constructed, were also included as a control. Cells were seeded in a 96-well plate and exposed to either 1μg/ml ionomycin or DMSO (vehicle control-ionomycin dissolved in DMSO) for 24 hours. Cell viability was measured using the CellTiter-Glo assay (Promega) which is based on the quantification of ATP contained in viable cells in culture. Cell survival was expressed as a percentage of the vehicle-treated controls. The experiment was conducted in triplicate with the mean and standard deviation of the three readings plotted as a histogram. The histrogram shown in Figure 3.3A is data from one experiment but is representative of two independent experiments. The same panel of cells were again exposed to 1μg/ml ionomycin for up to 48 hours and harvested at 24 hour intervals. Protein extracts from these cells were analysed by Western blotting for evidence of PARP cleavage (Figure 3.3B).

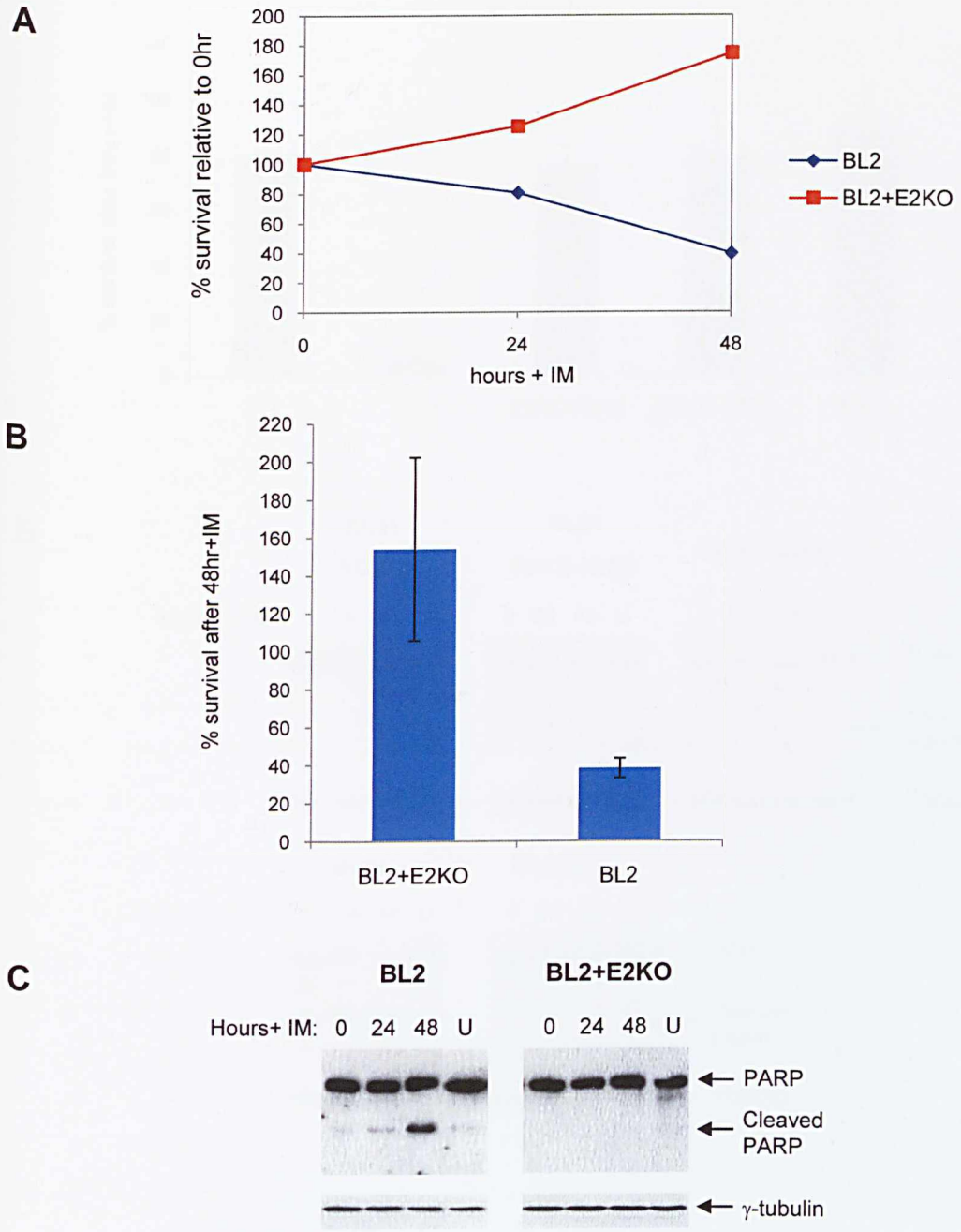


Figure 3-2 Resistance of BL2 cells recently infected with a recombinant EBNA2-knockout EBV to ionomycin-induced apoptosis. BL2 and its EBV-positive convert established with a recombinant EBNA2-knockout virus (BL2+E2KO) were treated with 1µg/ml ionomycin for up to 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Cell survival is expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments are shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control. IM=ionomycin

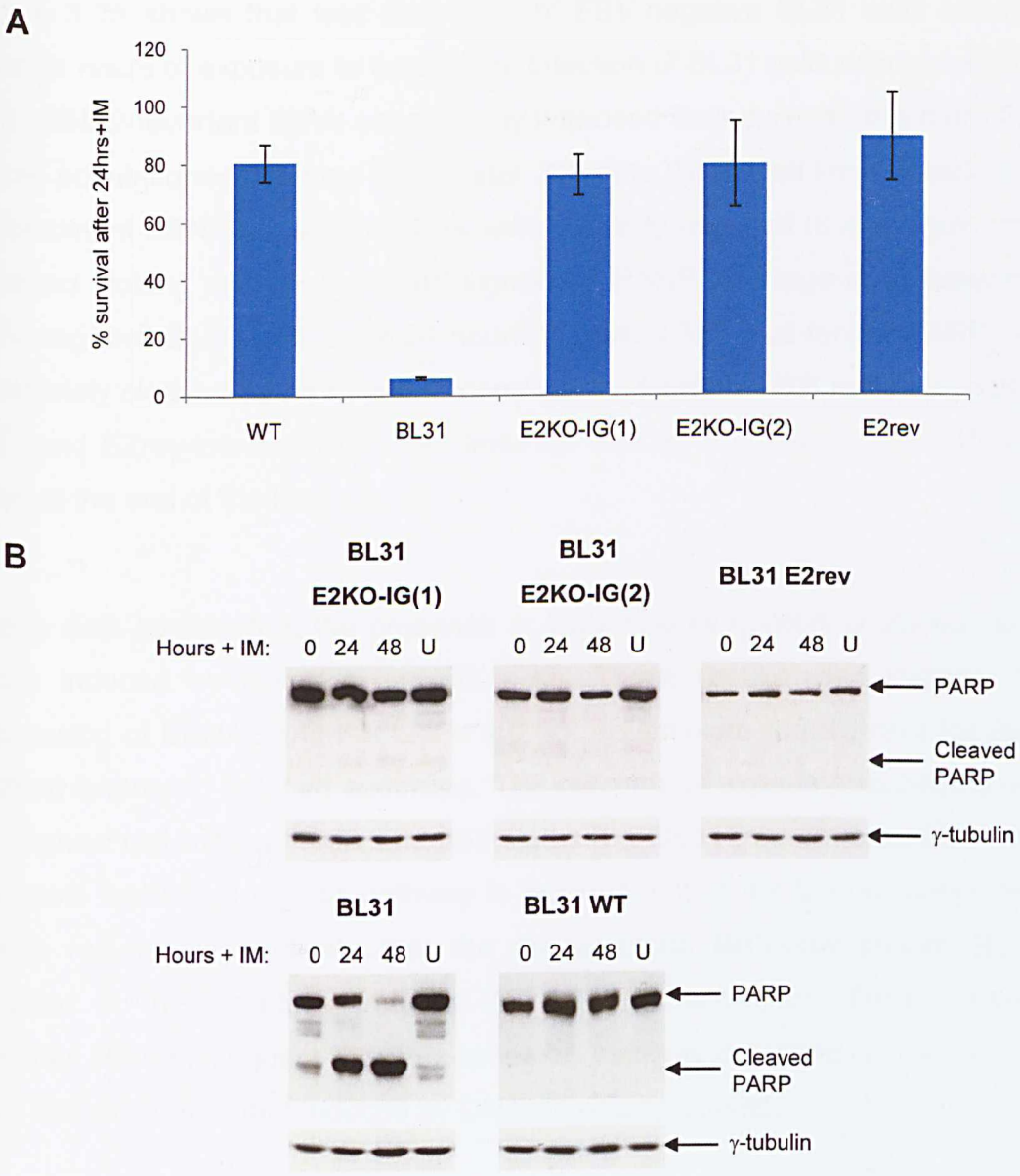


Figure 3-3 Resistance of BL31 cells recently infected with recombinant EBNA2-knockout EBVs to ionomycin-induced apoptosis. (A) EBV-negative BL31, its wild-type (WT)-infected counterpart and EBV-positive converts established with a recombinant EBNA2-knockout virus (BL31 E2KO-IG) and its revertant (BL31 E2rev) were treated with 1μg/ml ionomycin or DMSO (vehicle control) for 24 hours. Cell survival was determined using the Cell-TiterGlo assay and expressed as a percentage of vehicle-treated controls. **(B)** The same panel of cells was exposed to 1μg/ml ionomycin for up to 48 hours. Cells were harvested at 24 hour intervals for protein extraction. Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control. IM=ionomycin

Figure 3.3A shows that less than 10% of EBV-negative BL31 cells remain viable after 24 hours of exposure to ionomycin. Infection of BL31 cells with recombinant WT and EBNA2-revertant EBVs substantially improved their survival with more than 75% of cell populations remaining viable after 24 hours. BL31 cell lines established using recombinant EBNA2-knockout EBVs were similarly resistant to ionomycin treatment. Western blotting analysis revealed significant PARP cleavage in ionomycin-treated EBV-negative BL31 cells after 24 hours (Figure 3.3B). Full-length PARP is almost completely cleaved by 48 hours. In contrast, full-length PARP remains unaffected in WT- and E2rev-infected BL31 cell lines as well as both BL31 E2KO-IG cell lines even at the end of the time course.

These data confirm that the presence of EBV directly confers protection again cell death induced by ionomycin in BL cells. These results also suggest that the expression of EBNA2 and the LMPs are not an absolute requirement for resistance against ionomycin-induced apoptosis. The outcome of experiments carried out using BL-derived cell lines with different p53 status [i.e. BL31 (mutant) and BL2 (WT)] also suggests that this apoptotic pathway is independent of the tumour suppressor p53. These results also indicate that the pro-apoptotic BH3-only protein Bim is not involved in the apoptotic pathway induced by ionomycin. These observations together strongly suggest that the apoptotic pathway activated by ionomycin in BL cells is distinct from that induced by DNA-damaging agents.

3.1.2 LMP1 contributes to the resistance to ionomycin-induced apoptosis

Although the results above suggest that LMP1 is not necessary for ionomycin resistance, we decided to investigate the contribution of LMP1 further because there have been many reports documenting the anti-apoptotic properties of LMP1 (see Section 1.3). In order to directly assess the role of LMP1 in the EBV-mediated protection of BL cells against ionomycin, we decided to repeat these experiments using EBV-negative BL cells which express LMP1 from stably maintained plasmids (Cuomo *et al.*, 1992). The response of these LMP1-expressing BL41 cells were compared to that of the EBV-negative BL41 cells and the EBV-positive BL41/B95.8 cells which express the full set of latent proteins.

EBV-negative BL41, BL41/B95.8 and LMP1-expressing BL41 cells, BL41/MTLM5 and BL41/MTLM11, were treated with 1μg/ml ionomycin over a period of 48 hours. Cell survival was determined by counting viable cells that exclude trypan blue at 24 hour intervals throughout the course of treatment. Viable cell counts were plotted as a percentage of the starting population. The graph shown in Figure 3.4A was constructed using data from one experiment but is representative of data from three repeated experiments. Results from three independent experiments were pooled and the mean and standard deviation of viable cell counts after 48 hours of treatment with ionomycin were plotted as a histogram in Figure 3.4B. Cells were harvested at 24 hour intervals for protein extraction and Western blot analysis performed for PARP and LMP1.

Figure 3.4A&B show that less than 20% of EBV-negative BL41 cells had survived after 48 hours of exposure to ionomycin (Figures 3.4A&B). As before, viable cells counts show that BL41/B95.8 cells are resistant to ionomycin treatment with the population of cells growing to 150% after 48 hours. Viable cell counts show that more than 95% of both LMP1-expressing BL41 cells remain viable after 24 hours of exposure to ionomycin. However, survival drops to approximately 75% for BL41/MTLM5 and 55% for BL41/MTLM11 cells at the end of the time course. Figure 3.4C shows that in BL41 cells, significant cleavage of PARP is observed after 24 hours of exposure to ionomycin, and full-length PARP completely cleaved after 48 hours. In contrast, PARP cleavage is undetectable in EBV-positive BL41/B95.8 cells even after 48 hours of treatment. Very little cleaved PARP is detected in both BL41/MTLM5 and BL41/MTLM11 after 24 hours of exposure to ionomycin. However, an increase of cleaved PARP detected is observed after 48 hours, particularly in BL41/MTLM11. Figure 3.4D shows that LMP1 is expressed at similar levels in BL41/B95.8 and both LMP1-expressing BL41 cell lines.

These data suggest that LMP1 can provide some protection against ionomycin-induced apoptosis. However, it is likely that LMP1 is not the only EBV factor involved in the resistance to ionomycin as expression of LMP1 alone does not appear to be sufficient to mimic the level of protection seen in cells displaying type III latency. Furthermore, LMP1 is not expressed in BL cells shown to be resistant to ionomycin treatment such as BL41/P3HR1, Oku-BL and Sal-BL (see Section 3.1.1).

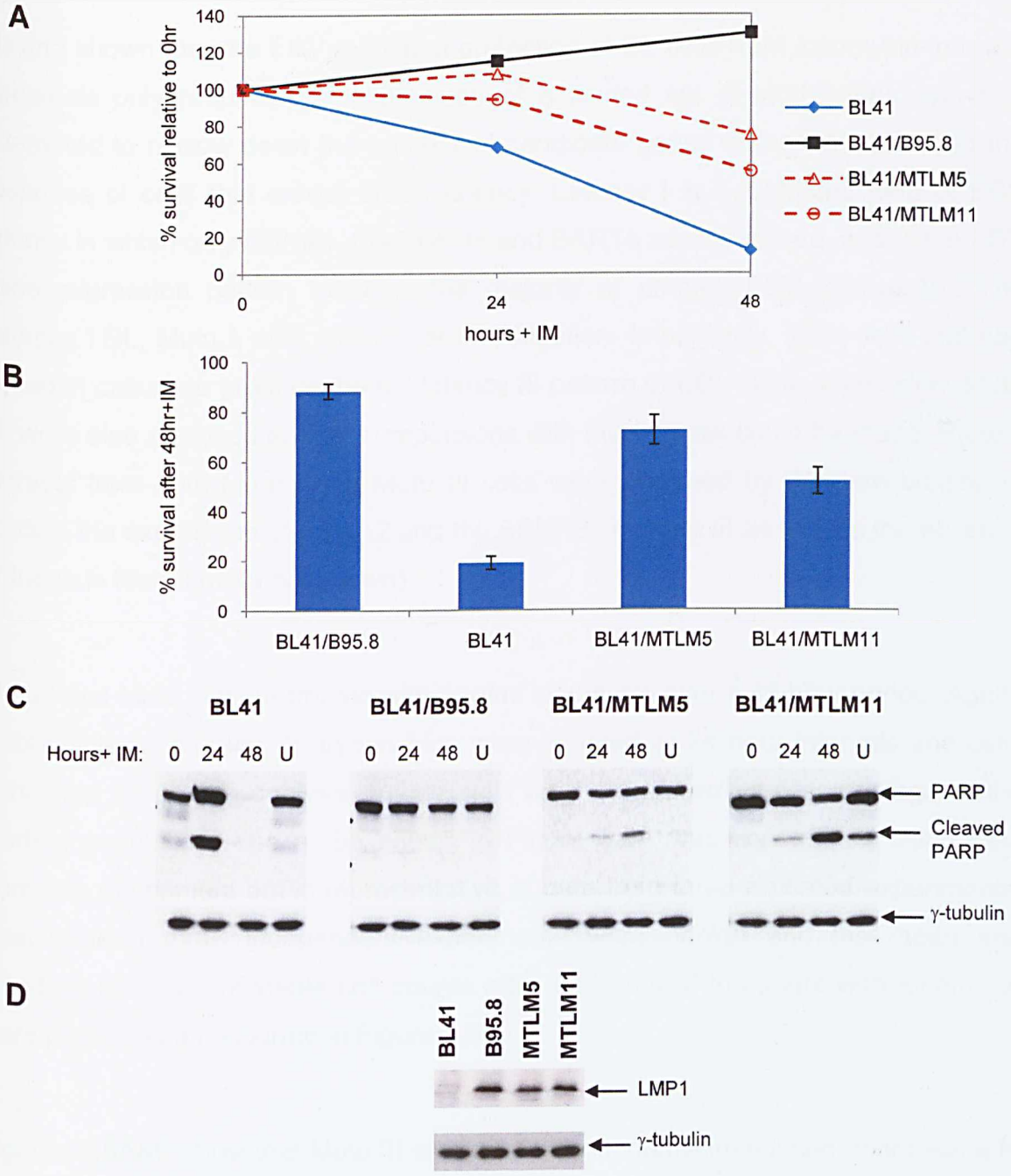


Figure 3-4 LMP1 contributes to the EBV-mediated protection of BL41 cells from ionomycin-induced apoptosis. BL41 and its EBV-positive counterpart, BL41/B95.8, as well as two EBV-negative BL41 cell lines transfected with LMP1 expression plasmids, BL41/MTLM5 and BL41/MTLM11, were treated with 1μg/ml ionomycin for up to 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Cell survival is expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. Protein analysis was carried out by Western blotting with an antibody directed against **(C)** PARP and **(D)** LMP1. Throughout, γ-tubulin was used as a loading control. IM = ionomycin

3.1.3 Type I latency not sufficient to protect against apoptosis induced by ionomycin

Having shown that the EBV-mediated protection of BL cells from ionomycin-induced apoptosis only requires the expression of a limited set of viral genes, we then attempted to narrow down the number of candidate genes further by assessing the response of cells that exhibit type I latency. Latency I is a restricted form of EBV latency in which only EBNA1, the EBERs and BARTs are expressed, and is the EBV gene expression pattern found in the majority of primary EBV-positive BL. The Latency I BL, Mutu I, was chosen for investigation. Importantly, Mutu cells that had drifted in culture to produce the full latency III pattern of EBV gene expression, Mutu III, were also analysed so that comparisons with Mutu I cells could be made. Protein extracts from both Mutu I and Mutu III cells were analysed by Western blotting to confirm the expression of EBNA2 and the EBNA3s in Mutu III as well as the absence of these in Mutu I (data not shown).

Mutu I and Mutu III were treated with 1μg/ml ionomycin over a 48 hour period. Again, viable cells which exclude trypan blue were counted at 24 hour intervals and cells harvested for protein analysis. Viable cells were expressed as a percentage of the starting population. The graph shown in Figure 3.5A was constructed using data from one experiment but is representative of data from three repeated experiments. Results from three independent experiments were pooled and the mean and standard deviation of viable cell counts after 48 hours of treatment with ionomycin were plotted as a histogram in Figure 3.5B.

Figures 3.5A&B show that Mutu III are resistant to ionomycin-induced apoptosis with the population of viable cells increasing to approximately 150% after 48 hours. Furthermore, PARP cleavage is undetectable in these cells by Western blotting (Figure 3.5C). Interestingly, viable cells counts show that less than 20% of Mutu I cells had survived after 48 hours of exposure to ionomycin. Complete cleavage of full-length PARP cleavage is observed in Mutu I cells after 48 hours (Figure 3.5C) indicating that Mutu I cells undergo apoptosis in response to ionomycin.

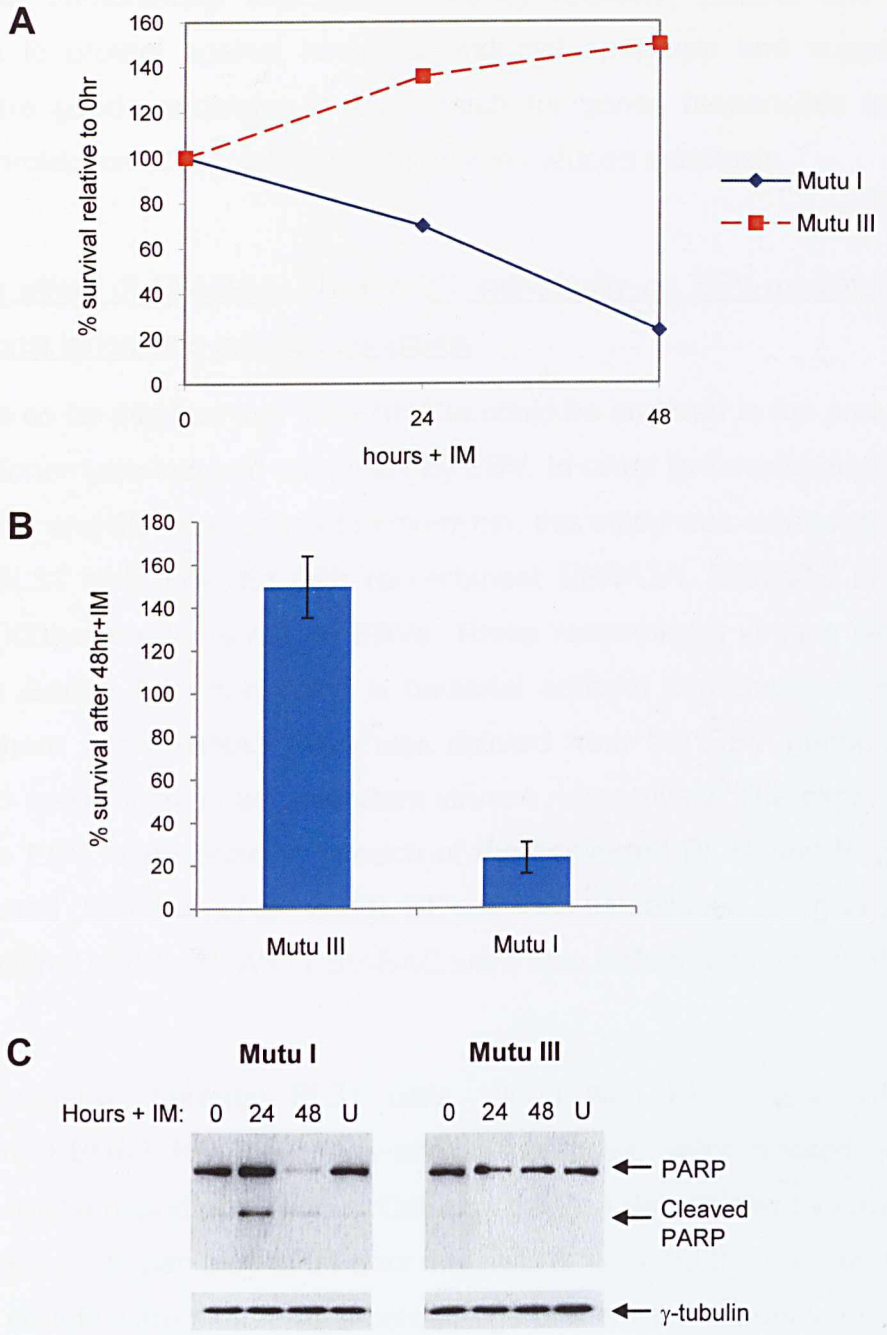


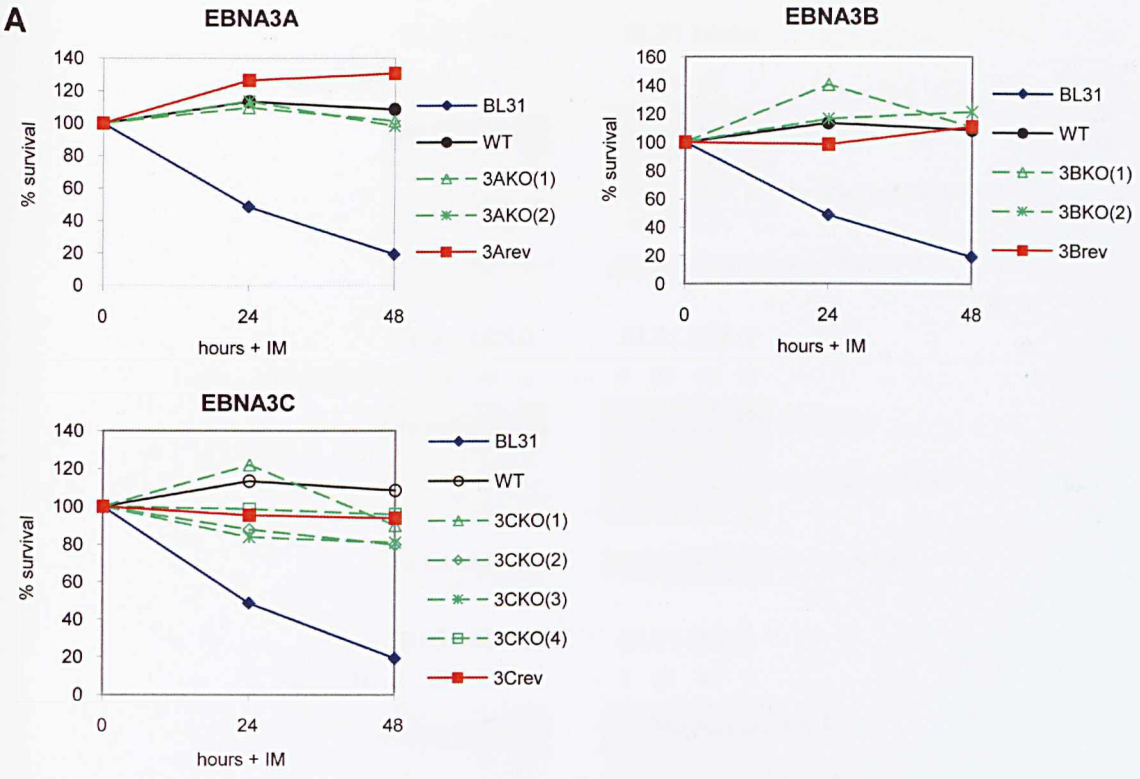
Figure 3-5 Latency I BL is not protected against ionomycin-induced apoptosis. Mutu I and its latency III counterpart, Mutu III, were treated with 1μg/ml ionomycin for up to 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Cell survival is expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control. IM=ionomycin

These data demonstrate that type I latency (EBNA1, EBERs and BARTs) is insufficient to protect against ionomycin-induced apoptosis and suggest that the EBNA3s are good candidates in our search for genes responsible for the EBV-mediated protection of BL cells from ionomycin-induced apoptosis.

3.1.4 The effect of EBNA3A, 3B and 3C individually on EBV-mediated protection against ionomycin-induced apoptosis

The results so far suggest that the EBNA3s could be involved in the protection of BL cells from ionomycin-induced apoptosis by EBV. In order to investigate the effects of EBNA3A, 3B and 3C in response to ionomycin, this study was extended to include a panel of BL31 cells infected with recombinant EBNA3A, EBNA3B and EBNA3C knockout (KO) and revertant (rev) EBVs. These recombinant viruses were made in the lab by Emma Anderton using a bacterial artificial chromosome (BAC)-based system, where each EBNA3 gene was deleted from the EBV genome and then replaced to make deletion and revertant viruses respectively. The expression of the appropriate EBV latent proteins in each of the converted BL31 and BL2 cells have been validated (Anderton *et al.*, 2008). BL cell lines established using virus produced from the original wild-type (WT) EBV-BAC were also included as a control.

The EBV-negative parental BL31 cells, along with BL31 cells infected with recombinant EBNA3 KO, revertant and WT viruses, were treated with 1µg/ml ionomycin over a period of 48 hours. Cell survival was determined by counting viable cells that exclude trypan blue at 24 hour intervals throughout the course of treatment. Viable cell counts were plotted as a percentage of the starting population. The graph shown in Figure 3.6A was constructed using data from one experiment but is representative of data from repeated experiments. Results from three independent experiments were pooled and the mean and standard deviation of viable cell counts after 48 hours of treatment with ionomycin were plotted as a histogram in Figure 3.6B. Cells were also harvested at 24 hour intervals for protein analysis.



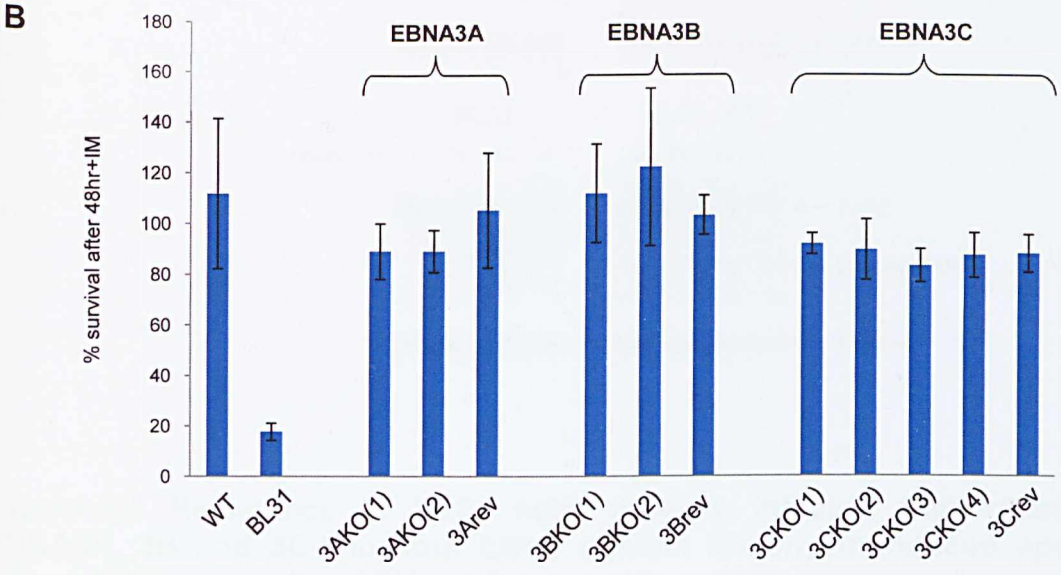


Figure 3-6 Resistance of BL31 cells recently infected with recombinant EBNA3A, 3B and 3C-knockout EBVs against ionomycin-induced apoptosis. (continued overleaf)

C

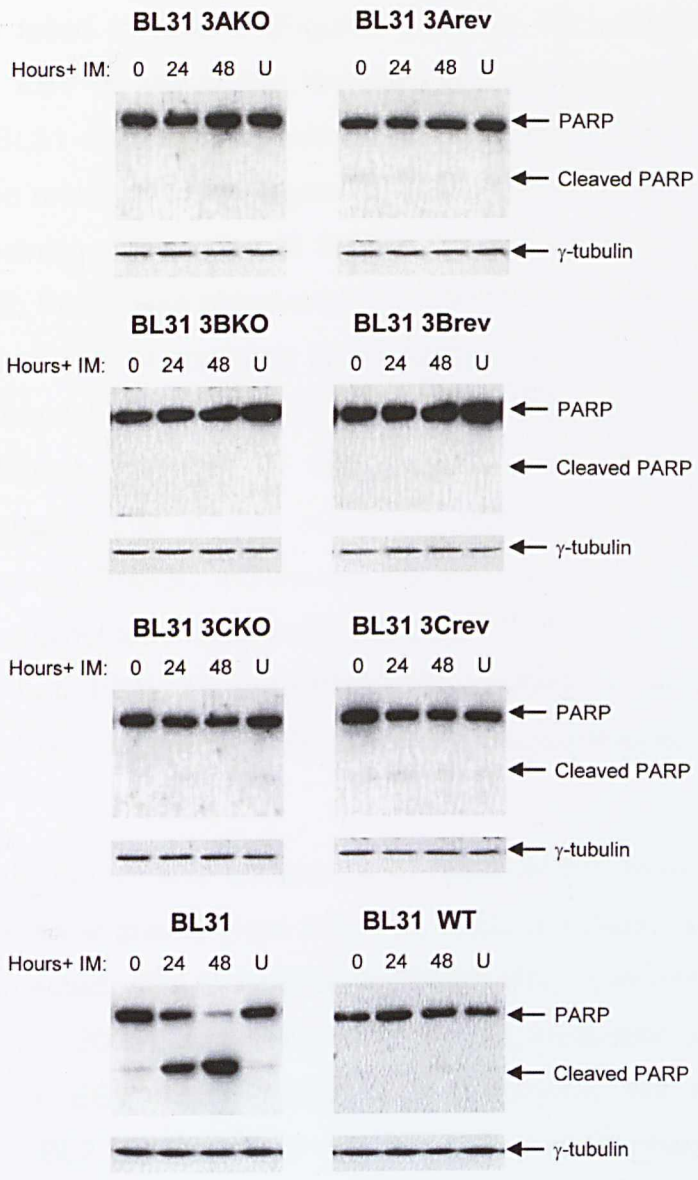


Figure 3.6 Resistance of BL31 cells recently infected with recombinant EBNA3A, 3B and 3C-knockout EBVs against ionomycin-induced apoptosis. Parental BL31 cells, their EBV-positive converts recently infected with recombinant EBNA3A, 3B and 3C-knockout and their respective revertants, as well as BL31 infected with the wild-type recombinant EBV were treated with 1μg/ml ionomycin for 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Cell survival is expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control. IM = ionomycin

After 48 hours of exposure to ionomycin, more than 80% of the parental EBV-negative BL31 cells failed to survive (Figures 3.6A&B). As expected, infection of BL31 cells with WT EBV or any of the revertant viruses improved the survival of cells. Interestingly, BL31 cells infected with the recombinant EBNA3A, 3B and 3C KO viruses were also resistant to ionomycin-induced apoptosis with at least 90% of cell populations remaining viable after 48 hours. Figure 3.6C shows that proteolytic cleavage of full length PARP was observed in the parental EBV-negative BL31 cells after 24 hours and was almost complete by 48 hours. Conversely, PARP cleavage was undetected or detectable at very low levels in BL31 cells infected with any of the EBNA3 KO recombinant, revertant or WT viruses even after 48 hours. This observation demonstrates that in p53 null BL31 cells, the EBV latent proteins EBNA3A, EBNA3B and EBNA3C individually do not have an effect on the EBV-mediated protection against ionomycin-induced apoptosis. This is in marked contrast to the contribution of both EBNA3A and EBNA3C to the EBV-mediated protection of BL cells against apoptosis induced by DNA damaging drugs (Anderton *et al.*, 2008).

In order to investigate further and ensure these observations were not specific to BL31 cells, the same experiments were repeated using a similar panel of WT p53 carrying BL2 cells infected with recombinant EBNA3 KO, revertant and wild-type EBVs (Anderton *et al.*, 2008). Following 48 hours of treatment with ionomycin, approximately 60% of EBV-negative BL2 cells underwent cell death (Figures 3.7A&B). In contrast, BL2 cells infected with the WT and revertant viruses were resistant to ionomycin-induced apoptosis. Infection of BL2 cells with any of the EBNA3A, 3B and 3C KO recombinant viruses also appear to protect the cells from cell death with populations growing to more than 100% even after 48 hours of exposure to ionomycin. This was confirmed by Western blot analysis showing that while significant cleavage of full length PARP was observed in the parental BL2 cells after 48 hours of exposure to ionomycin, PARP cleavage was undetectable in the panel of EBV-positive BL2 cells at the end of the experiment (Figure 3.7C).

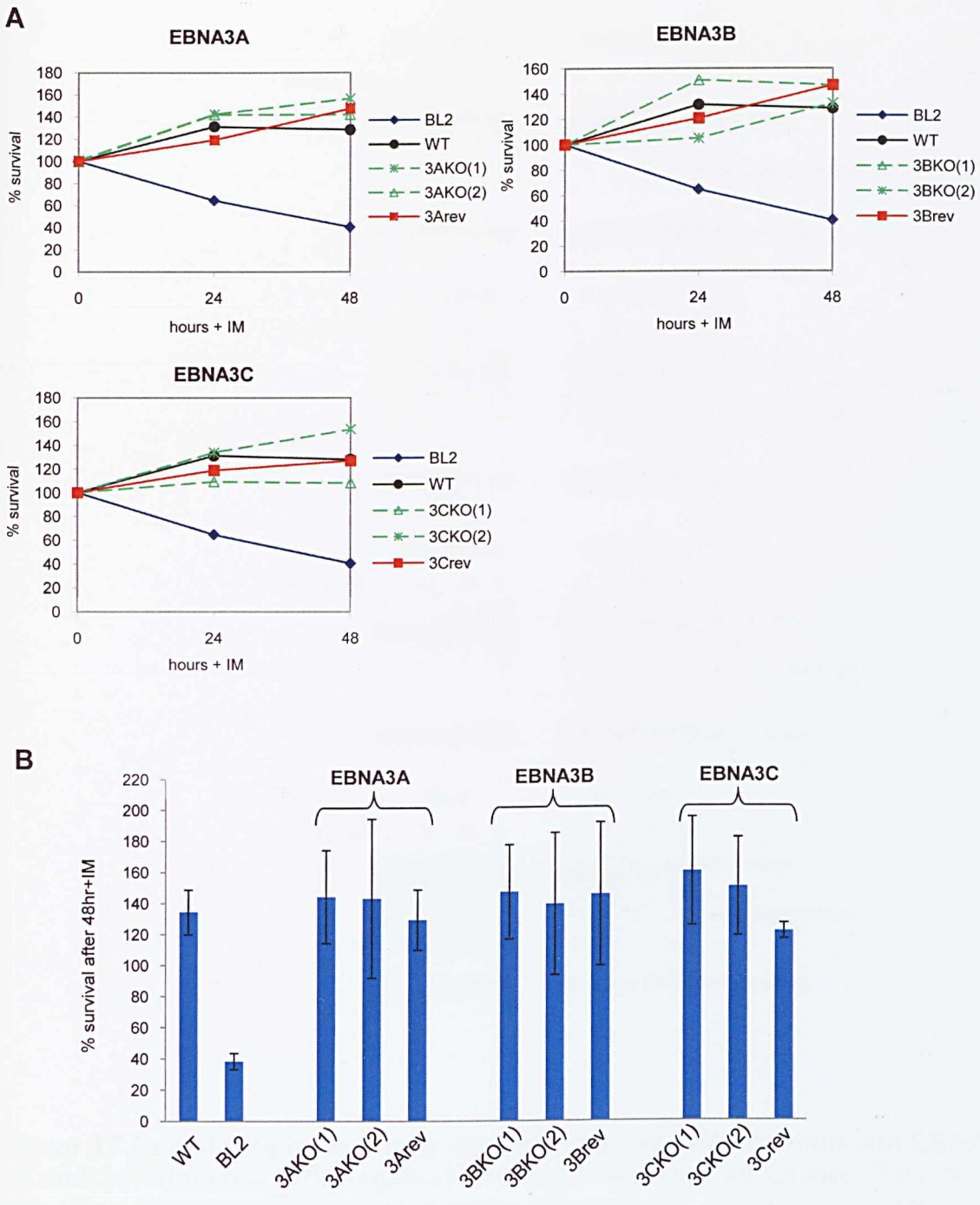


Figure 3-7 Resistance in BL2 cells recently infected with recombinant EBNA3A, 3B and 3C-knockout EBVs against ionomycin-induced apoptosis. (continued overleaf)

C



Figure 3.7 Resistance in BL2 cells recently infected with recombinant EBNA3A, 3B and 3C-knockout EBVs against ionomycin-induced apoptosis. Parental BL2 cells, their EBV-positive converts recently infected with recombinant EBNA3A, 3B and 3C-knockout and their respective revertants, as well as BL2 infected with the wild-type recombinant EBV were treated with 1µg/ml ionomycin for 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Cell survival is expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control. IM=ionomycin

All these results indicate that individually EBNA3A, 3B and 3C do not play a role in the protection conferred by EBV against apoptosis induced by ionomycin. These data also confirm earlier observations that the apoptotic pathway induced by ionomycin is independent of both p53 and Bim.

3.2 EBV-mediated protection against ionomycin-induced apoptosis not due to induction of lytic cycle

Certain inducers of apoptosis including phorbol esters, sodium butyrate and calcium ionophores such as ionomycin have been reported to increase the proportion of EBV-positive BL cells entering the lytic program (Faggioni *et al.*, 1986; Borras *et al.*, 1996). Studies have shown that some activators of the EBV lytic program can concomitantly induce apoptosis, but that EBV lytic gene expression protects cells containing replicating virus from cell death (Inman *et al.*, 2001). At least two lytic proteins have been reported to inhibit apoptosis: the BHRF1 protein is a homologue of the anti-apoptotic protein Bcl-2 (Henderson et al., 1993) and the product of BALF1 also has weak homology to Bcl-2 and has been shown to bind the pro-apoptotic proteins Bax and Bak (Marshall *et al.*, 1999). Reports have also suggested a role for other EBV lytic proteins in modulating apoptotic responses (Rodriguez *et al.*, 1999; Swenson *et al.*, 1999). Hence, it was important investigate if lytic gene products were involved in the protection of EBV-positive BL cells against ionomycin-induced apoptosis.

Protein extracts from a selection of EBV-positive BL cells resistant to ionomycin treatment (based on experiments described in Section 3.1) were analysed by Western blotting for evidence of activation of the EBV lytic cycle. Western blotting was carried out using the extremely sensitive human EE serum (from a patient with chronic infectious mononucleosis) which detects EBV early lytic agents including BZLF1. Protein extracts from Akata cells treated with anti-Ig to induce the lytic cycle were included (provided by Carol McDonald) as a reference point for the levels of EBV lytic proteins found in cells containing replicating virus. The proportion of anti-Ig treated Akata cells expressing lytic antigens was approximately 15%, as determined

by anti-BZLF1 immunofluorescence staining (Carol McDonald - personal communication). Figure 3.8A shows that lytic antigens were detectable in some EBV-positive BL cell lines resistant to ionomycin treatment. However, the expression levels of lytic antigens were much lower than that seen in the control anti-Ig induced Akata cells, where it has been determined that the lytic cycle has been activated in only approximately 15% of the population. Expression of lytic antigens also appears to be present at the start of the time course, with the levels generally remaining constant through the 48 hour treatment with ionomycin. Early lytic antigens were generally undetectable by Western blotting using the human serum EE in the majority of the EBV-positive BL cell lines surveyed.

Protein extracts were also analysed by Western blotting using a monoclonal antibody against the viral Bcl-2 homologue, BHRF1 (Figure 3.8B). Protein extracts from anti-Ig induced Akata cells were included as a positive control. BHRF1 protein was undetectable in all the EBV-positive BL cells studied with the exception of Oku-BL and Sal-BL (Emma Anderton – personal communication). The potential role of BHRF1 will be discussed further in Chapter 4.

These data show that ionomycin treatment failed to consistently induce expression of the EBV early lytic antigens and suggest that the rescue of BL cells from ionomycin-induced apoptosis is not dependent on expression of the EBV lytic genes.

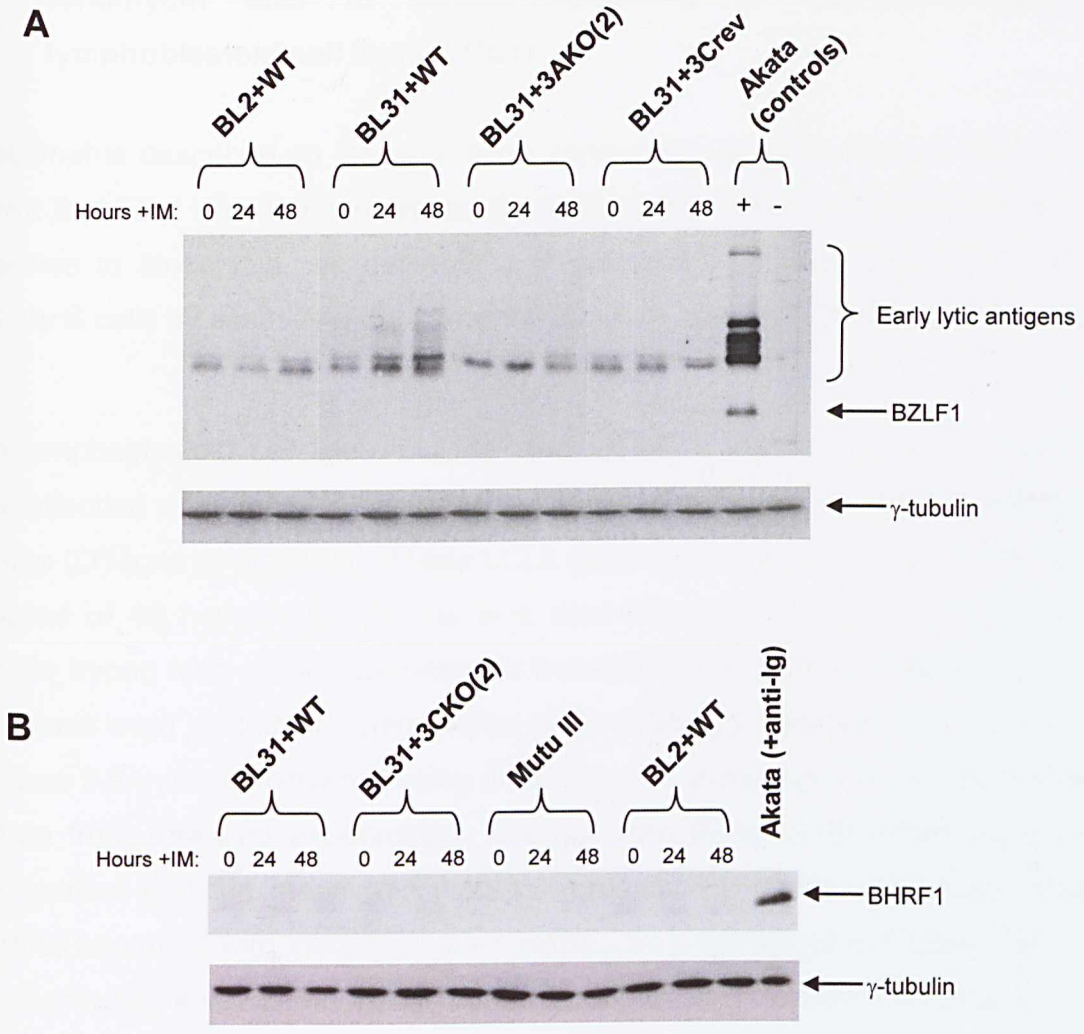


Figure 3-8 Rescue from apoptosis induced by ionomycin is not dependent on EBV lytic gene expression. Various EBV-positive BL cells were treated with 1µg/ml ionomycin for 48 hours and harvested at the times indicated. Protein extracts from treated cells were analysed by Western blotting using **(A)** the human serum EE which detects early lytic antigens and **(B)** a monoclonal antibody against BHRF1. Extracts from Akata cells left untreated (–) or treated with anti-Ig (+) to stimulate the expression of EBV lytic proteins were included as a control. γ-tubulin was used a loading control. IM = ionomycin

3.3 Ionomycin fails to induce apoptosis in EBV-immortalised B-lymphoblastoid cell lines (LCLs)

Experiments described so far have been conducted using BL-derived cells. Having shown that EBV infection can protect BL-derived cells from undergoing apoptosis in response to ionomycin, we decided to investigate if this was also the case with primary B cells by assessing the response of newly established LCLs to ionomycin.

Two lymphoblastoid cell lines, LCL-BF and LCL-CH, were produced in the lab by EBV infection of primary B cells from mixed donors and cultured for less than 4 months (O'Nions *et al.*, 2006). These LCLs were treated with 1µg/ml ionomycin over a period of 48 hours. Cell survival was determined by counting viable cells that exclude trypan blue at 24 hour intervals throughout the course of treatment. Viable cell counts were plotted as a percentage of the starting population. The graph shown in Figure 3.9A was constructed using data from one experiment but is representative of data from repeated experiments. Results from three independent experiments were pooled and the mean and standard deviation of viable cell counts after 48 hours of treatment with ionomycin were plotted as a histogram in Figure 3.9B. Cells were harvested every 24 hours for protein extraction and Western blot analysis.

Viable cells counts show that at least 90% of both LCLs survived after 48 hours of exposure to ionomycin (Figures 3.9A&B). Western blotting analysis shows that full-length PARP is unaffected in both LCLs, indicating that these LCLs did not undergo apoptosis even after 48 hours of exposure to ionomycin (Figure 3.9C).

All these data demonstrate that LCLs do not undergo apoptosis in response to ionomycin treatment. This is in marked contrast to the response of LCLs to genotoxic drugs. It has been shown that EBV cannot protect against genotoxin-induced p53-mediated apoptosis in LCLs (Allday *et al.*, 1995a; O'Nions and Allday, 2003).

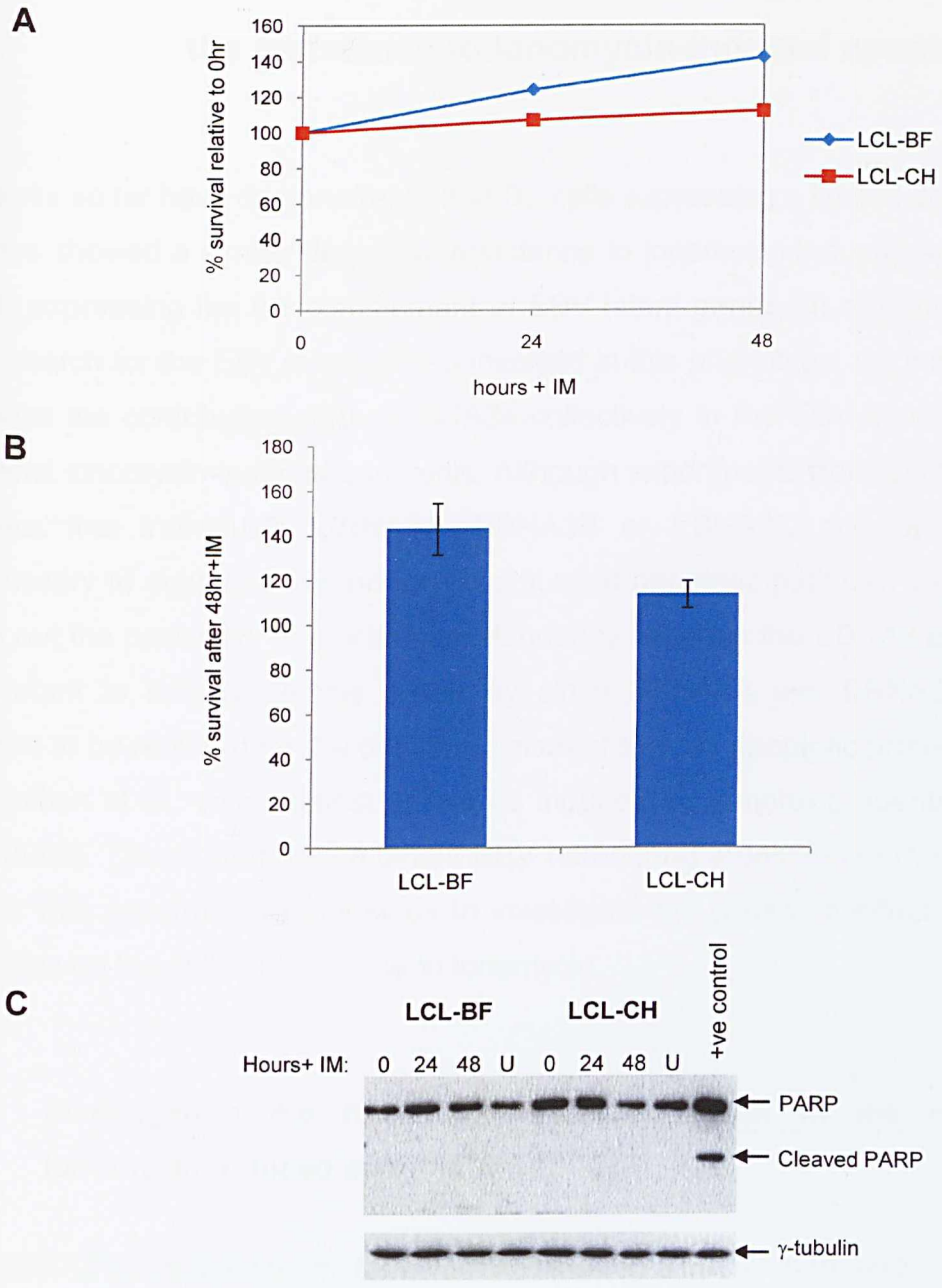


Figure 3-9 EBV infection protects primary B cells from apoptosis induced by ionomycin. Two lymphoblastoid cell lines, LCL-BF and LCL-CH, were treated with 1µg/ml ionomycin for up to 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Cell survival is expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. +ve control= ionomycin-treated BL31 cells used as a positive control for cleaved PARP. γ-tubulin was used as a loading control. IM = ionomycin

Chapter 4 Identifying the EBV latent gene product(s) involved in the resistance to ionomycin-induced apoptosis

Results so far have demonstrated that BL cells expressing a limited set of EBV latent genes showed a similar degree of resistance to ionomycin-induced apoptosis as BL cells expressing the full complement of EBV latent genes. In order to narrow down the search for the EBV latent genes involved in this phenotype, we initially wanted to assess the contribution of the EBNA3s collectively in the EBV-mediated protection against ionomycin-induced apoptosis. Although experiments from Section 3.1.4 have shown that individually EBNA3A, EBNA3B or EBNA3C do not appear to be necessary to suppress the ionomycin activated apoptotic pathway, we could not yet rule out the possibility of functional redundancy between the EBNA3 proteins. It was important to investigate this possibility since EBNA3A and EBNA3C have been shown to be required for the downregulation of the pro-apoptotic protein Bim and the protection of BL cells against apoptosis induced by genotoxic agents (Anderton *et al.*, 2008). Therefore, a recombinant EBV harbouring a deletion of the entire EBNA3 locus was constructed to allow us to investigate the collective effect of the EBNA3 proteins on the cellular response to ionomycin.

4.1 Investigating the role of the EBNA3 locus in the resistance to ionomycin-induced apoptosis

An EBNA3 locus deletion mutant was made using a bacterial recombinase A (RecA)-based markerless recombination system (Lalioti and Heath, 2001; White *et al.*, 2003) whereby the entire EBNA3 locus was deleted from a bacterial artificial chromosome (BAC) harbouring the EBV genome (Delecluse *et al.*, 1998) via homologous recombination between the EBV-BAC and a shuttle vector containing a fragment of the EBV genome with an internal deletion of EBNA3A, EBNA3B and EBNA3C (Figure 4.1). In order to control for any second-site mutations which may have occurred during the recombination process, revertants were generated by restoration of the EBNA3 locus to the EBNA3-deleted EBV-BAC during a second round of homologous recombination.

The BAC used for these recombinations (pHB9) was derived from the original EBV-BAC constructed by Delecluse, Hammerschmidt and colleagues which harbours the B95.8 strain of EBV (p2089, Delecluse *et al.*, 1998). The only difference between these two EBV-BACs is that the original p2089 BAC is reported to contain 11 copies of the 3072bp *Bam*HI W (*Bam* W) repeat, whereas pHB9 contains only 6 copies of this repeat. The pHB9 EBV-BAC contains a chloramphenicol (Cm) resistance gene for selection in prokaryotic cells, hygromycin (Hyg) resistance gene for selection in eukaryotic cells and a green fluorescent protein (GFP) expression cassette for visualisation in eukaryotic cells.

The targeting constructs were made in the context of the shuttle plasmid p4487.1, pKOV-Kan-ΔCm (White *et al.*, 2003) modified to include a different polylinker. The shuttle plasmid contains some positive and negative selection markers - kanamycin (Kan) resistance gene, a temperature-sensitive origin of replication with permissive temperature of 30-33°C and *SacBII* gene of *Bacillus amyloliquefaciens* - colonies containing this gene do not grow in medium containing sucrose (Gay *et al.*, 1983). The generation of targeting constructs is described in Section 2.5.1.

Homologous recombination was mediated by RecA expressed from the plasmid pDF25-Tet which contains a tetracycline (Tet) resistance gene for positive selection and a temperature-sensitive origin of replication with permissive temperature of 30-33°C. Deletion and revertant EBV-BACs were then transfected into HEK293 cells and stable producer cell lines established. Infectious virus was generated by transient transfection of 293 producer cells with expression plasmids carrying the viral lytic switch gene *BZLF1* and the *BALF4* gene which encodes the glycoprotein gp110 shown to enhance the ability of 293-produced EBV to infect human cells (Neuhierl *et al.*, 2002). The recombinant EBNA3 knockout (E3KO) and revertant (E3rev) EBVs were used to infect EBV-negative BL31 and BL2 cells. The response of these newly established BL cell lines to ionomycin were then examined.

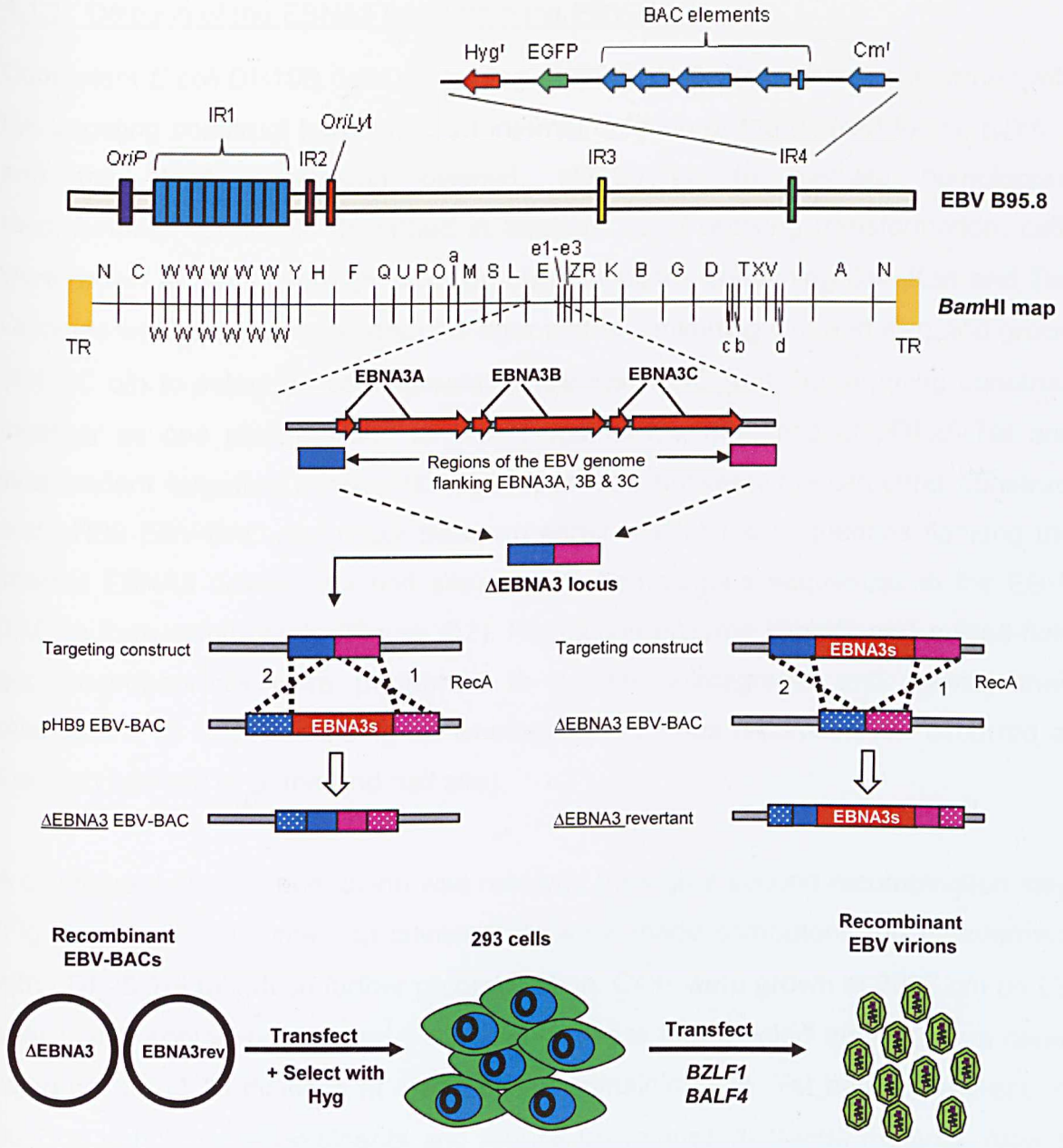


Figure 4-1 Construction of EBNA3 locus knockout and revertant EBV-BACs. An enlargement of the *Bam*HI E fragment containing the EBNA3 genes is shown. Sequences of the EBV genome flanking the EBNA3 locus were cloned contiguously into targeting constructs. The EBNA3 locus in the EBV-BAC is replaced by the targeting construct by homologous recombination between first one pair of homologous regions followed by the second. A revertant was generated by homologous recombination between the EBNA3-knockout BAC and a targeting construct harbouring the EBNA3 locus. Both EBNA3-knockout and -revertant BACs were transfected into HEK293 cells and stable producer lines were generated following selection with hygromycin (Hyg). Recombinant virions were produced by transfecting producer lines with *BZLF1* and *BALF4* expression plasmids. TR=terminal repeat, IR=internal repeat.

4.1.1 Deletion of the EBNA3 locus from the EBV-BAC

Competent *E.coli* DH10B cells containing pHB9 EBV-BAC were co-transformed with the targeting construct harbouring an internal deletion of the EBNA3 locus, pJY6.1, and the RecA expression plasmid, pDF25-Tet, to mediate homologous recombination (protocols described in Section 2.5). Following transformation, cells were grown at 30°C overnight (o/n) on LB agar plates containing Cm, Kan and Tet. Colonies were then replated onto LB agar plates containing Cm and Kan and grown at 43°C o/n to select for cointegrants (pHB9 EBV-BAC and the targeting construct together as one plasmid) and to select against the presence of pDF25-Tet and independent targeting constructs. Recombination between the targeting construct and pHB9 EBV-BAC can occur between either end of the sequences flanking the internal EBNA3 deletion (or half site) and its homologous sequences in the EBV-BAC to form cointegrants (Figure 4.2). Restriction enzyme digests and pulsed-field get electrophoresis were performed to screen cointegrants and identify their orientations (S or E depending on whether homologous recombination occurred at the Start half site or at the End half site).

A cointegrant of each orientation was resolved through a second recombination step (Figure 4.3). *E.coli* containing cointegrants were made competent and transformed with pDF25-Tet to induce further recombination. Cells were grown at 30°C o/n on LB agar plates containing Cm and Tet. Colonies were then pooled and grown in serial liquid culture (1:10 dilutions at 4 and 8 hrs) containing Cm, Tet and 5% sucrose at 30°C to enrich for recombinants and reduce prevalence of *SacBII* mutants. After 8 hrs, the culture was left to grow in fresh LB media containing Cm, Tet and 5% sucrose at 30°C o/n. 5µl of the o/n culture was streaked onto LB Agar containing Cm and 5% sucrose and left to grow at 43°C o/n. This selects for resolved cointegrants, from which the *SacBII* gene is lost (along with the targeting construct), as a result of the second recombination event. Colonies were replica plated onto LB Agar plates containing both Cm and Kan or Cm alone to identify potential recombinants (which are Kan sensitive) and to eliminate cointegrant colonies which have survived sucrose selection due to mutation of the *SacBII* gene (and thus retain Kan resistance). Recombinants which have successfully resolved to yield the EBNA3-deleted (E3KO) EBV-BAC were identified by restriction enzyme digests.

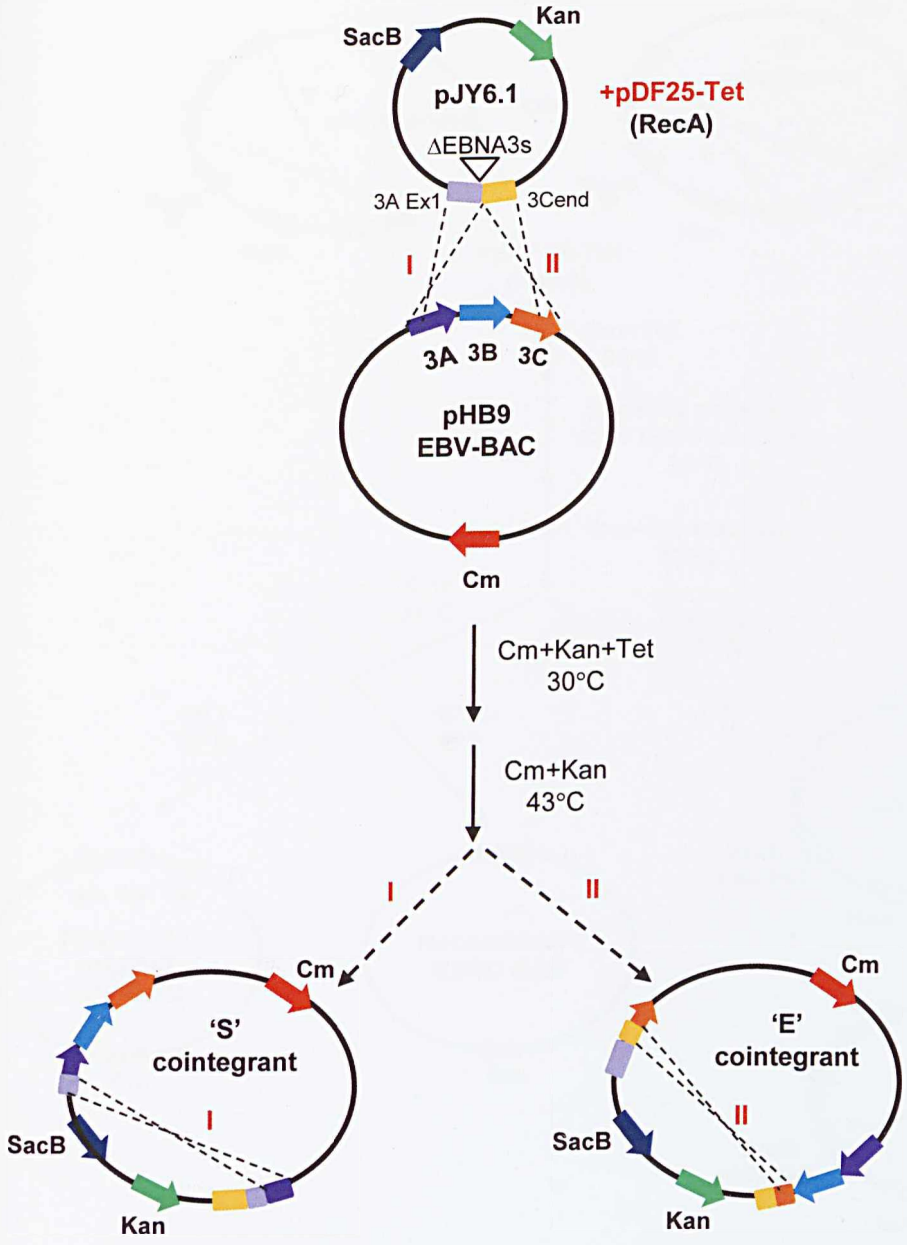


Figure 4-2 Making cointegrants: Homologous recombination between pHB9 EBV-BAC and the targeting construct containing an internal deletion of the EBNA3 locus. The targeting construct harbouring an internal deletion of the EBNA3 locus (pJY6.1) and the RecA expression plasmid (pDF25-Tet) were used to transform *E.coli* DH10B cells containing pHB9 EBV-BAC. Cells were plated on LB agar containing chloramphenicol (Cm), kanamycin (Kan) and tetracycline (Tet) and left to grow overnight at 30°C. Colonies were re-plated onto LB agar containing Cm and Kan and left to grow overnight at 43°C to select for cointegrants and to select against pDF25-Tet or independent targeting construct plasmids. Homologous recombination between pHB9 EBV-BAC and the targeting construct can occur either at the start half-site (S - in this case, the sequences overlapping the start of EBNA3A exon 1 or 3Aex1, represented by I) or the end half-site (E- the sequences overlapping the end of EBNA3C exon 2 or 3Cend, represented by II).

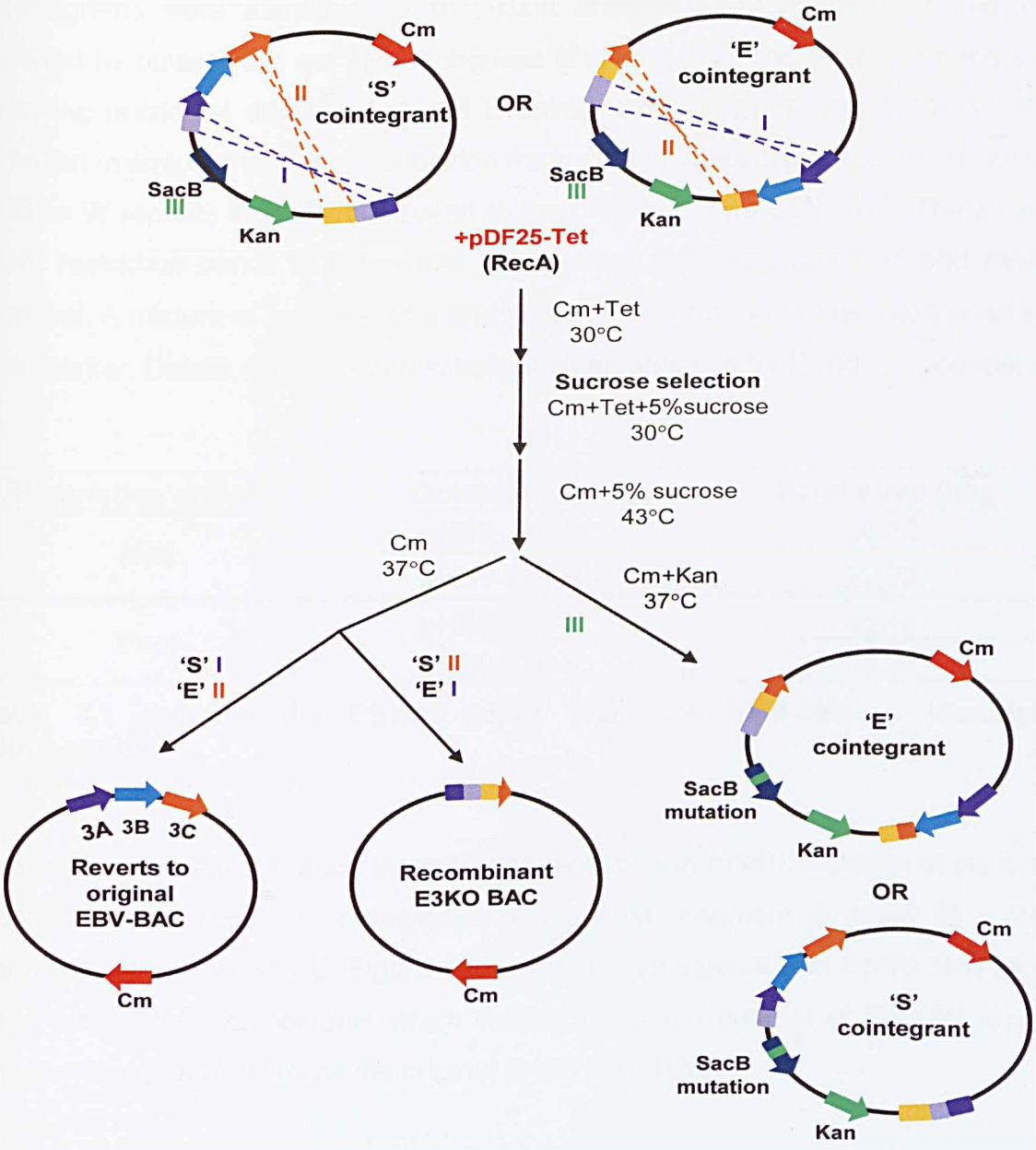


Figure 4-3 Resolving cointegrants to generate recombinant EBNA3-knockout BAC. *E.coli* containing cointegrants were made competent and transformed with the RecA expression plasmid, pDF25-Tet, to induce a second round of recombination. To select against the *SacBII* gene in the targeting construct, colonies were pooled and grown overnight in culture containing 5% sucrose. 5µl of culture was streaked onto LB agar plates containing chloramphenicol (Cm) and 5% sucrose and left to grow overnight at 43^{0}C. Colonies were replica-plated onto LB agar plates containing Cm and kanamycin (Kan) or just Cm to eliminate cointegrants which have survived sucrose selection due to mutations in their *SacBII* gene (represented by III). This second round of homologous recombination can again occur at either the 'S' half-site (represented by I) or 'E' half-site (represented by II). If a cointegrant undergoes recombination at the same half-site as the first recombination event, the cointegrant will revert to the original pHB9 EBV-BAC (along with the targeting construct which is not retained under these selection conditions). Recombination at a different half-site to the first recombination will result in the desired EBNA3-knockout (E3KO) BAC.

Cointegrants were identified by restriction enzyme digests with *Not*I and *Pvu*I followed by pulsed-field gel electrophoresis (Figure 4.4A). The expected band sizes following restriction digests of S and E cointegrants are shown in Table 4.1. The variation in size of the largest restriction fragments is indicative of the lesser number of *Bam* W repeats in pHB9 compared to the original p2089 EBV-BAC. The sizes of other restriction bands in pHB9 and p2089 when analysed with *Not*I and *Pvu*I is identical. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders was used as a size marker. Details of band sizes in both DNA ladders can be found in Appendix A.

Restriction enzyme	Cointegrants	Band sizes (kb)
***Not*I**	pHB9-JY6-S	30,13
	pHB9-JY6-E	22,22
***Pvu*I**	pHB9-JY6-S	43,14
	pHB9-JY6-E	35,22

Table 4.1 Deleting the EBNA3 locus from the EBV-BAC – Identifying cointegrants

Recombinants were screened by restriction digests with *Eco*RI. Deletion of the entire EBNA3 locus resulted in a decrease of the 30kb fragment in pHB9 to a 24kb fragment in the E3KO-BAC (Figure 4.4B). Restriction digests with *Eco*RI also allows us to screen for recombinants which contain the same number of Bam W repeats and terminal repeats (TR) as the original pHB9 EBV-BAC.

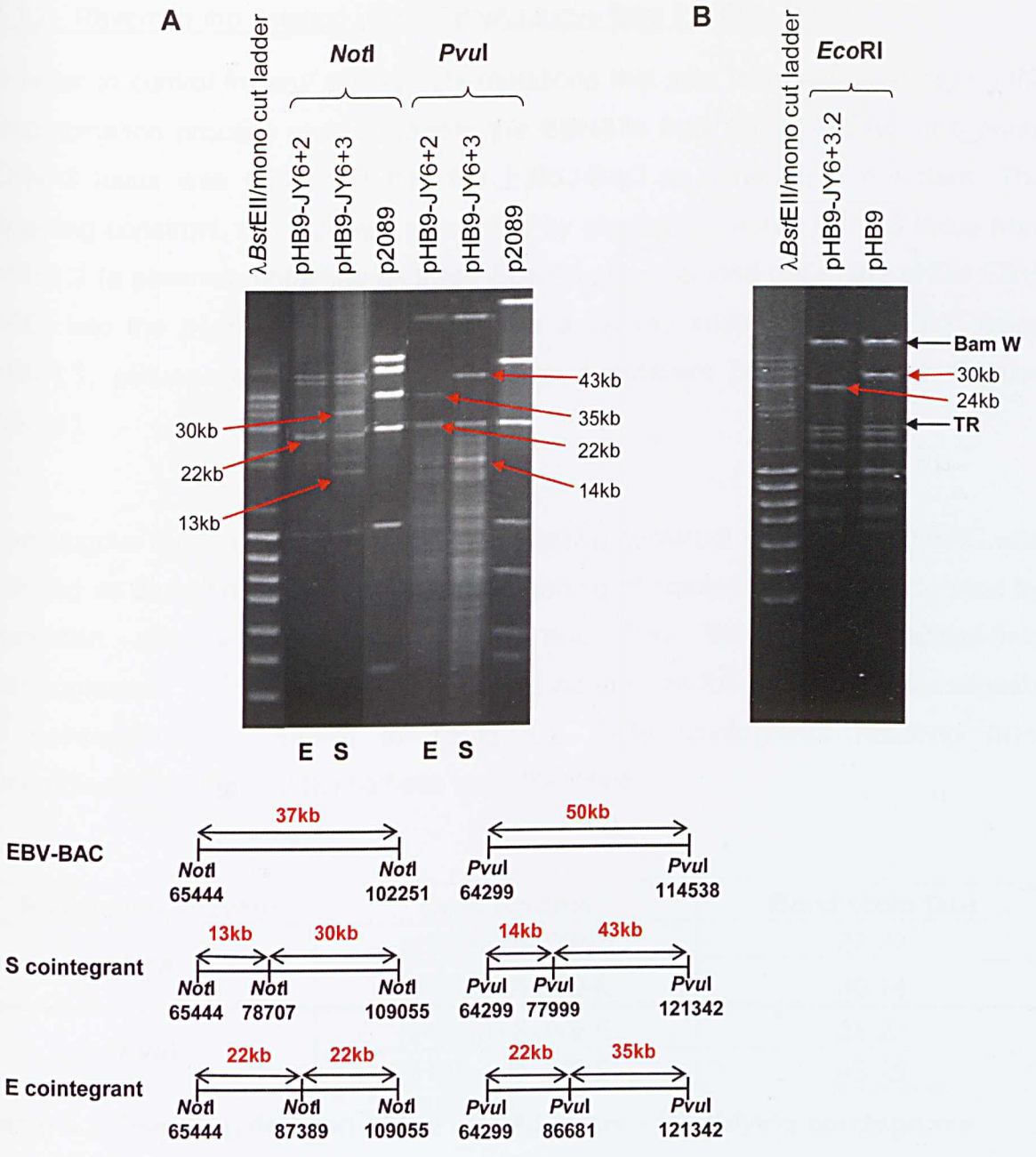


Figure 4-4 Deleting the EBNA3 locus from the EBV-BAC. (A) Following the first recombination event during the construction of the EBNA3 locus-knockout (E3KO) EBV-BAC, restriction enzyme digests were performed with *Not*I and *Pvu*I to screen for cointegrants. Cointegrants were identified as either S or E depending on whether recombination occurred at the start (S) half-site or the end (E) half-site. **(B)** Cointegrants were resolved following a second recombination event. Recombinants were analysed by restriction digest with *Eco*RI followed by pulsed-field gel electrophoresis to identify the E3KO EBV-BAC. DNA from the pHB9 EBV-BAC was digested with the same restriction enzymes and included as a control. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders was used as a size marker. The *Eco*RI restriction fragments corresponding to the *Bam* W repeats and the terminal repeats (TR) are shown.

4.1.2 Reverting the deletion of the EBNA3 locus from the EBV-BAC

In order to control for any second site mutations that may have occurred during the recombination process used to delete the EBNA3s from the EBV-BAC, the entire EBNA3 locus was reinserted into the E3KO-BAC to generate a revertant. The targeting construct, pJY9.2, was generated by cloning the entire EBNA3 locus from p4852.3 (a plasmid containing all three EBNA3 genes cloned out of the pHB9 EBV-BAC) into the p4487.1 shuttle plasmid via a cloning intermediate (pJY7.1, using p4674.1, pBluescriptII® modified to include a different polylinker) (see Section 2.5.1.1).

Homologous recombination between the targeting construct and the E3KO-BAC was induced as described in Section 4.1.1. Screening of cointegrants was performed by restriction enzyme digests with *Not*I and *Pvu*I followed by pulsed-field electrophoresis (Figure 4.5A). The expected band sizes following restriction digests of cointegrants are shown in Table 4.2. Only cointegrants resulting from recombination at the end (E) half-site were identified.

Restriction enzyme	Cointegrants	Band sizes (kb)
***Not*I**	pHB9-JY6-JY9-S	22,22
	pHB9-JY6-JY9-E	30,14
***Pvu*I**	pHB9-JY6-JY9-S	35,23
	pHB9-JY6-JY9-E	43,15

Table 4.2 Reverting deletion of the EBNA3 locus – Identifying cointegrants

The cointegrant was resolved as previously described through a second recombination step. Recombinant BACs were screened by restriction digests with *Eco*RI (Figure 4.5B). An increase of the 24kb band in the E3KO-BAC to 30kb in the E3rev-BAC was observed as expected. The bands corresponding to *Eco*RI restriction fragments containing the *Bam* W repeat and the terminal repeat (TR) are identical in both the E3KO-BAC and the E3rev-BAC.

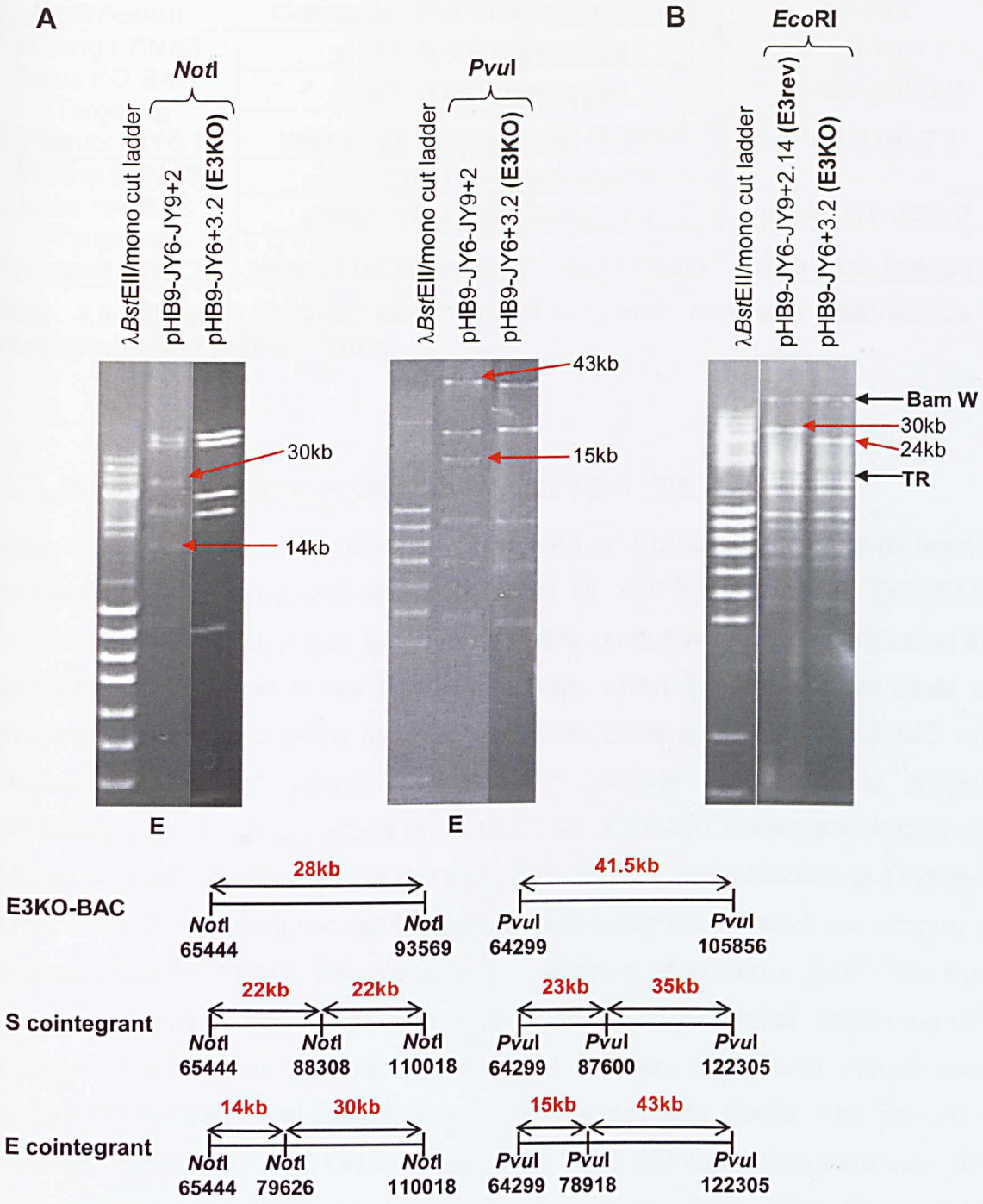


Figure 4-5 Reverting deletion of the EBNA3 locus. (A) Following the first recombination event during the construction of the EBNA3 locus-revertant (E3rev) EBV-BAC, restriction enzyme digests were performed with *Not*I and *Pvu*I to identify cointegrants. Only cointegrants resulting from recombination at the end (E) half-site were identified. **(B)** Cointegrants were resolved following a second recombination event. Recombinants were analysed by restriction digest with *Eco*RI followed by pulsed-field gel electrophoresis to identify the E3rev EBV-BAC. DNA from the EBNA3 locus-knockout (E3KO) EBV-BAC was digested with the same restriction enzymes and included as a control. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders was used as a size marker. The *Eco*RI restriction fragments corresponding to the *Bam* W repeats and the terminal repeats (TR) are shown.

Description	Cointegrants and Recombinants	Names
Making EBNA3 locus KO-BAC (Targeting construct pJY6.1)	pHB9-JY6-S cointegrant	pHB9-JY6+3
	pHB9-JY6-E cointegrant	pHB9-JY6+2
	pHB9-JY6 recombinant (E3KO)	**pHB9-JY6+3.2**
Making EBNA3 locus rev-BAC (Targeting construct pJY9.2)	pHB9-JY6-JY9-S cointegrant	-
	pHB9-JY6-JY9-E cointegrant	pHB9-JY6-JY9+2
	pHB9-JY6-JY9 recombinant (E3rev)	**pHB9-JY6-JY9+2.14**

Table 4.3 Making EBNA3 locus knockout and revertant EBV-BACs - Cointegrants and recombinants

4.1.3 Making stable producer cell lines and infectious virus

Figure 4.6 shows a schematic diagram of the method and screening strategy used to generate infectious virus and establish stable BL cell lines from the EBV-BACs constructed. The first step was to generate stable producer cell lines harbouring the recombinant E3KO and E3rev EBV-BACs from which infectious virus could be generated. DNA was isolated by maxiprep from *E.coli* containing the E3KO and E3rev EBV-BACs and subjected to caesium chloride (CsCl) density gradient centrifugation (protocols described in Section 2.4). EBV-BACs were transfected into HEK293 cells and stable producer cell lines generated by Hyg selection and isolation by ring cloning. 293 producer cell lines were then screened to check the integrity of the transfected EBV-BAC. This involved the recovery of episomal BAC DNA from cells, transformation into *E.coli* and growth on LB agar plates containing Cm (protocols described in Section 2.6). Several colonies from each rescue were screened by miniprep and restriction digests analysis with *Eco*RI. The pattern of restriction fragments of BAC DNA rescued from three 293 cell clones harbouring the E3KO-BAC (Figure 4.7A) and two clones harbouring the E3rev-BAC (Figure 4.7B) was identical to that of the EBV-BACs originally transfected, indicating that there have been no major rearrangements of the transfected BAC. The bands corresponding to *Eco*RI restriction fragments containing the *Bam* W repeats and terminal repeats are shown.

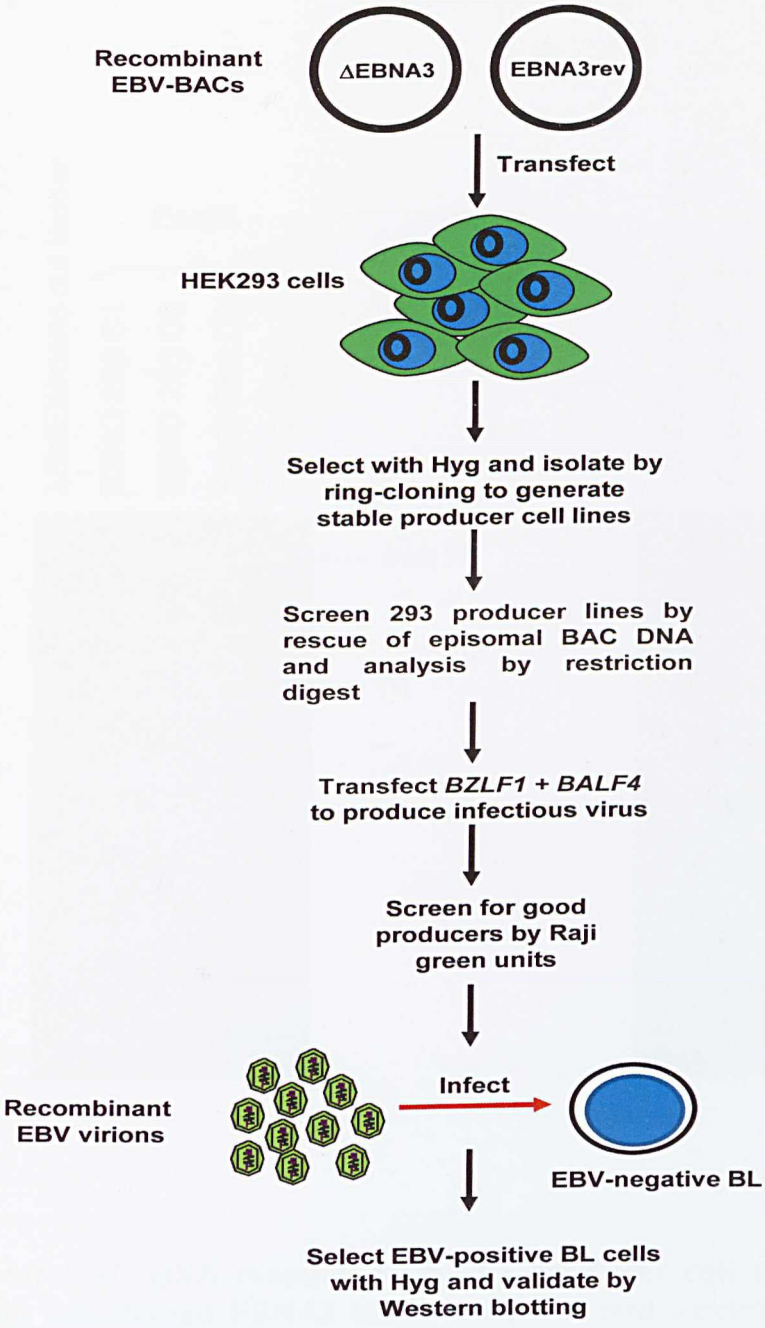


Figure 4-6 Generating infectious virus from recombinant EBV-BACs and establishing stable BL cell lines. Recombinant EBV-bacterial artificial chromosomes (BACs) are transfected into HEK293 cells and stable producer cell lines generated after selection with hygromycin (Hyg) and isolation by ring-cloning. Producer cell lines are then screened for integrity of transfected EBV-BACs by rescue of episomal BAC DNA followed by restriction enzyme digests analysis. Infectious virus is generated by co-transfection of plasmids expressing *BZLF1* and *BALF4*. High-titre producer cell lines were selected by infection of Raji cells followed by induction of the lytic cycle and counting of GFP-positive cells. Recombinant EBVs were then used to infect EBV-negative BL cells. Stable BL cell lines were generated by selection with Hyg and validated for expression of EBV latent proteins by Western blotting.

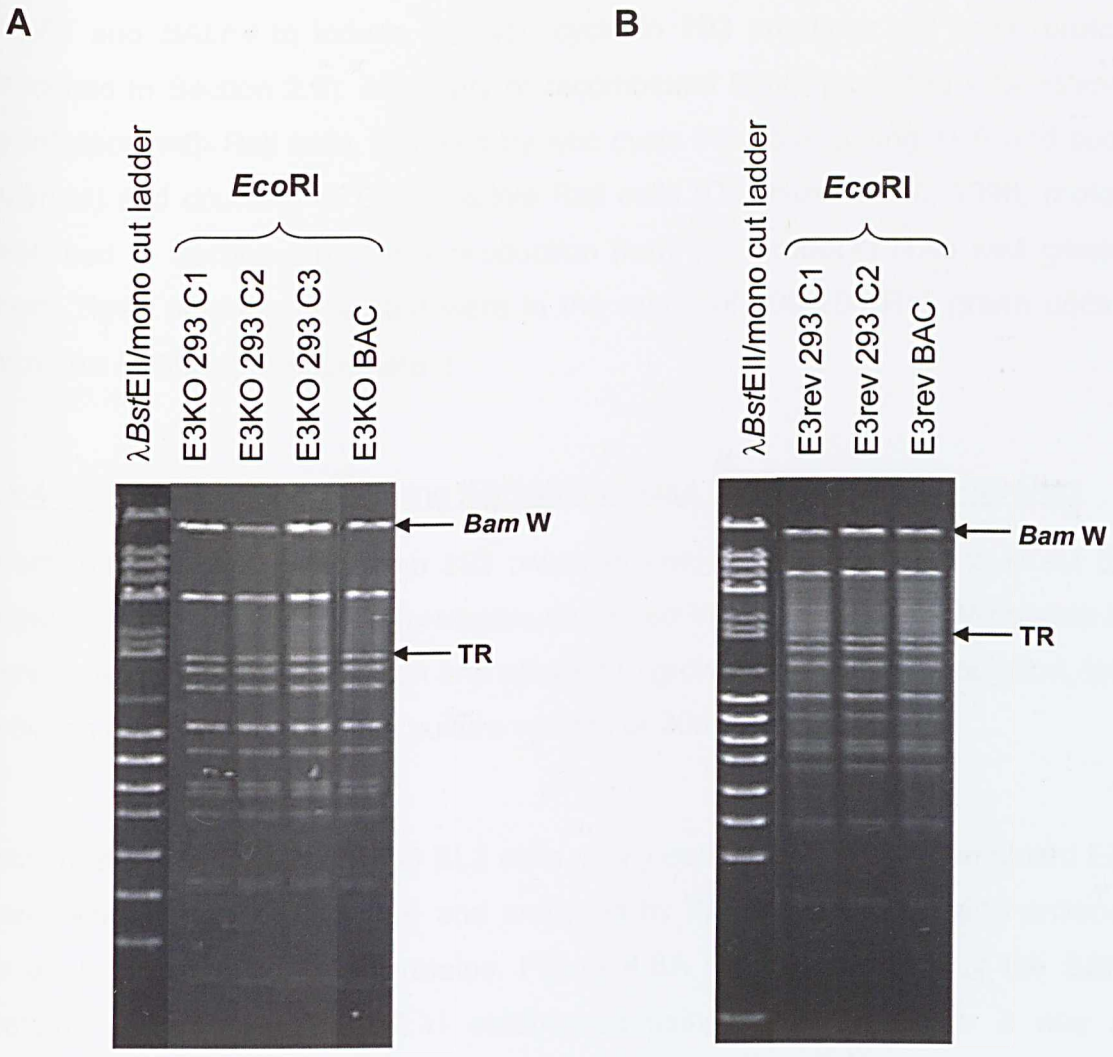


Figure 4-7 BAC DNA rescued from 293 producer cell lines are identical to originally transfected EBNA3 locus-knockout and -revertant EBV-BACs. BAC DNA was recovered from 293 producer cell clones harbouring recombinant **(A)** EBNA3 locus-knockout (E3KO) and **(B)** EBNA3 locus-revertant (E3rev) EBV-BACs. The BAC DNA rescued and the originally transfected EBV-BACs were compared by restriction digest with *Eco*RI followed by pulsed-field gel electrophoresis. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders were used as a size marker. The *Eco*RI restriction fragments corresponding to the *Bam* W repeats and the terminal repeats (TR) are shown.

Infectious virus was produced by transient transfection of plasmids expressing *BZLF1* and *BALF4* to induce the lytic cycle in 293 producer cell lines (protocols described in Section 2.6). Infectivity of recombinant EBVs produced was estimated by infection with Raji cells, followed by lytic cycle induction (using TPA and sodium butyrate) and counting of GFP-positive Raji cells (Delecluse *et al.*, 1998; protocols described in Section 2.6). Virus production from 293 producer lines was generally good. Titres of virus generated were in the range of 100-200 Raji green units per microlitre (RGU/μl) of supernatant.

4.1.4 Establishing and validating EBNA3-knockout and -revertant BL cell lines

Infectious virus generated from 293 producer lines were then used to infect EBV-negative BL31 and BL2 cells (protocols described in Section 2.6). EBV-positive cells were selected using hygromycin and allowed to grow out as a bulk population, taking between 4-8 weeks to reach a culture volume of 30ml.

Protein extracts from BL31 and BL2 cells newly established with recombinant EBVs were separated by SDS-PAGE and analysed by Western blotting using antibodies for each of the EBV latent proteins. Figure 4.8A shows that none of the EBNA3 proteins are expressed in BL31 established using the E3KO virus. It was also confirmed that these cells express the other EBV latent proteins – EBNA1, EBNA2, EBNA-LP and LMP1. It was interesting to note that longer isoforms of EBNA-LP were observed in some cell lines established using the E3KO virus, particularly in BL2. LMP2A- and LMP2B-specific antibodies are not available. It was also noted that the BH3-only pro-apoptotic protein Bim is expressed at a high level in BL31 E3KO cells, similar to those observed in the parental EBV-negative BL31 cells. This is consistent with the observation that EBNA3A and EBNA3C are responsible for the EBV-mediated downregulation of Bim (Anderton *et. al.*, 2008). It was confirmed that by Western blotting that BL2 cells infected with the E3KO recombinant virus express all the EBV latent proteins except for EBNA3A, 3B and 3C (Figure 4.8B).

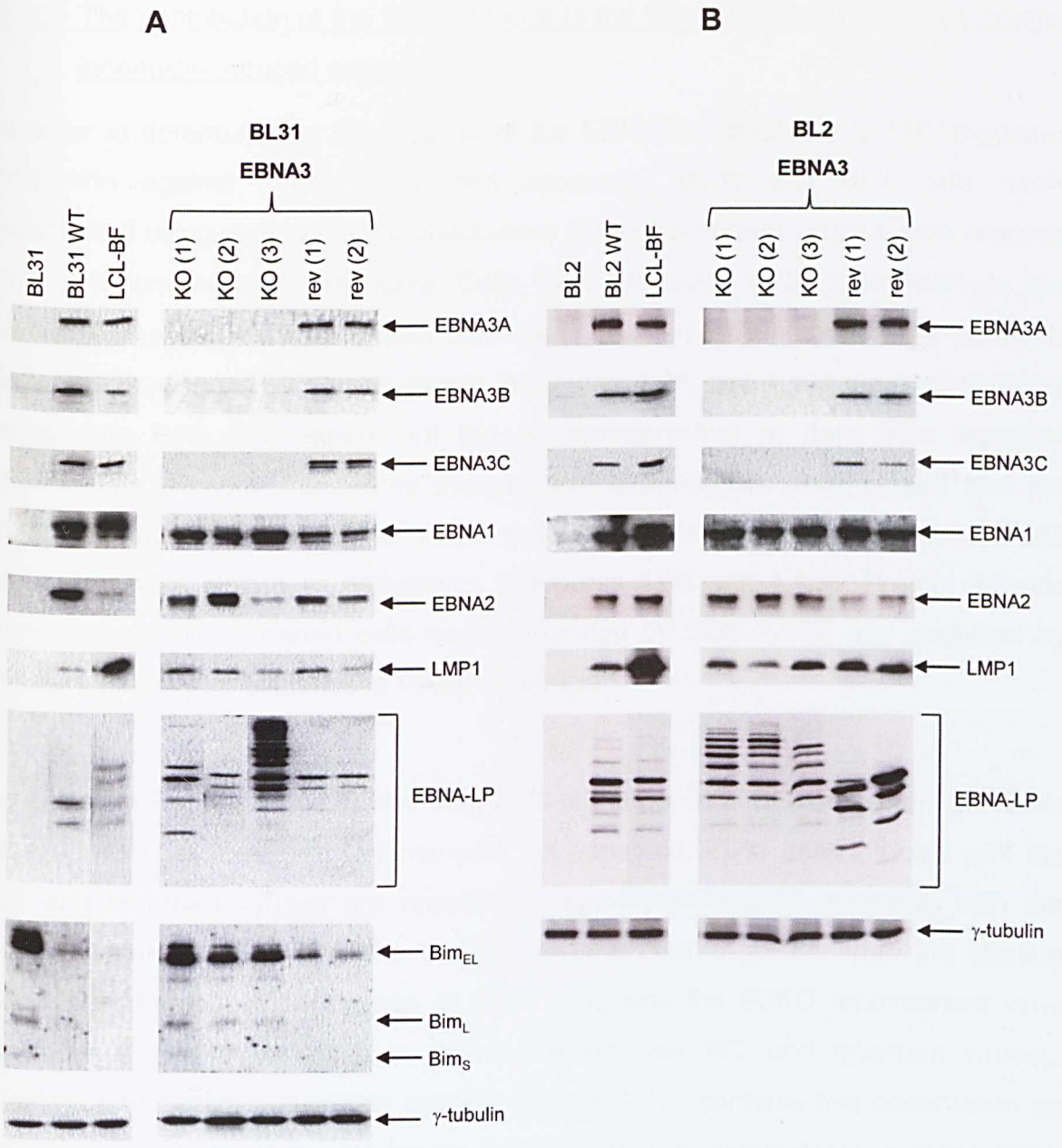


Figure 4-8 Expression of the EBV latent proteins in BL31 and BL2 cells newly established with recombinant EBNA3-knockout and revertant EBVs. Protein extracts prepared from **(A)** BL31 and **(B)** BL2 cells infected with recombinant EBVs, and from parental BL31 and BL2 cells as well as a lymphoblastoid cell line (LCL) were separated by SDS–PAGE followed by Western blotting with antibodies against EBV latent proteins and the pro-apoptotic protein Bim. γ-tubulin was used as a loading control.

4.1.5 The contribution of the EBNA3 locus to the EBV-mediated resistance against ionomycin-induced apoptosis

In order to determine the contribution of the EBNA3s collectively to EBV-mediated protection against ionomycin-induced apoptosis, BL31 and BL2 cells newly established using recombinant EBNA3 locus KO and revertant viruses were exposed to 1μg/ml ionomycin for 48 hours. Cells were harvested at 24 hour intervals and viable cells which exclude trypan blue were counted at the same time points to determine survival. The graphs shown in Figures 4.9A and 4.10A were constructed using data from one experiment but is representative of data from repeated experiments. Results from three independent experiments were pooled and the mean and standard deviation of viable cell counts after 48 hours of treatment with ionomycin were plotted as histograms in Figures 4.9B and 4.10B. Protein extracts from treated and untreated cells were separated by SDS-PAGE and analysed by Western blotting for evidence of PARP cleavage.

As shown in Figures 4.9A&B, less than 20% of the parental BL31 cells remain viable after 48 hours of exposure to ionomycin. As expected, BL31 cells infected with the WT and revertant viruses are resistant to ionomycin-induced apoptosis with the population of viable cells at the end of the time course greater than the starting population. Interestingly, infection of BL31 cells with the E3KO recombinant virus produces a similar response to those infected with WT and revertant viruses. Western blot analysis of protein extracts (Figure 4.9C) confirms this observation as proteolytic cleavage of the full length PARP protein is undetectable in these EBV-positive BL31 cells even after 48 hours of exposure to ionomycin.

Similarly, BL2 cells established with any of the recombinant E3KO, revertant or WT virus are significantly more resistant to ionomycin-induced apoptosis than the parental EBV-negative cell line (Figures 4.10A&B). This is supported by Western blot analysis showing no evidence of PARP cleavage in these EBV-positive BL2 cells (Figure 4.10C).

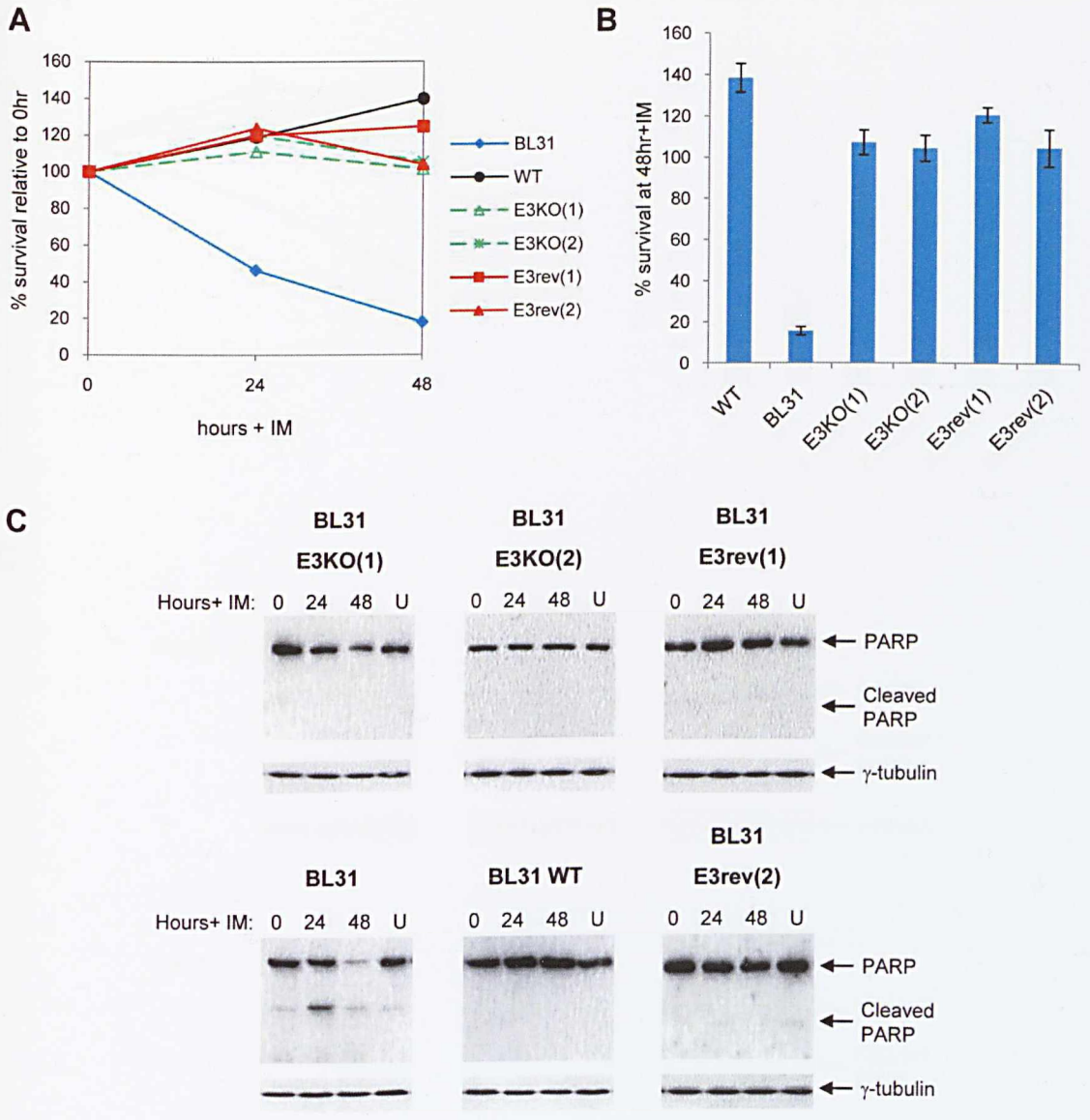


Figure 4-9 Resistance in BL31 cells newly infected with recombinant EBNA3-knockout (E3KO) EBV to ionomycin-induced apoptosis. EBV-negative BL31 cells, their EBV-positive converts newly infected with recombinant wild-type (WT), EBNA3 knockout (E3KO) and revertant (E3rev) EBVs were treated with 1μg/ml ionomycin for 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Viable cells are expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control.

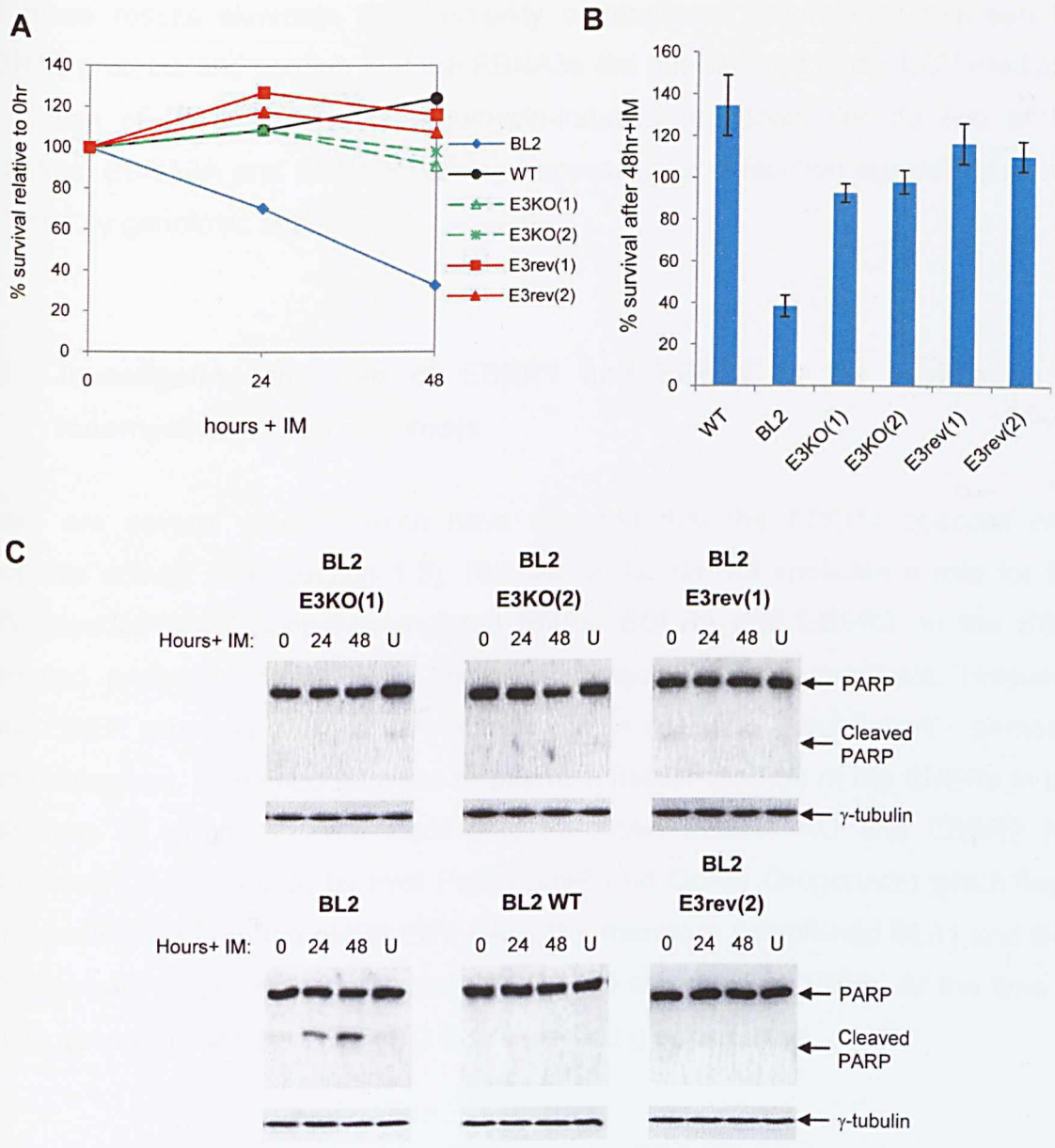


Figure 4-10 Resistance in BL2 cells newly infected with recombinant EBNA3-knockout (E3KO) EBV to ionomycin-induced apoptosis. EBV-negative BL2 cells, their EBV-positive converts infected with recombinant wild-type (WT), EBNA3 knockout (E3KO) and revertant (E3rev) EBVs were treated with 1μg/ml ionomycin for 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Viable cells are expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control.

All these results eliminate the possibility of functional redundancy between the EBNA3 proteins and confirm that the EBNA3s are not involved in the EBV-mediated protection of BL cells against ionomycin-induced apoptosis despite two of the EBNA3s, EBNA3A and EBNA3C, being necessary for protection against apoptosis induced by genotoxic agents.

4.2 Investigating the role of EBER1 and EBER2 in the resistance to ionomycin-induced apoptosis

There are several studies which have reported that the EBERs possess anti-apoptotic activity (see Section 1.3). Results so far do not implicate a role for the EBV-encoded small nonpolyadenylated RNAs, EBER1 and EBER2, in the EBV-mediated protection of BL cells against ionomycin-induced apoptosis. However, since EBER expression levels vary greatly across cell lines (Paul Farrell – personal communication), it seemed sensible to examine further the role of the EBERs in the resistance to ionomycin. We were able to obtain EBER1 KO and EBER2 KO recombinant EBVs (made by Prof Paul Farrell and Goran Gregorovic) which were also constructed from the pHB9 EBV-BAC. We therefore established BL31 and BL2 cell lines with these EBER1 KO and EBER2 KO recombinant EBVs. At the time of writing, revertants for the EBER KO EBVs are being constructed.

4.2.1 Validation of BL cell lines established with EBER KO EBVs

Protein extracts from BL31 and BL2 cell lines recently established with the EBER1 KO and the EBER2 KO recombinant EBVs analysed by Western blotting using antibodies for each of the EBV latent proteins. Figure 4.11 shows that BL31 and BL2 cells infected with the EBER1 KO and EBER2 KO viruses express the full set of latent proteins. Northern blot analysis carried out by Goran Gregorovic confirmed the absence of viral RNAs EBER1 or EBER2 expression in the respective EBER1 KO and EBER2 KO converts (data not shown).

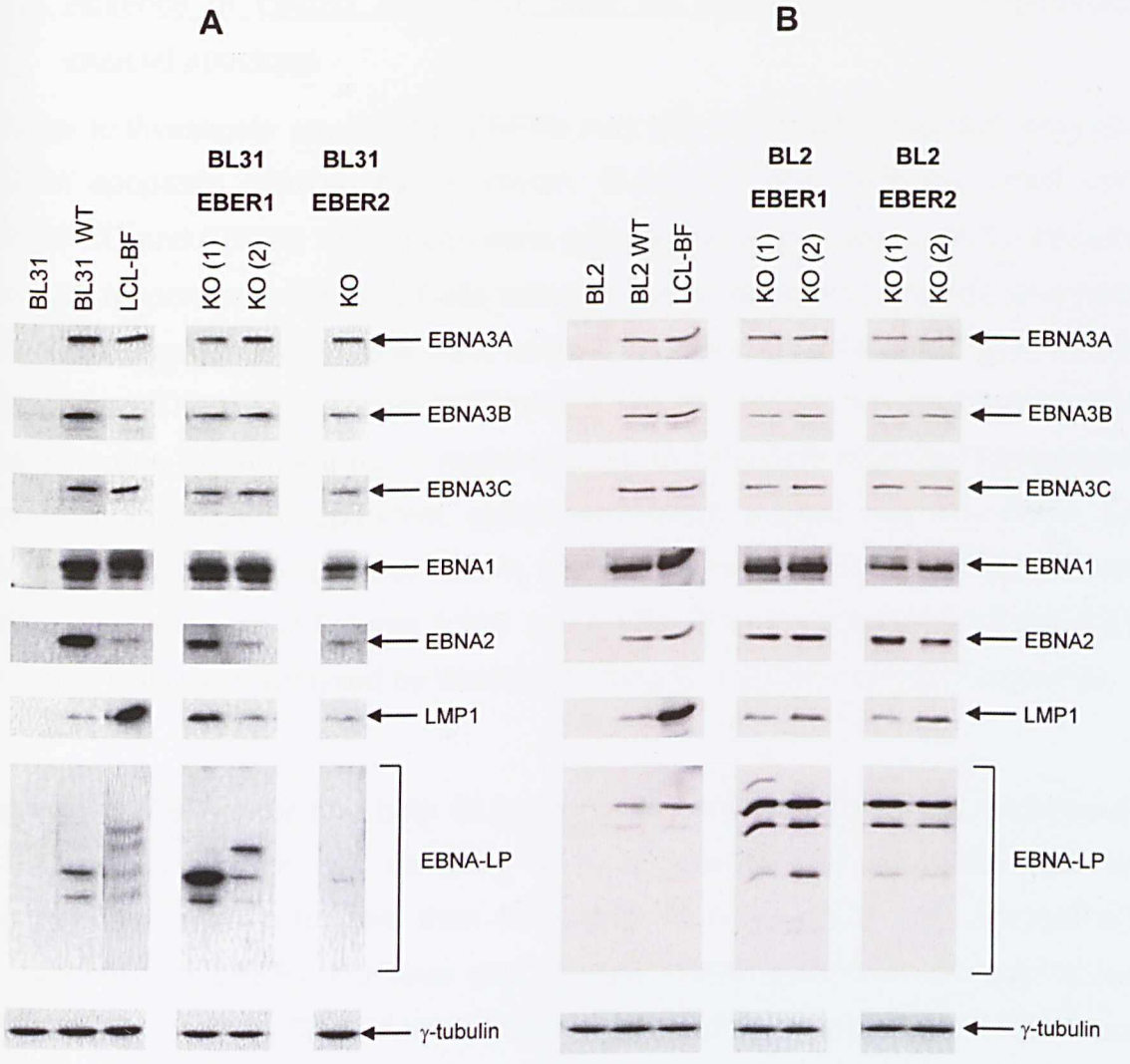


Figure 4-11 Expression of the EBV latent proteins in BL31 and BL2 cells newly established with recombinant EBER1-knockout and EBER2-knockout EBVs. Protein extracts prepared from **(A)** BL31 and **(B)** BL2 cells infected with EBER1-knockout (EBER1 KO) and EBER2-knockout (EBER2 KO) recombinant EBVs, parental, wild-type (WT)-infected BL31 and BL2 cells as well as a lymphoblastoid cell line (LCL) were separated by SDS–PAGE followed by Western blotting with antibodies against EBV latent proteins. γ-tubulin was used as a loading control.

4.2.2 Absence of EBER1 or EBER2 does not affect resistance to ionomycin-induced apoptosis

In order to investigate any role the EBERs may play in the EBV-mediated protection against apoptosis induced by ionomycin, BL31 and BL2 cells converted using EBER1 KO and EBER2 KO viruses were exposed to 1μg/ml ionomycin for 48 hours and their responses examined. Cells were harvested at 24 hour intervals and viable cells which exclude trypan blue were counted at the same time points to determine cell survival. The graphs shown in Figures 4.12A and 4.13A was constructed using data from one experiment but is representative of data from repeated experiments. Results from three independent experiments were pooled and the mean and standard deviation of viable cell counts after 48 hours of ionomycin treatment were plotted as histograms in Figures 4.12B and 4.13B. Protein extracts from treated and untreated cells were analysed by Western blotting for evidence of PARP cleavage.

Figures 4.12A&B show that both BL31 cell lines established with the recombinant EBER1 KO virus are not sensitive to ionomycin-induced apoptosis with cell populations increasing to more than 100% after 48 hours. BL31 cells infected with the recombinant EBER2 KO virus was similarly protected from ionomycin-induced apoptosis. Western blotting analysis showed that proteolytic cleavage of PARP was not detectable in BL31 cells infected with either recombinant EBER1 or EBER2 KO viruses confirming that these cells are resistant to apoptosis induced by ionomycin (Figure 4.12C).

Similarly, viable cells counts reveal that BL2 cells infected with either EBER KO virus are also resistant to ionomycin treatment (Figure 4.13A&B). This is confirmed by Western blot analysis which showed no evidence of PARP cleavage in these EBV-positive converts even after 48 hours of exposure to ionomycin (Figure 4.13C).

These data suggest that EBER1 and EBER2 are not required for EBV-mediated protection against ionomycin-induced apoptosis.

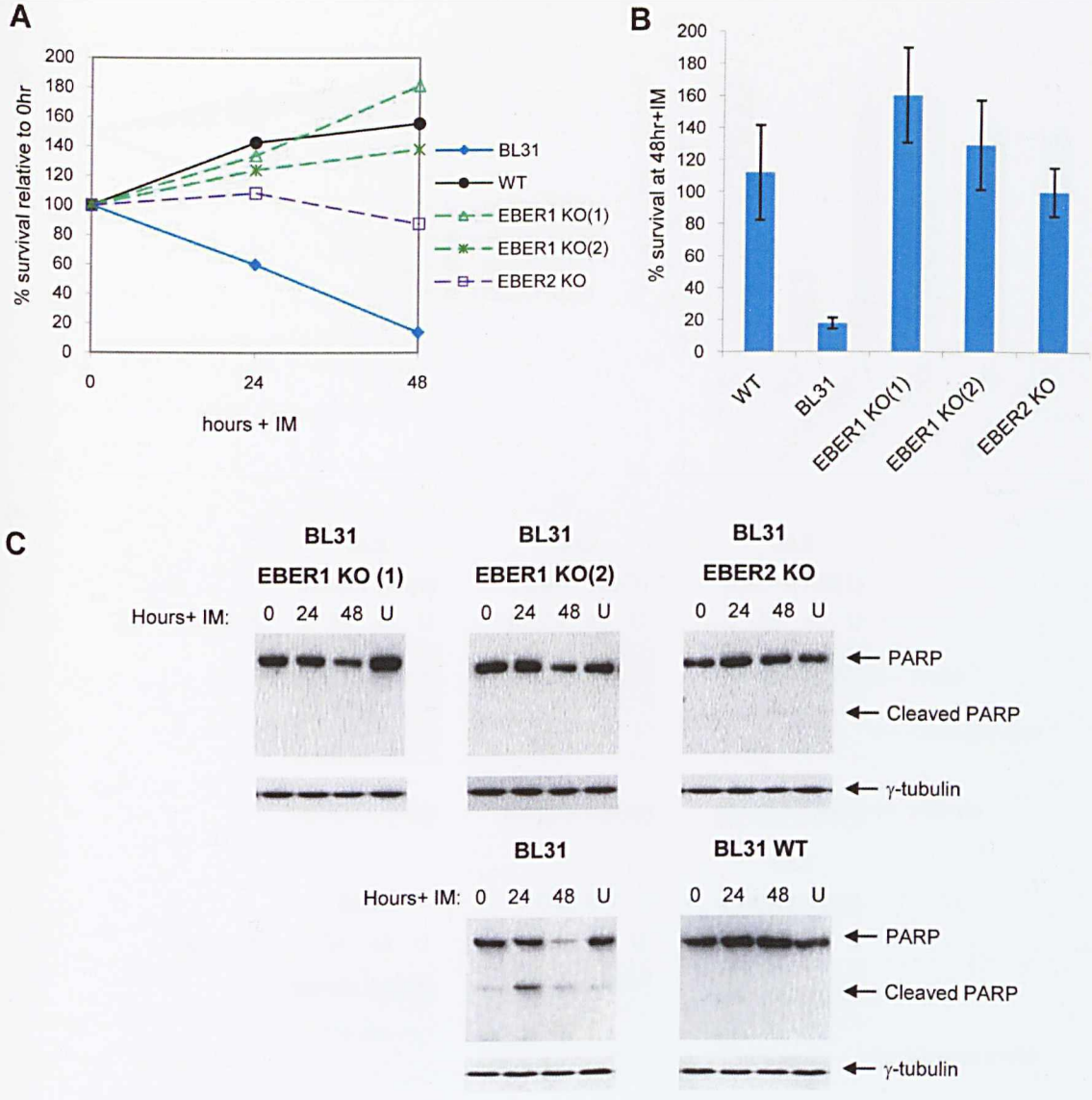


Figure 4-12 Resistance of BL31 cells newly infected with recombinant EBER1-knockout and EBER2-knockout EBVs to ionomycin-induced apoptosis. EBV-negative BL31, their EBV-positive converts infected with recombinant wild-type (WT), EBER1-knockout (EBER1 KO) and EBER2-knockout (EBER2 KO) EBVs were treated with 1μg/ml ionomycin for 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Viable cells are expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control.

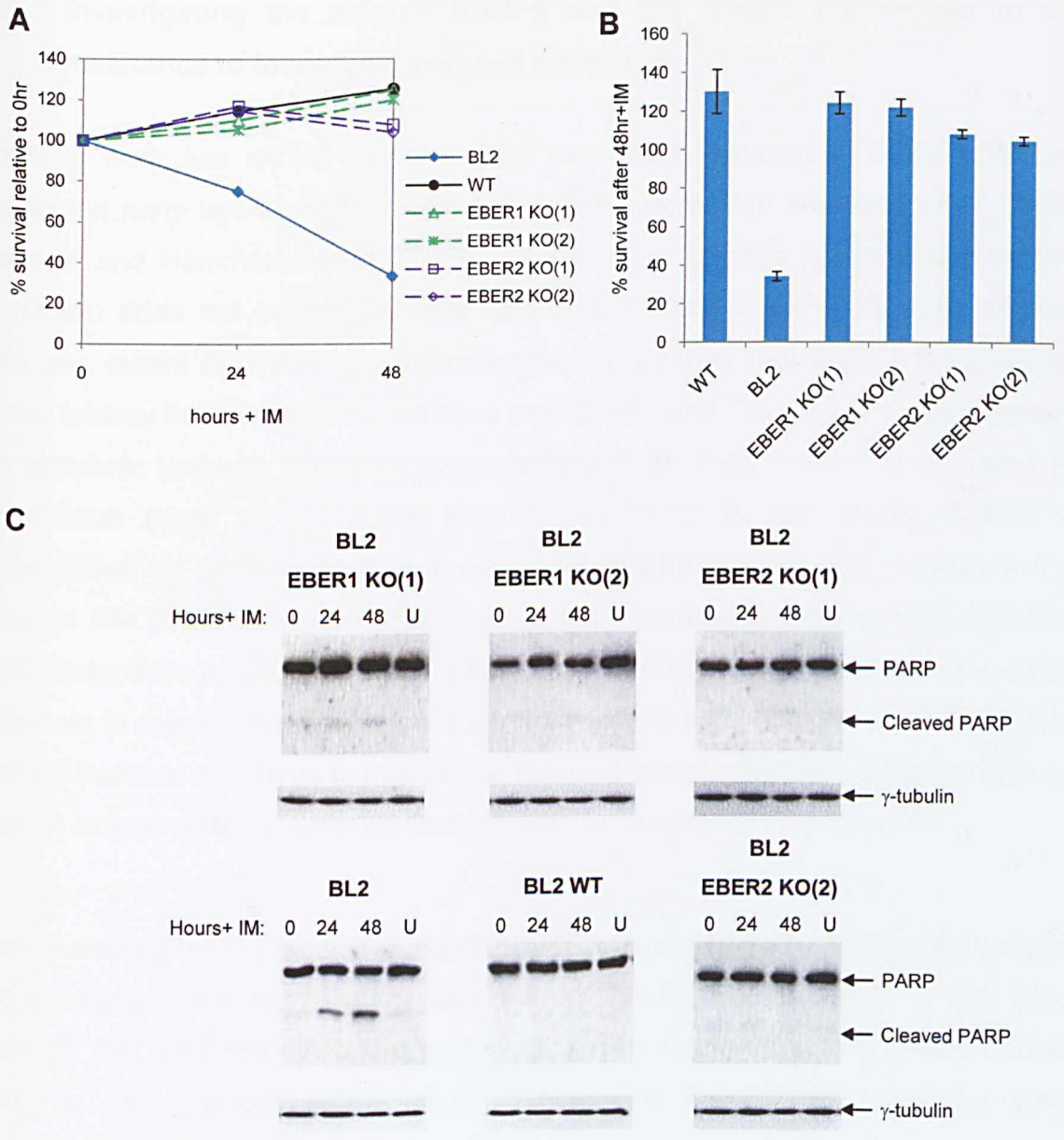


Figure 4-13 Resistance of BL2 cells newly infected with recombinant EBER1-knockout and EBER2-knockout EBVs to ionomycin-induced apoptosis. EBV-negative BL2 cells, their EBV-positive converts infected with recombinant wild-type (WT), EBER1-knockout (EBER1 KO) and EBER2-knockout (EBER2 KO) EBVs were treated with 1μg/ml ionomycin for 48 hours. Viable cells that exclude trypan blue were counted and cells were harvested at the times indicated. **(A)** Viable cells are expressed as a percentage of the starting population. **(B)** The mean and standard deviation of viable cell counts at the 48 hour time point from three independent experiments is shown. **(C)** Protein analysis was carried out by Western blotting with an antibody directed against PARP. γ-tubulin was used as a loading control.

4.3 Investigating the role of BHRF1 and the BHRF1 microRNAs in the resistance to ionomycin-induced apoptosis

Previous work has shown that the EBV viral Bcl-2 homologue, BHRF1, is only expressed early upon infection and during lytic reactivation (Pearson *et al.*, 1987; Altmann and Hammerschmidt, 2005). Results from Section 3.2 demonstrate that ionomycin does not induce the viral lytic cycle in the panel of BL cells studied. However, recent work by Kelly and colleagues has shown that BHRF1 is expressed during latency in Wp-restricted cell lines (eg. Sal-BL and Oku-BL) and can suppress the apoptotic pathway activated by ionomycin in BL cells when it is delivered by transfection (Kelly *et al.*, 2009) (see Section 1.3). In our hands, BHRF1 is undetectable by Western blotting in our panel of EBV-positive cells. However, it is possible that BHRF1 may be expressed at such low levels in these cells that they are undetectable by Western blotting but is still functionally relevant. As such, it was important to construct a recombinant EBV containing a deletion of the BHRF1 open reading frame to enable us to investigate the contribution of BHRF1 to the resistance against ionomycin in the cells we have chosen to investigate (Figure 4.14).

Recent studies have shown that some microRNAs encoded by herpesviruses have anti-apoptotic functions (Skalsky and Cullen, 2010). For example, it has been reported that EBV mir-BART5 downregulates expression of the BH3-only protein Puma in epithelial cells (Choy *et al.*, 2008). The BHRF1 locus encodes three miRNAs which have recently been shown to inhibit apoptosis and promote cell proliferation early after infection (Seto *et al.*, 2010). In order to investigate whether the BHRF1 miRNAs are involved in the EBV-mediated protection against ionomycin, a second recombinant EBV harbouring a deletion of the whole BHRF1 locus (and thus BHRF1 and the BHRF1 miRNA cluster) was constructed (Figure 4.14). We decided against constructing a mutant virus harbouring deletions of the BHRF1 miRNAs because of the complexity involved. As miR-BHRF1-1 overlaps with the BHRF1 mRNA transcriptional start site, deleting miR-BHRF1-1 may interfere with BHRF1 transcription or translation.

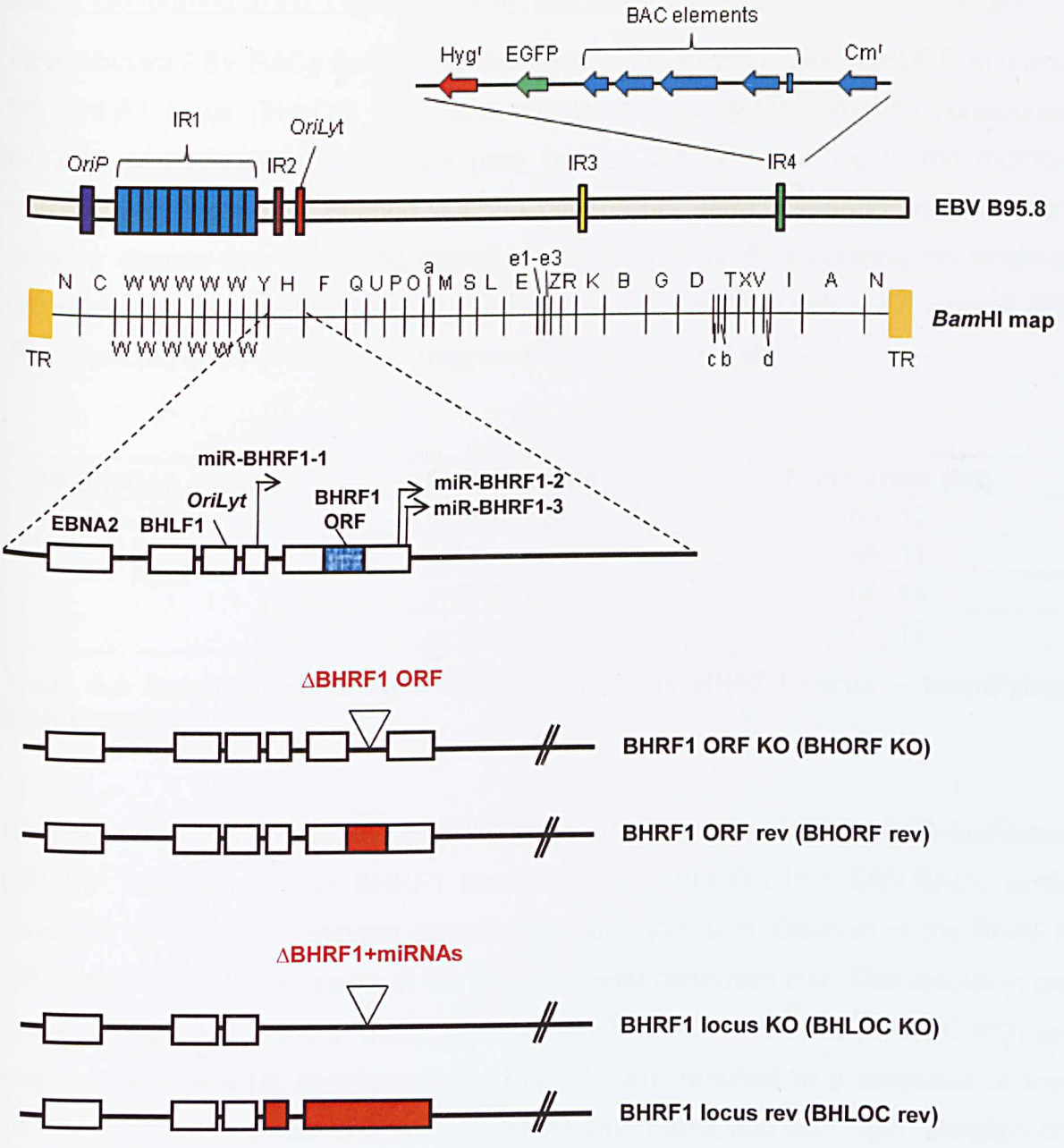


Figure 4-14 Schematic representation of the deletions made in the BHRF1 region of the EBV-BAC. Recombinant EBV-BACs harbouring deletions of the BHRF1 open reading frame (ORF) and the full BHRF1 locus were constructed. The regions deleted in each BHRF1 knockout EBV-BAC are reinserted to generate revertants. Figure is not drawn precisely to scale.

Recombinant EBVs generated were then used to infect EBV-negative BL31 and BL2 cells. The response of these newly established BL converts to ionomycin was assessed to determine the contribution of BHRF1 and the BHRF1 miRNAs to the EBV-mediated protection against ionomycin-induced apoptosis.

4.3.1 Generating BHRF1 ORF-knockout and BHRF1 locus-knockout EBV-BACs

Recombinant EBV-BACs harbouring deletions of the BHRF1 ORF (BHORF KO) and the BHRF1 locus (BHLOC KO) were constructed using the targeting constructs, pJY24.1 and pJY28.1 respectively (see Section 2.5.1), according to the method previously described in Section 4.1.1. Cointegrants were screened by restriction enzyme digests with *Pvu*I and identified as either S or E depending on whether recombination occurred at the start (S) half-site or the end (E) half-site (Figure 4.15). The expected sizes of restriction fragments are shown in Table 4.4.

Restriction enzyme	Cointegrants	Band sizes (kb)
***Pvu*I**	pHB9-JY24-S	65, 12
	pHB9-JY24-E	66, 11
	pHB9-JY28-S	65, 14
	pHB9-JY28-E	67, 12

Table 4.4 Deleting the BHRF1 ORF and the full BHRF1 locus – Identifying cointegrants.

Recombinants which have successfully resolved to yield the BHRF1 ORF-knockout (BHORF KO) and the full BHRF1 locus-knockout (BHLOC KO) EBV-BACs were screened by restriction enzyme digests with *Bgl*II and *Age*I. Deletion of the BHRF1 ORF and BHRF1 locus results in the loss of a *Bgl*II restriction site. This results in an increase of the 9kb band in pHB9 to 11kb (BHORF KO) and 10kb (BHLOC KO) as shown in Figure 4.16. Deletion of the BHRF1 ORF resulted in a decrease of the 14.7kb fragment in pHB9 to a 14kb fragment when analysed with *Age*I. Deletion of the whole BHRF1 locus resulted in a 1.7kb decrease in the same fragment to give a band of 13kb. Recombinants were also screened for BAC integrity by restriction enzyme digests with *Eco*RI. This allowed us to identify recombinants which contain the same number of *Bam* W repeats and terminal repeats (TR) as pHB9. Small variations in the size of the *Eco*RI restriction fragment containing the Bam W repeats is observed because the 0.7kb and 1.7kb deletions of the BHRF1 ORF and BHRF1 locus are within the same restriction fragment.

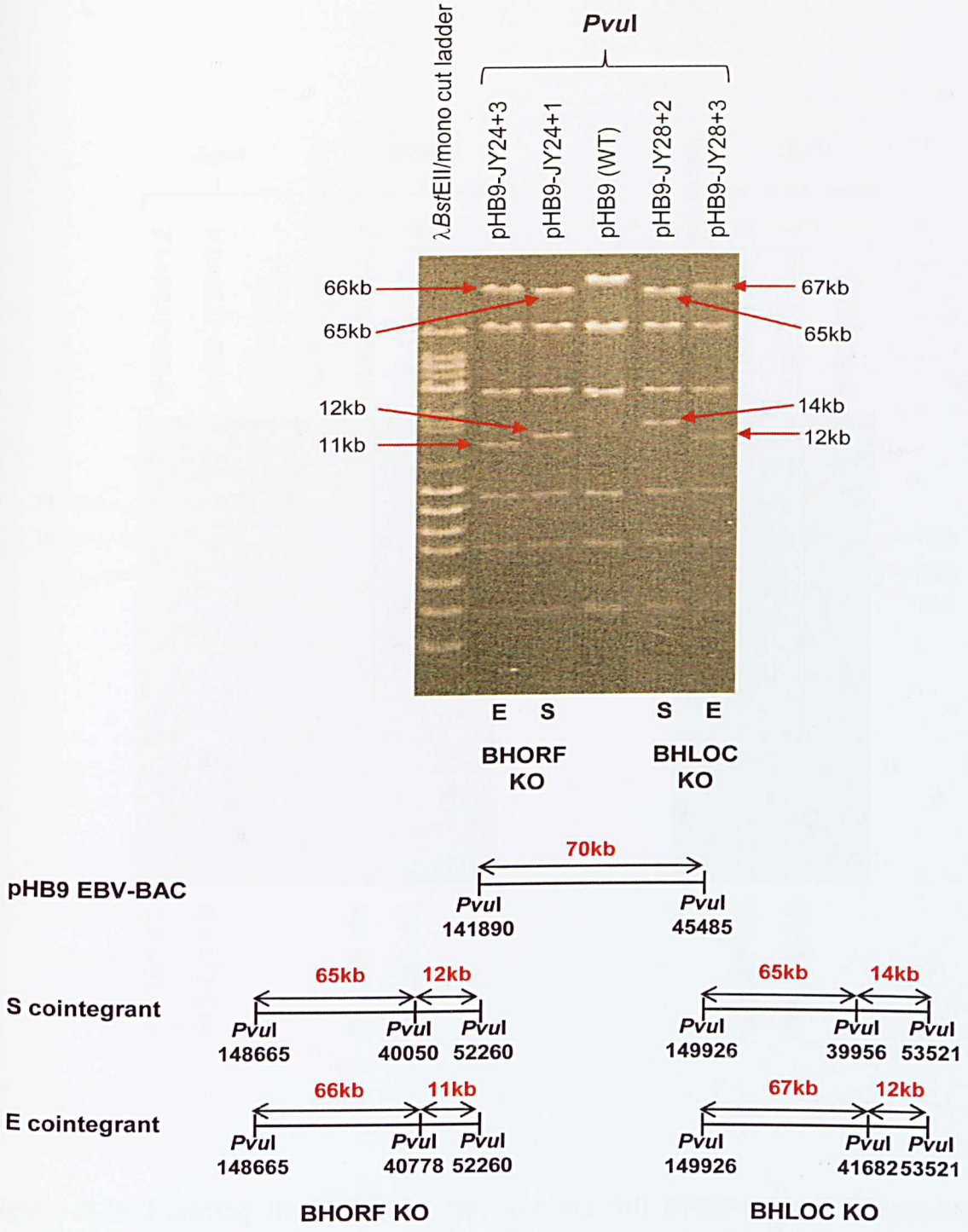


Figure 4-15 Deleting the BHRF1 ORF and the full BHRF1 locus – cointegrants. Cointegrants formed by recombination between WT EBV-BAC (pHB9) and the targeting constructs harbouring deletions of the BHRF1 open reading frame (ORF) and the full BHRF1 locus were screened by restriction digests with *Pvu*I followed by pulsed-field gel electrophoresis. DNA from the wild-type (WT) EBV-BAC (pHB9) was digested with the same restriction enzyme and included as a control. Cointegrants were identified as either S or E depending on whether recombination occurred at the start (S) half-site or the end (E) half-site. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders were used as a size marker.

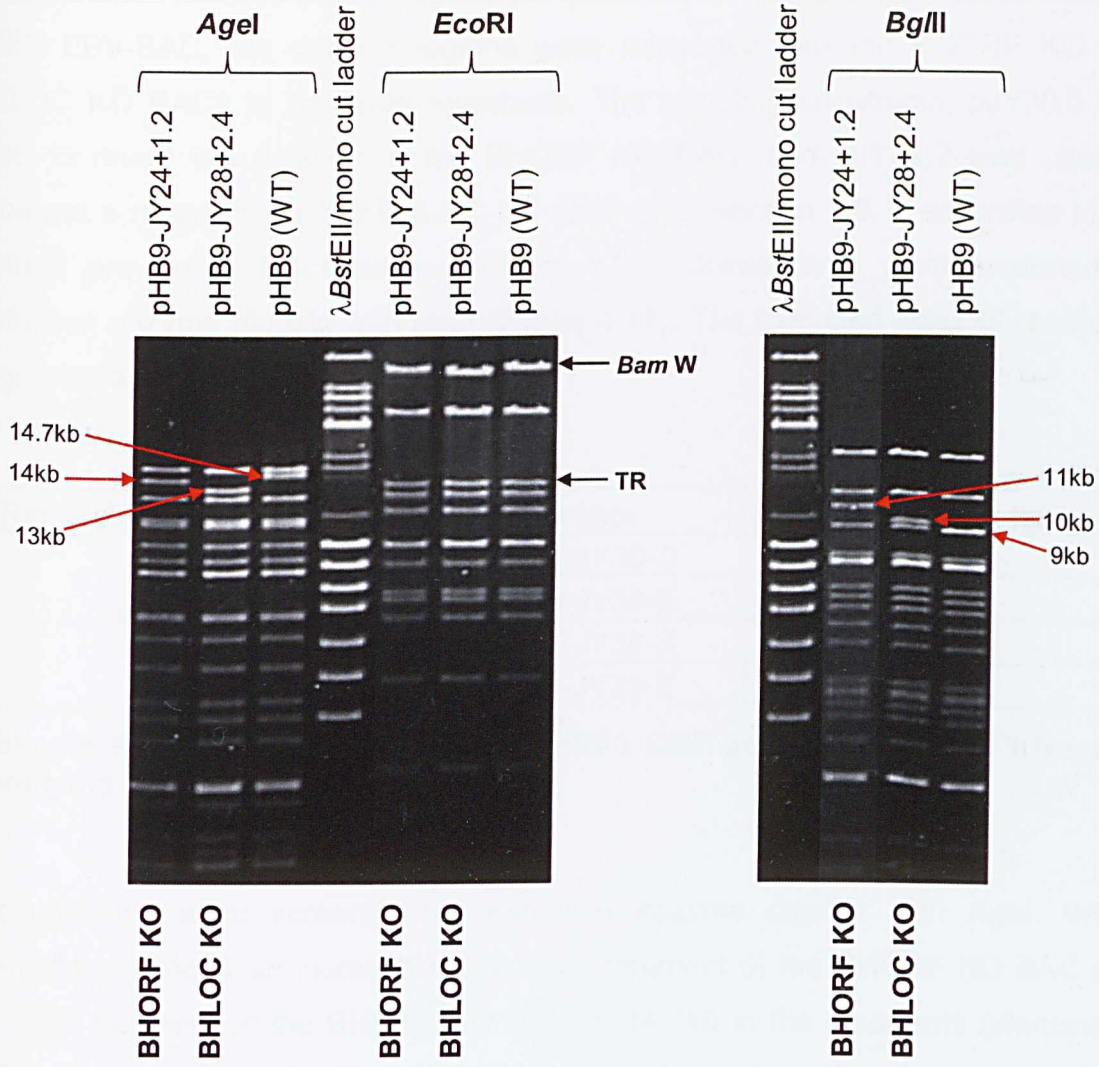


Figure 4-16 Deleting the BHRF1 ORF and the full BHRF1 locus – resolving cointegrants. Cointegrants were resolved following a second recombination event. DNA from resolved cointegrants were analysed by restriction enzyme digests with *Age*I, *Bgl*II and *Eco*RI followed by pulsed-field gel electrophoresis. DNA from the wild-type (WT) EBV-BAC (pHB9) was digested with the same restriction enzymes and included as a control. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders were used as a size marker. The *Eco*RI restriction fragments corresponding to the *Bam* W repeats and the terminal repeats (TR) are also shown.

4.3.2 Generating BHRF1 ORF-revertant and BHRF1 locus-revertant EBV-BACs

In order to control for any second site mutations which may have occurred during the recombination process used to delete the BHRF1 ORF or the BHRF1 locus from the pHB9 EBV-BAC, the deleted regions were reinserted into the BHORF KO and BHLOC KO BACs to generate revertants. The targeting constructs, pJY30.5 was used to revert the deletion in the BHORF KO BAC, and pJY32.2 was used to generate a revertant for the BHLOC KO BAC (see Section 2.5.1) according to the method previously described in Section 4.1.1. Cointegrants were screened by restriction enzyme digests with *Pvu*I (Figure 4.17). The expected sizes of restriction fragments are shown in Table 4.5.

Restriction enzyme	Cointegrants	Band sizes (kb)
***Pvu*I**	pHB9-JY24-JY30-S	66,11
	pHB9-JY24-JY30-E	65,12
	pHB9-JY28-JY32-S	71,7
	pHB9-JY28-JY32-E	69, 9

Table 4.5 Reverting deletions of the BHRF1 ORF and the full BHRF1 locus – Identifying cointegrants

Recombinants were screened by restriction enzyme digests with *Age*I. When analysed with *Age*I, an increase of the 14kb fragment of the BHORF KO BAC and the 13kb fragment of the BHLOC KO BAC to 14.7kb in the revertants (identical to pHB9) is observed (Figure 4.18). Recombinants were also screened for BAC integrity by restriction enzyme digests with *Eco*RI. Recombinants containing the same number of Bam W repeats and TR as pHB9 were identified as shown in Figure 4.18.

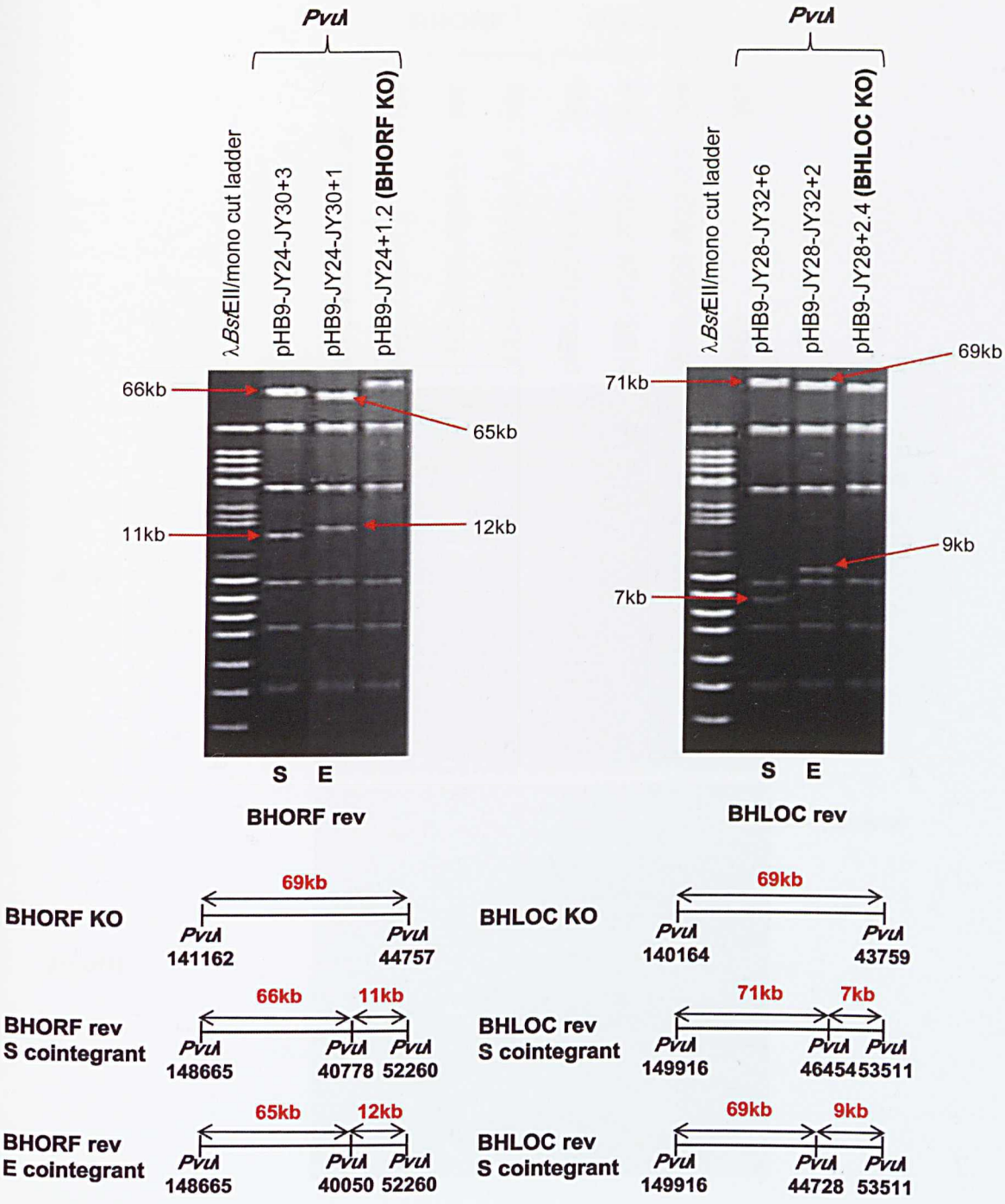


Figure 4-17 Reverting deletions of the BHRF1 ORF and the full BHRF1 locus – cointegrants. Cointegrants formed by recombination between the BHRF1 ORF-knockout (BHORFKO) and BHRF1 locus-knockout (BHLOCKO) EBV-BACs and targeting constructs harbouring the corresponding deleted sequences were screened by restriction enzyme digests with *Pvu*I followed by pulsed-field gel electrophoresis. DNA from the relevant BHRF1-deleted EBV-BACs were digested with the same restriction enzyme and included as controls. Cointegrants were identified as either S or E depending on whether recombination occurred at the start (S) half-site or the end (E) half-site. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders were used as a size marker.

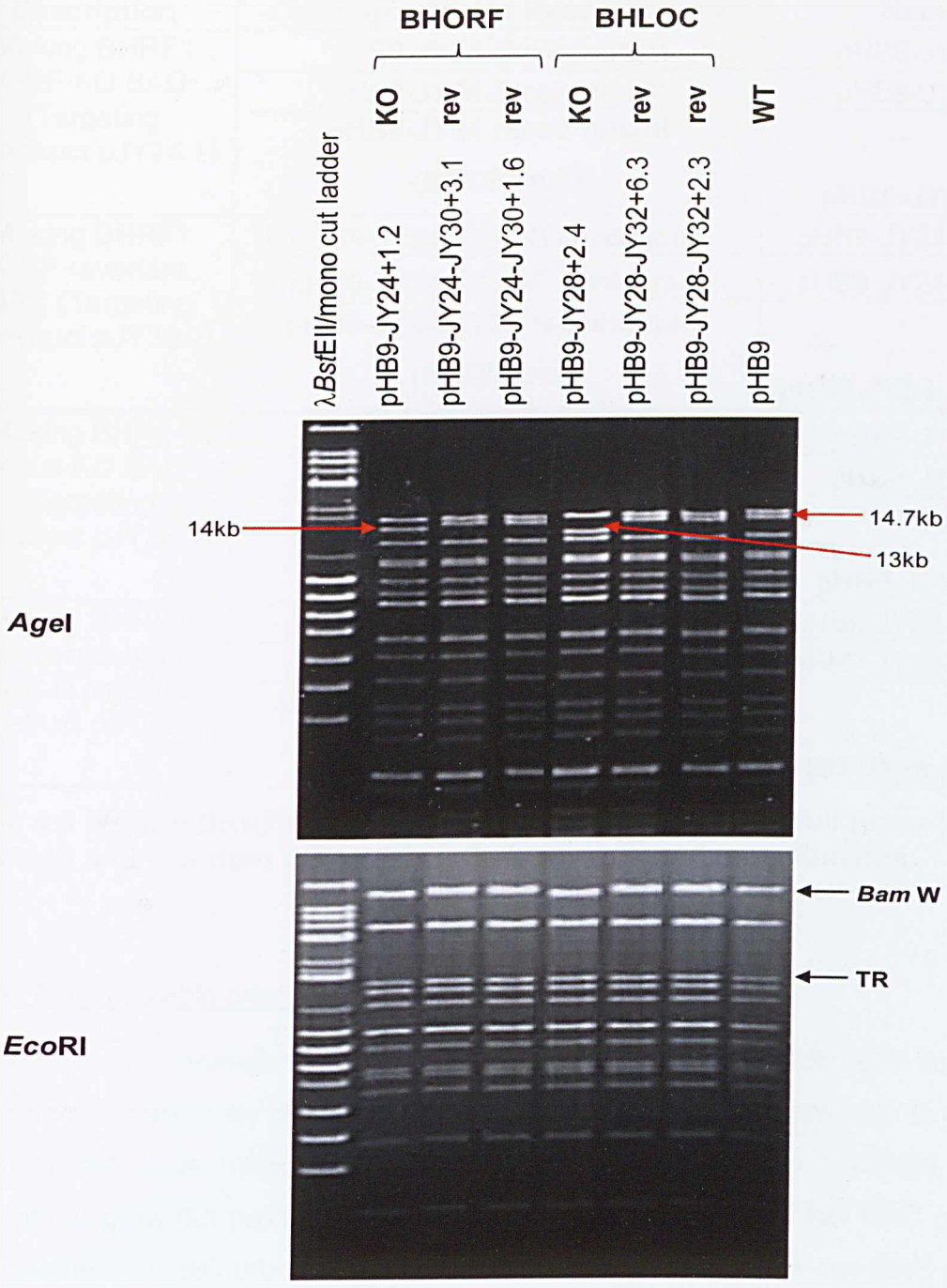


Figure 4-18 Reverting deletions of the BHRF1 ORF and the full BHRF1 locus-resolving cointegrants. Cointegrants were resolved following a second recombination event. DNA from resolved cointegrants were analysed by restriction digests with *Age*I and *Eco*RI followed by pulsed-field gel electrophoresis. DNA from the BHRF1 ORF-knockout (BHORFKO) and BHRF1 locus-knockout (BHLOCKO) EBV-BACs as well as the wild-type (WT) EBV-BAC (pHB9) were digested with the same restriction enzymes and included as controls. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders were used as a size marker. The *Eco*RI restriction fragments corresponding to the *Bam* W repeats and the terminal repeats (TR) are also shown.

Description	Cointegrants and Recombinants	Names
Making BHRF1 ORF-KO BAC (Targeting construct pJY24.1)	pHB9-JY24-S cointegrant	pHB9-JY24+1
	pHB9-JY24-E cointegrant	pHB9-JY24+3
	pHB9-JY24 recombinant (BHORFKO)	**pHB9-JY24+1.2**
Making BHRF1 ORF-revertant BAC (Targeting construct pJY30.2)	pHB9-JY24-JY30-S cointegrant	pHB9-JY24-JY30+3
	pHB9-JY24-JY30-E cointegrant	pHB9-JY24-JY30+1
	pHB9-JY24-JY30 recombinant (BHORFrev)	**pHB9-JY24-JY30+1.6**
Making BHRF1 locus-KO BAC (Targeting construct pJY28.1)	pHB9-JY28-S cointegrant	pHB9-JY28+2
	pHB9-JY28-E cointegrant	pHB9-JY28+3
	pHB9-JY28 recombinant (BHLOCKO)	**pHB9-JY28+2.4**
Making BHRF1 locus-revertant BAC (Targeting construct pJY32.2)	pHB9-JY28-JY32-S cointegrant	pHB9-JY28-JY32+6
	pHB9-JY28-JY32-E cointegrant	pHB9-JY28-JY32+2
	pHB9-JY28-JY32 recombinant (BHLOCrev)	**pHB9-JY28-JY32+2.3**

Table 4.6 Making BHRF1 open reading frame (BHORF) and full locus (BHLOC) knockout and revertant EBV-BACs - Cointegrants and recombinants

4.3.3 Making stable producer cell lines and infectious virus

EBV-BACs were transfected into 293 cells and stable producer cell lines were generated as previously described in Section 4.1.3. While we were able to generate producer cell lines harbouring the BHLOC KO BAC and its revertant, several attempts to grow out producer cell lines harbouring the BHORF KO BAC proved to be unsuccessful. 293 producer cells lines harbouring the BHORF rev BAC grew out fine, thereby eliminating the possibility that a second site mutation is suppressing the growth of 293 cells harbouring the BHORF KO BAC.

Producer cell lines were screened by episomal DNA recovery and restriction enzyme digests with *Eco*RI and *Age*I (as previously described in Section 4.1.3). Restriction digests analysis shows that BAC DNA rescued from 293 producer cells lines harbouring both the BHLOC KO (Figure 4.19A) and BHLOC rev (Figure 4.19B) EBV-BACs are identical to the EBV-BAC DNA originally transfected.

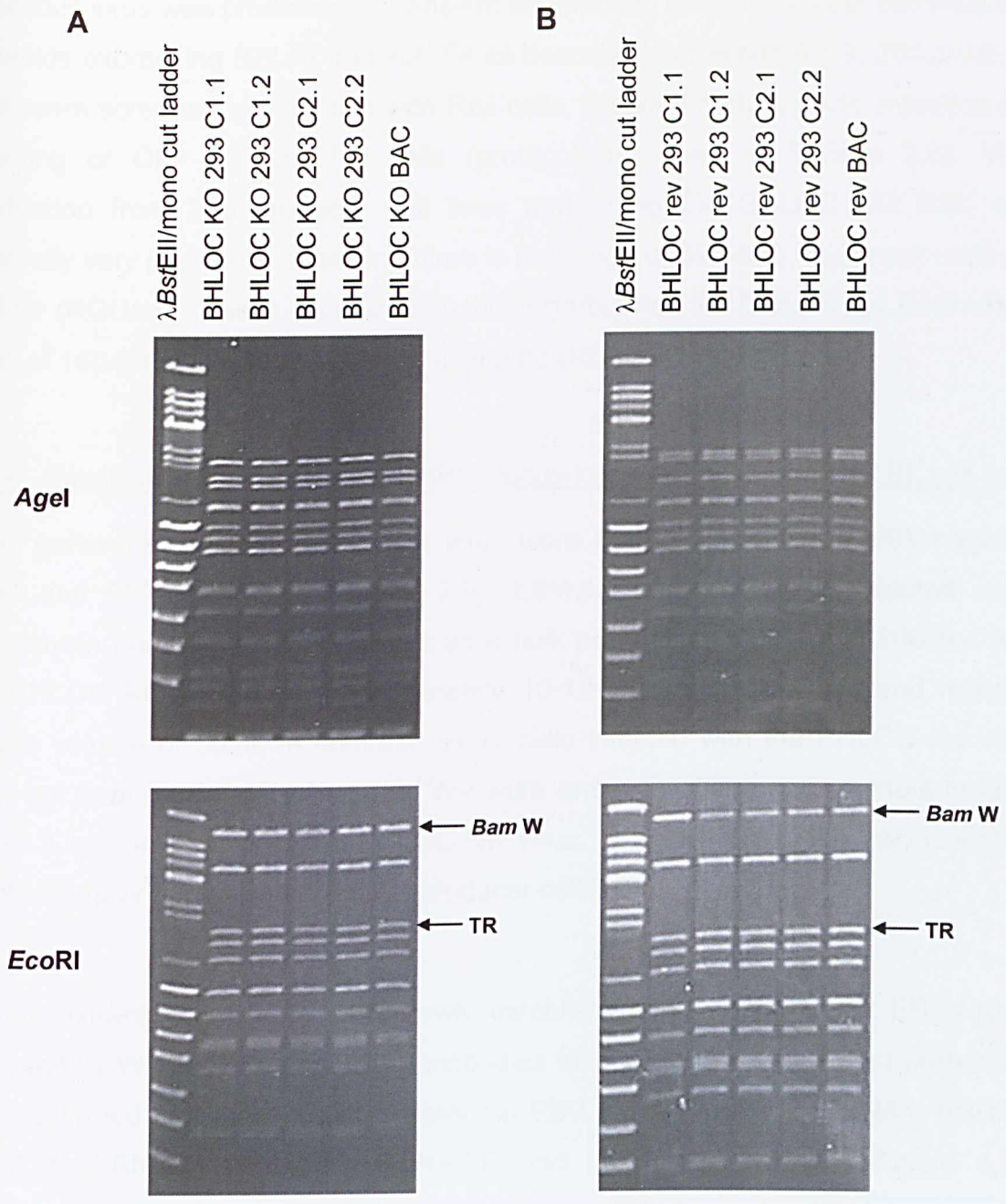


Figure 4-19 BAC DNA rescued from 293 producer cell lines are identical to originally transfected EBV-BACs. BAC DNA was recovered from 293 producer cell clones harbouring the **(A)** BHRF1 locus-knockout (BHLOCKO) and **(B)** -revertant (BHLOCrev) EBV-BACs. The BAC DNA rescued and the originally transfected EBV-BACs were compared by restriction enzyme digests with *Age*I and *Eco*RI followed by pulsed-field gel electrophoresis. A mixture of λ-DNA *Bst*EII and λ-DNA mono cut mix ladders were used as a size marker. The *Eco*RI restriction fragments corresponding to the *Bam* W repeats and the terminal repeats (TR) are also shown.

Infectious virus was produced by transient transfection of 293 producer cell lines with plasmids expressing *BZLF1* and *BALF4* as described in Section 4.1.3. 293 producer cells were screened by infection with Raji cells, followed by lytic cycle induction and counting of GFP-positive Raji cells (protocol described in Section 2.6). Virus production from 293 producer cell lines harbouring the BHLOC KO BAC was generally very poor, only achieving titres in the range of 300-800 Raji green units per mililiter (RGU/ml). This is in contrast to those harbouring the BHLOC rev BAC where titres of 150-200 Raji green units per microlitre (RGU/μl) were obtained.

4.3.4 Establishing and validating BHRF1 locus-knockout and -revertant BL cell lines

Virus generated from 293 producer lines were then used to infect EBV-negative BL31 and BL2 cells (see Section 2.6). EBV-positive cells were selected using hygromycin and allowed to grow out as a bulk population. BL31 cells infected with the BHLOC KO virus took approximately 10-12 weeks to grow out and reach a culture volume of 30ml. In contrast, BL31 cells infected with the BHLOC rev virus grew out in approximately 4 weeks. We were unsuccessful in our attempts to grow out BL2 cells infected with the BHLOC KO virus, probably due to the very low titres of infectious virus generated by 293 producer cell lines.

Protein extracts from BL31 cells newly established with recombinant EBVs were analysed by Western blotting using antibodies for each of the EBV latent proteins. It was confirmed that these cells express the EBV latent proteins – EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP and LMP1 as shown in Figure 4.20. LMP2A- and LMP2B-specific antibodies are not available.

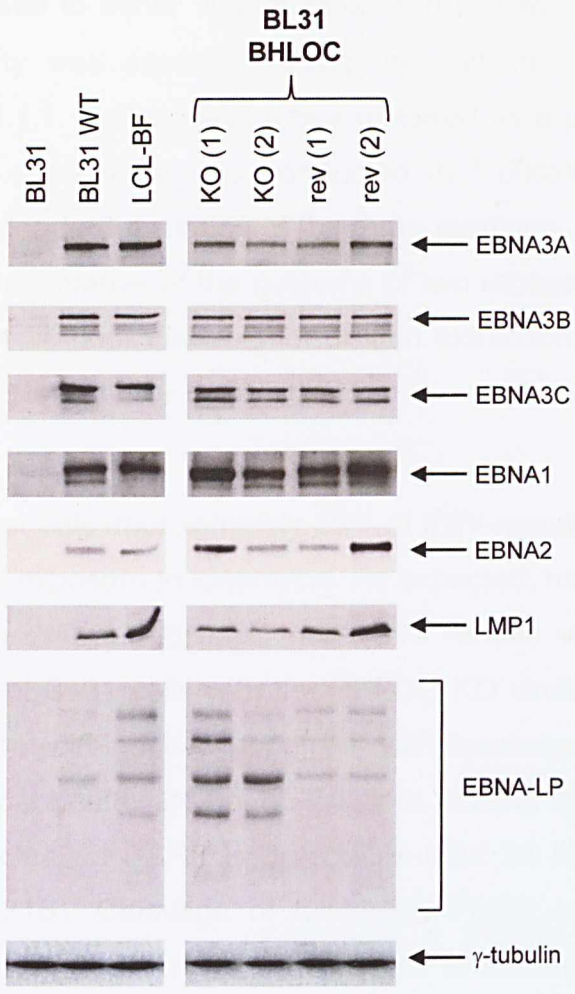


Figure 4-20 Expression of EBV latent proteins in BL31 cells newly established with recombinant BHRF1 locus-knockout and -revertant EBVs. Protein extracts prepared from BL31 cells infected with recombinant wild-type (WT), BHRF1 locus-knockout (BHLOCKO) and -revertant (BHLOCrev) EBVs, EBV-negative BL31 cells and a lymphoblastoid cell line (LCL) were separated by SDS-PAGE followed by Western blotting using antibodies against EBV latent proteins. γ-tubulin was used as a loading control.

4.3.5 The contribution of the BHRF1 locus to the resistance against ionomycin-induced apoptosis

In order to determine the contribution of the BHRF1 locus to EBV-mediated protection against ionomycin-induced apoptosis, the response of BL31 cells newly established using recombinant BHLOC KO and revertant viruses to ionomycin was

examined. Cells exposed to either 1μg/ml ionomycin or DMSO (vehicle control) for 24 hours. Cell viability was assessed using the CellTiter-Glo assay previously described in Section 3.1.1. Cell survival was expressed as a percentage of vehicle-treated controls. The experiment was conducted in triplicate and the histogram shows the mean and standard deviation of the three readings. The histogram shown in Figure 4.21A is representative of the outcome of two repeated experiments. Cells were also harvested at 24 hour intervals for protein extraction and Western blotting analysis.

Figure 4.21A shows that only approximately 25% of EBV-negative BL31 cells remain viable after 24 hours of exposure to ionomycin. As expected, more than 80% of BL31 cells infected with the WT and BHLOC rev EBVs remain viable after 24 hours. Interestingly, infection of BL31 cells with the BHLOC KO virus only provided partial protection against ionomycin-induced apoptosis with approximately 50% of the cell population remaining viable after 24 hours. Western blotting for PARP shows that a significant amount of cleaved PARP is detectable after 24 hours in EBV-negative BL31 cells (Figure 4.21B). Cleavage of full-length PARP appears to be almost complete in BL31 cells after 48 hours of exposure to ionomycin. Very little or no cleaved PARP was detectable in BL31 cells infected with the WT and BHLOC rev EBVs even after 48 hours of exposure to ionomycin. Cleaved PARP was detectable in BL31 cells infected with the BHLOC KO virus at both 24hr and 48hr time points. However, the amount of cleaved PARP detected in BL31 BHLOC KO cell lines was less than that observed in ionomycin-treated EBV-negative BL31 cells.

These data suggest that the BHRF1 locus is involved in the EBV-mediated protection of BL cells against ionomycin-induced apoptosis. However, since the loss of the BHRF1 locus only resulted in a partial loss of protection, it is likely that other EBV gene product(s) are also involved in the resistance to ionomycin.

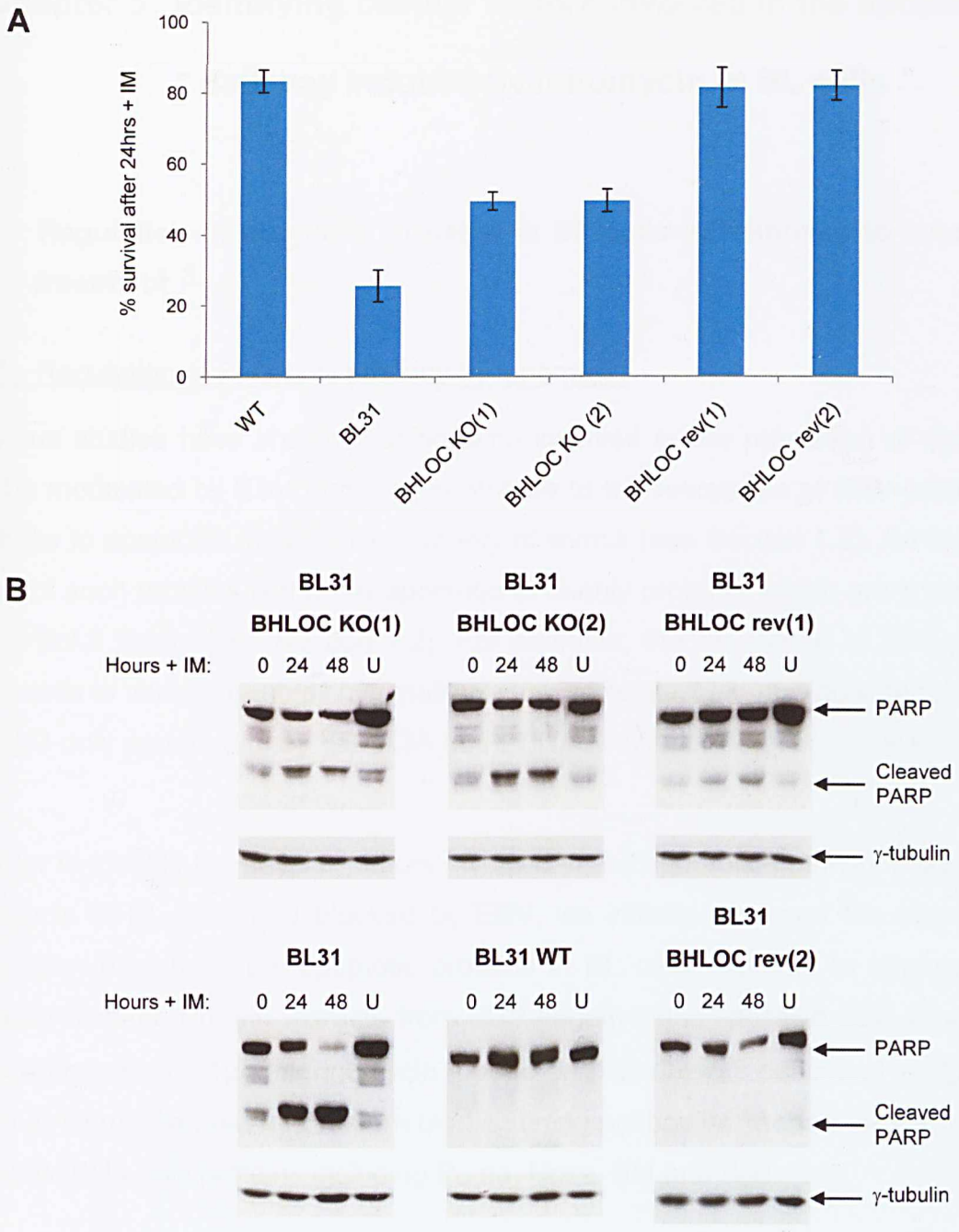


Figure 4-21 BL31 cells established using BHRF1 locus-knockout recombinant EBVs are only partially resistant to ionomycin treatment. EBV-negative BL31 and its EBV-positive converts established using recombinant wild-type (WT), BHRF1 locus-knockout (BHLOC KO) and –revertant (BHLOC rev) EBVs were treated with 1µg/ml ionomycin or DMSO (vehicle control) for up to 48 hours. **(A)** Cell survival was determined after 24 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at 24 hour intervals. Protein extracts from treated cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. γ-tubulin was used as a loading control. IM=ionomycin

Chapter 5 Identifying cellular factors involved in the apoptotic pathway induced by ionomycin in BL cells

5.1 Regulation of apoptotic proteins in BL cells in response to ionomycin treatment

5.1.1 Regulation of BH3-only proteins by ionomycin

Previous studies have shown that proteins involved in the regulation of apoptosis can be modulated by EBV and may contribute to the resistance of EBV-positive BL cell lines to apoptosis induced by a variety of stimuli (see Section 1.3). An important group of such proteins is the pro-apoptotic BH3-only proteins, which are a subgroup of the Bcl-2 family (see Section 1.2). For example, the resistance of EBV-positive BL31 cells to various genotoxic stimuli strongly correlates with the downregulation of the BH3-only protein Bim by EBNA3A and EBNA3C (Anderton *et al.*, 2008).

In order to identify the cellular factors involved in the apoptotic pathway triggered by ionomycin in BL cells and blocked by EBV, we initially surveyed the changes in expression of various pro-apoptotic proteins in BL cells exposed to ionomycin by Western blotting. Protein extracts from EBV-negative and -positive cells treated for up to 48 hours with 1μg/ml ionomycin (based on experiments described in Chapters 3 and 4) were analysed by Western blotting using antibodies specific for various pro-apoptotic BH3-only proteins including Puma, Noxa, Bid and Bad.

5.1.1.1 Induction of Noxa by ionomycin is inhibited by EBV

The gene encoding *Noxa* is a known transcriptional target of the tumour suppressor p53 and its expression has been shown to be upregulated during p53-mediated cell death (Oda *et al.*, 2000a). Western blot analysis revealed a steady increase of Noxa protein expression in EBV-negative BL41 cells in response to ionomycin challenge (Figure 5.1A). In contrast, Noxa protein expression was unaffected in BL41/B95.8 and BL41/P3HR1 cells which are resistant to ionomycin. Interestingly, an increase in

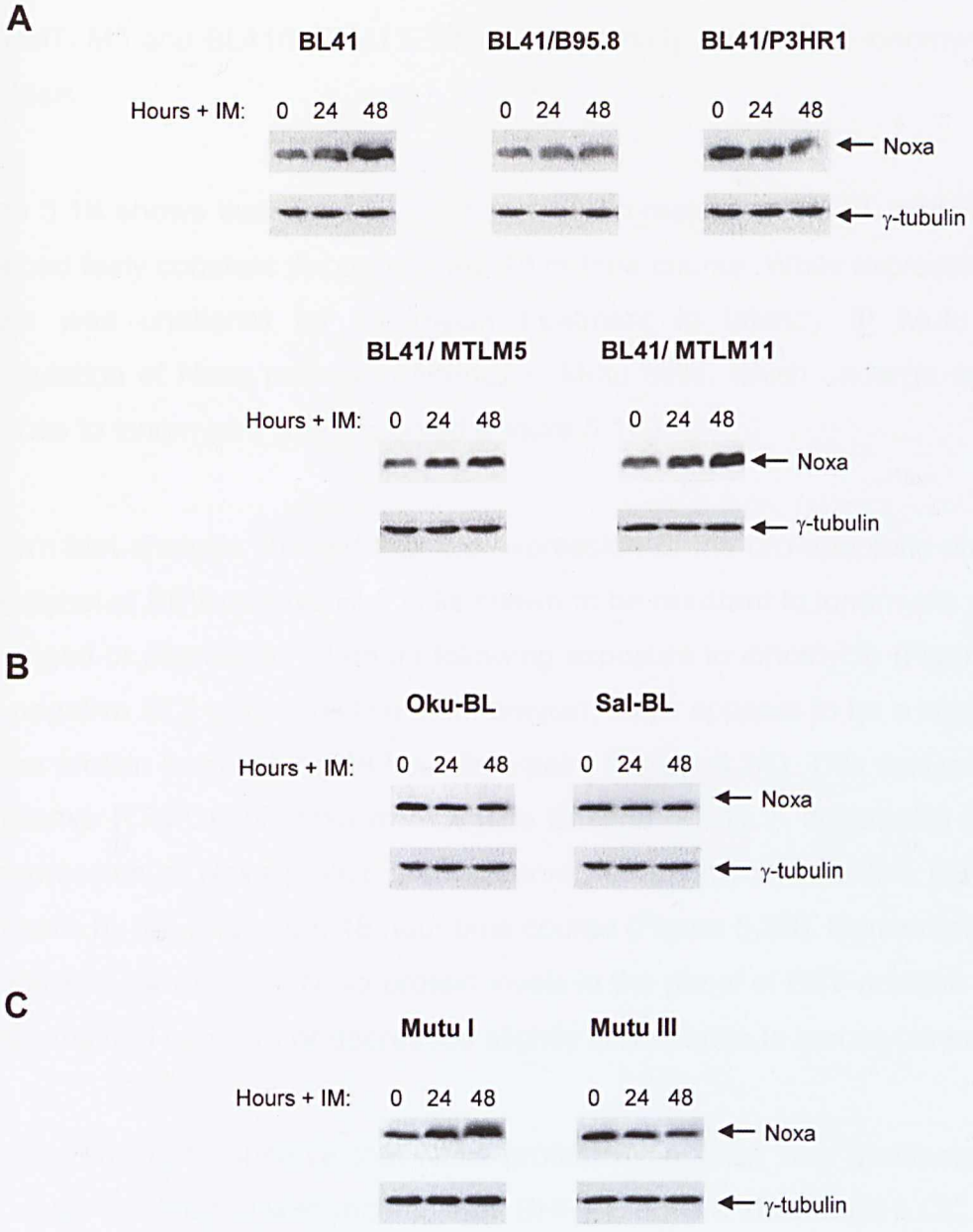


Figure 5-1 Ionomycin consistently induces the pro-apoptotic protein Noxa in EBV-negative and latency I BL cells but not in EBV-positive cells resistant to ionomycin-induced apoptosis. Protein extracts from **(A)** EBV-negative BL41 and its EBV-positive converts, BL41/B95.8 and BL41/P3HR1, **(B)** two recently isolated EBV-positive P3HR1-like BL-derived cell lines, Oku-BL and Sal-BL, and **(C)** latency I and latency III Mutu cells exposed to 1μg/ml ionomycin for up to 48 hours were analysed by Western blotting using antibodies directed against Noxa. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

Noxa protein was observed in two BL41 cell lines constitutively expressing LMP1, BL41/MTLM5 and BL41/MTLM11, which are partially resistant to ionomycin-induced apoptosis.

Figure 5.1B shows that Noxa levels in ionomycin-resistant Oku-BL and Sal-BL cells remained fairly constant throughout the 48 hr time course. While expression of Noxa protein was unaltered by ionomycin treatment in latency III Mutu cells, an accumulation of Noxa protein in latency I Mutu cells, which undergo apoptosis in response to ionomycin, was observed (Figure 5.1C).

Western blot analysis showed that the expression of the pro-apoptotic protein Noxa in the panel of EBV-positive BL2 cells shown to be resistant to ionomycin were either unchanged or decreased 48 hours following exposure to ionomycin (Figure 5.2A). In EBV-negative BL2 cells sensitive to ionomycin, there appears to be a slight increase in Noxa protein levels at the 48 hour time point (Figure 5.2A). This was confirmed by quantitative RT-PCR of Noxa mRNA (see Section 5.3.1). A substantial increase in the expression of Noxa protein was observed in ionomycin-sensitive EBV-negative BL31 cells by the end of the 48 hour time course (Figure 5.2B). Conversely, Western blot analysis showed that Noxa protein levels in the panel of EBV-positive BL31 cells either remained constant or decreased slightly in response to ionomycin treatment.

It was surprising to observe that Noxa protein expression was unaffected in BL31 cells newly established with recombinant BHRF1 locus-knockout (BHLOC KO) EBVs which are partially sensitive to ionomycin treatment (Figure 5.3).

These data suggest that Noxa is consistently upregulated in EBV-negative and latency I cells sensitive to ionomycin treatment. This induction of Noxa is independent of p53 and can be inhibited by latent EBV encoded factor(s). Two observations made suggest that there are other pro-apoptotic factors involved in mediating apoptosis induced by ionomycin: firstly, LMP1 confers partial resistance against ionomycin but does not block Noxa induction and secondly, loss of the BHRF1 locus partially removes protection against ionomycin but does not relieve EBV-mediated inhibition of Noxa induction.

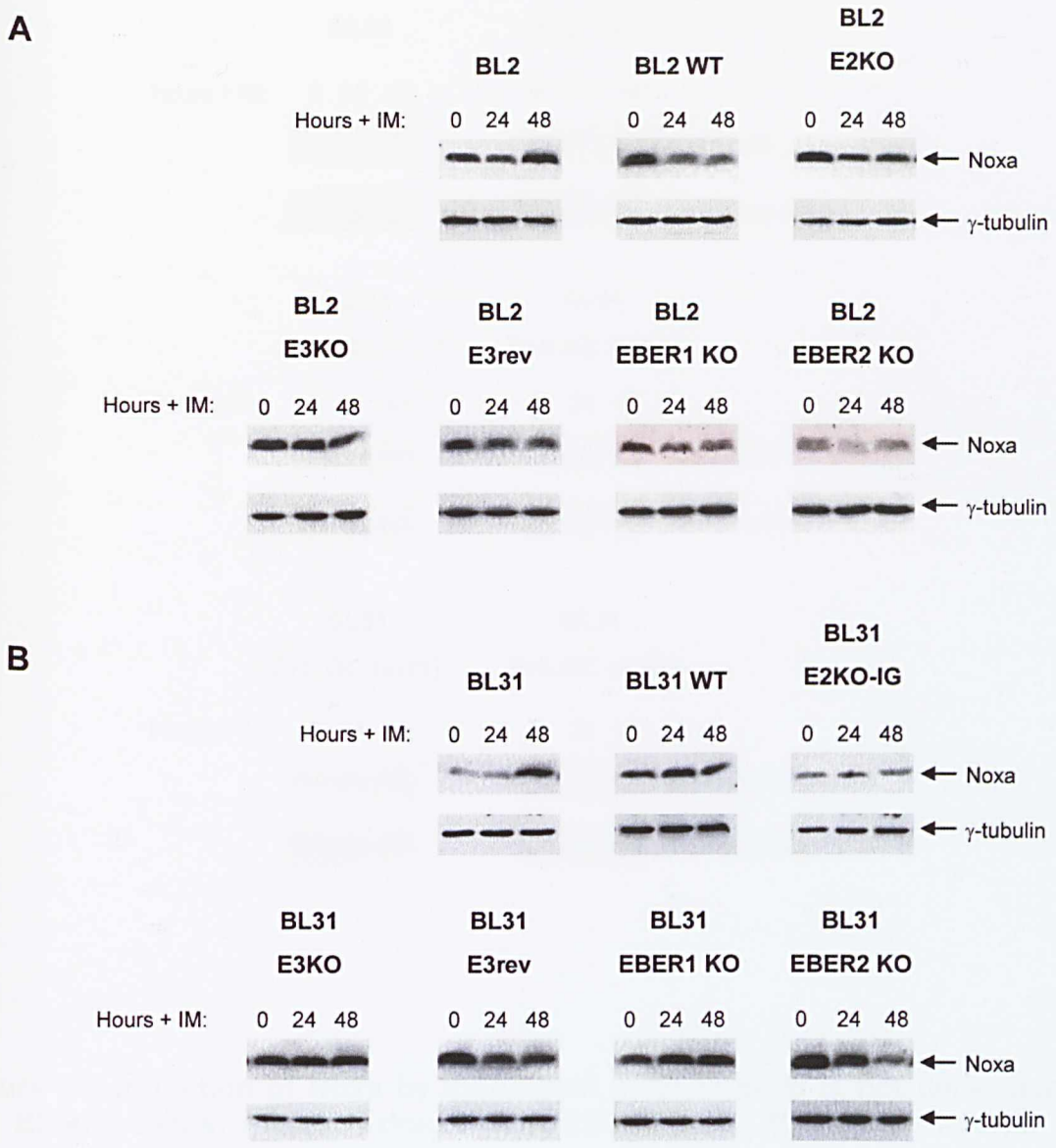


Figure 5-2 Ionomycin consistently induces the pro-apoptotic protein Noxa in EBV-negative BL2 and BL31 cells but not in EBV-positive cells resistant to ionomycin-induced apoptosis. Protein extracts from EBV-negative **(A)** BL2 and **(B)** BL31 and several of their EBV-positive converts recently infected with recombinant EBNA2-knockout (E2KO), EBNA3 locus-knockout (E3KO) and revertant (E3rev), EBER1-knockout (EBER1 KO), EBER2-knockout (EBER2 KO) and wild-type (WT) EBVs exposed to 1µg/ml ionomycin for up to 48 hours were analysed by Western blotting using antibodies directed against Noxa. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

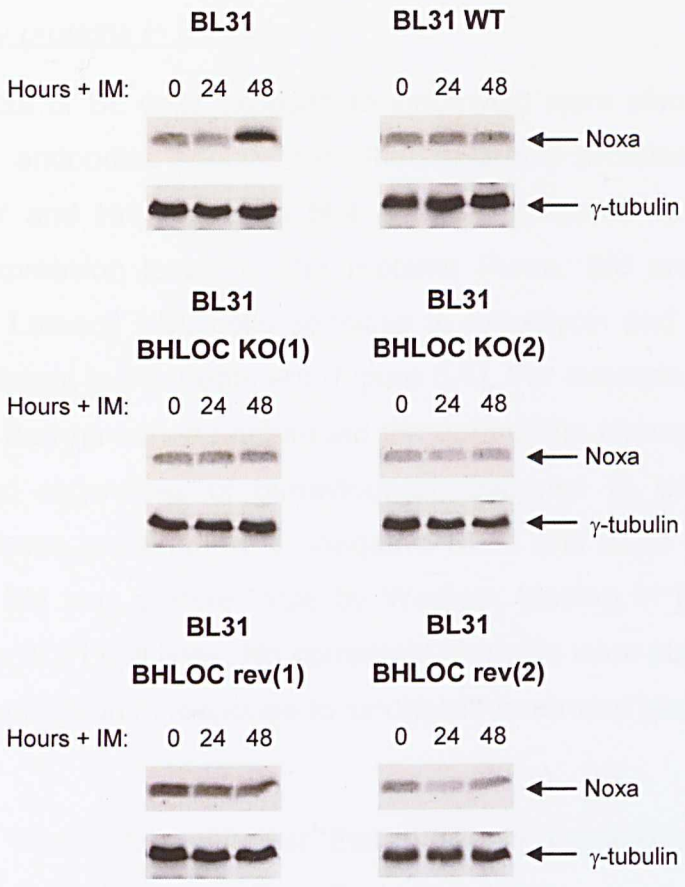


Figure 5-3 Induction of Noxa by ionomycin in BL31 cells is not dependent on the BHRF1 locus. Protein extracts from EBV-negative BL31, its wild-type (WT) recombinant EBV convert and BL31 established with recombinant BHRF1 locus-knockout (BHLOC KO) and -revertant EBV (BHLOC rev) exposed to 1µg/ml ionomycin for up to 48 hours were analysed by Western blotting using antibodies directed against Noxa. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

5.1.1.2 *Ionomycin does not consistently influence the expression of other BH3-only proteins in BL cells*

Protein extracts of BL cells exposed to ionomycin were also analysed by Western blotting using antibodies specific for other BH3-only proteins such as Bad, Puma, Bid, Bik, Bmf and Hrk. Western blot analysis revealed no consistent pattern of change in expression levels of the proteins Puma, Bid and Bad between EBV-negative and Latency I BL cells sensitive to ionomycin and EBV-positive BL cells which are resistant to the treatment (Figure 5.4). For example, the expression levels of Puma and Bad remained unchanged throughout the course of treatment in all BL cells surveyed regardless of behaviour in response to ionomycin except for a decrease of these proteins in EBV-negative BL41 and BL31 cells at the end of the time course. Bid was undetectable by Western blotting in both EBV-positive and EBV–negative BL31 cell lines. No consistent changes were observed in Bik, Bmf and Hrk protein expression in response to ionomycin treatment (data not shown).

In summary, these data suggest that the only pro-apoptotic BH3-only protein consistently induced by ionomycin in BL cells is Noxa.

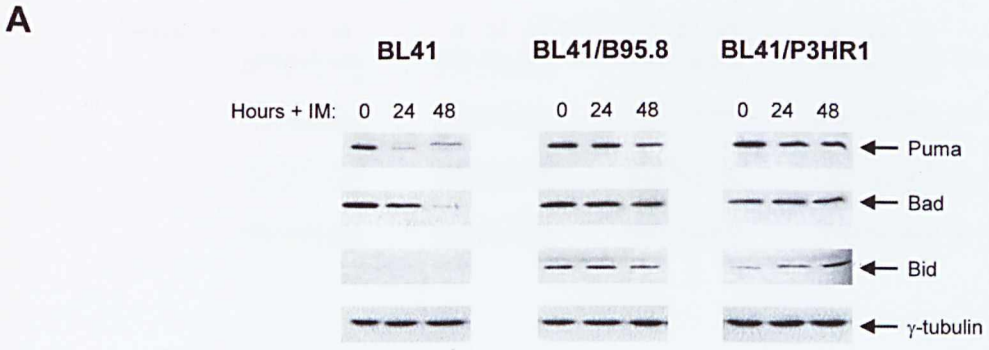


Figure 5-4 Changes in expression levels of the BH3-only proteins Puma, Bid and Bad do not correlate to the sensitivity of BL cells to ionomycin-induced apoptosis. (continued overleaf)

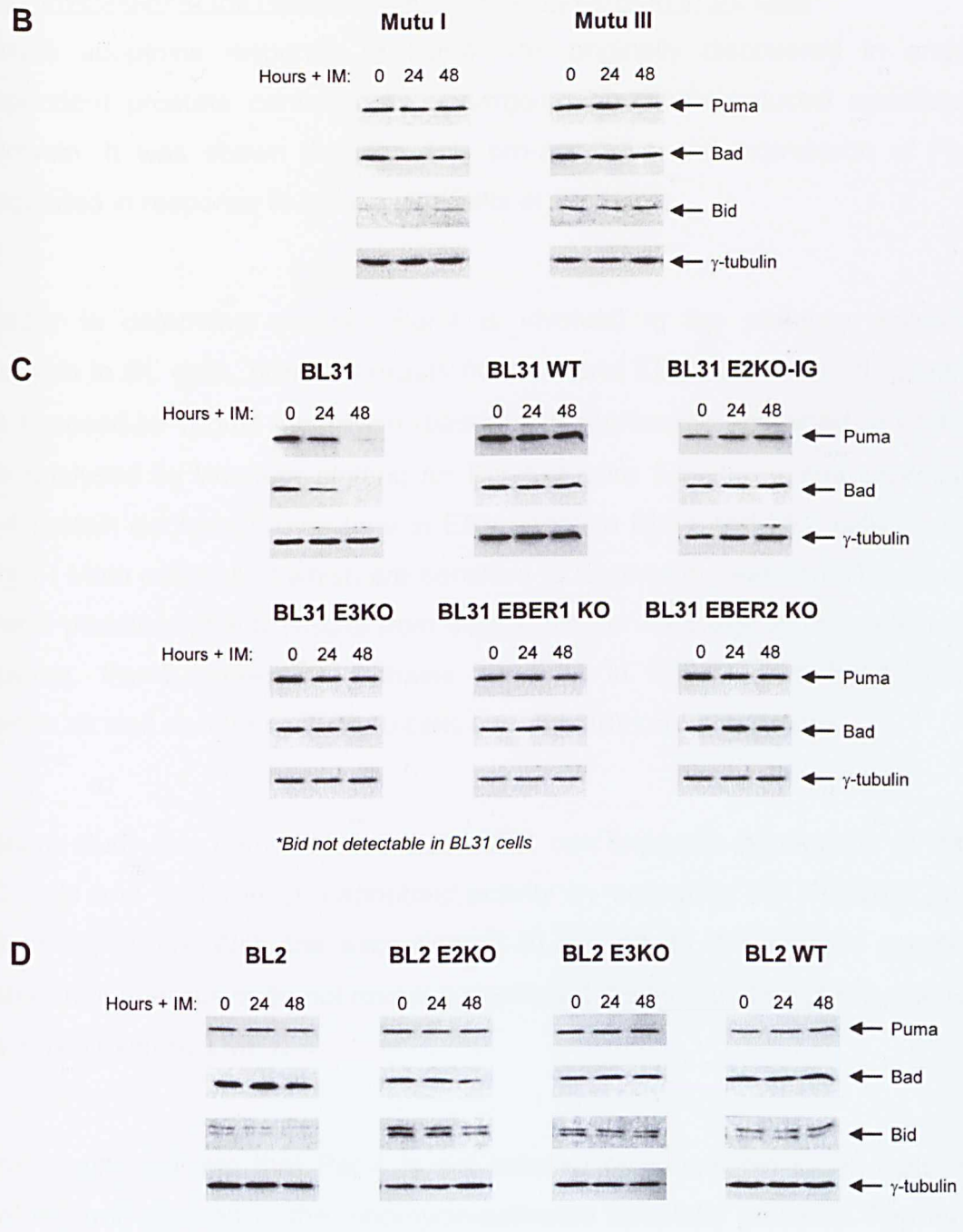


Figure 5.4 Changes in expression levels of the BH3-only proteins Puma, Bid and Bad do not correlate to the sensitivity of BL cells to ionomycin-induced apoptosis. Protein extracts from **(A)** EBV-negative BL41 and its EBV-positive converts BL41/B95.8 and BL41/P3HR1, **(B)** latency I and latency III Mutu, **(C)** BL31 and **(D)** BL2 and several of their EBV-positive converts recently infected with recombinant EBNA2-knockout (E2KO), EBNA3 locus-knockout (E3KO) and revertant (E3rev), EBER1-knockout (EBER1 KO), EBER2-knockout (EBER2 KO) and wild-type (WT) EBVs exposed to 1µg/ml ionomycin for up to 48 hours were analysed by Western blotting using antibodies directed against Puma, Bid and Bad. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

5.1.2 Investigating the regulation of Par-4 by ionomycin in BL cells

Prostate apoptosis response or Par-4 was originally discovered in androgen-independent prostate cancer cells undergoing apoptosis induced specifically by ionomycin. It was shown that Par-4 is pro-apoptotic and expression of Par-4 is upregulated in response to ionomycin (Sells *et al.*, 1994).

In order to determine whether Par-4 is involved in the pathway activated by ionomycin in BL cells, protein extracts from several EBV-negative and -positive BL cells exposed to 1μg/ml ionomycin (based on experiments described in Chapter 3) were analysed by Western blotting for Par-4. Figure 5.5 shows that expression of Par-4 protein decreases over time in EBV-negative BL31 and BL2 cells as well as latency I Mutu cells, all of which are sensitive to ionomycin treatment. This decrease of Par-4 protein probably results from degradation or cleavage as the cells undergo apoptosis. Par-4 expression remains constant in WT-infected BL31 and BL2 converts as well as latency III Mutu cells over the 48 hour time course.

A recent study has demonstrated that LMP1 can suppress expression of Par-4 in NPC cells and inhibit its pro-apoptotic activity by activating the PI3K/Akt pathway (Lee *et al.*, 2009). With the exception of BL31 and its WT-infected counterpart, Western blot analysis does not reveal a significant downregulation of Par-4 in BL cell lines which express LMP1.

These results indicate that Par-4 is not induced by ionomycin in BL cells and is therefore, not involved in the ionomycin-activated apoptotic pathway. Furthermore, there is no evidence of downregulation of Par-4 in BL cells which express LMP1.

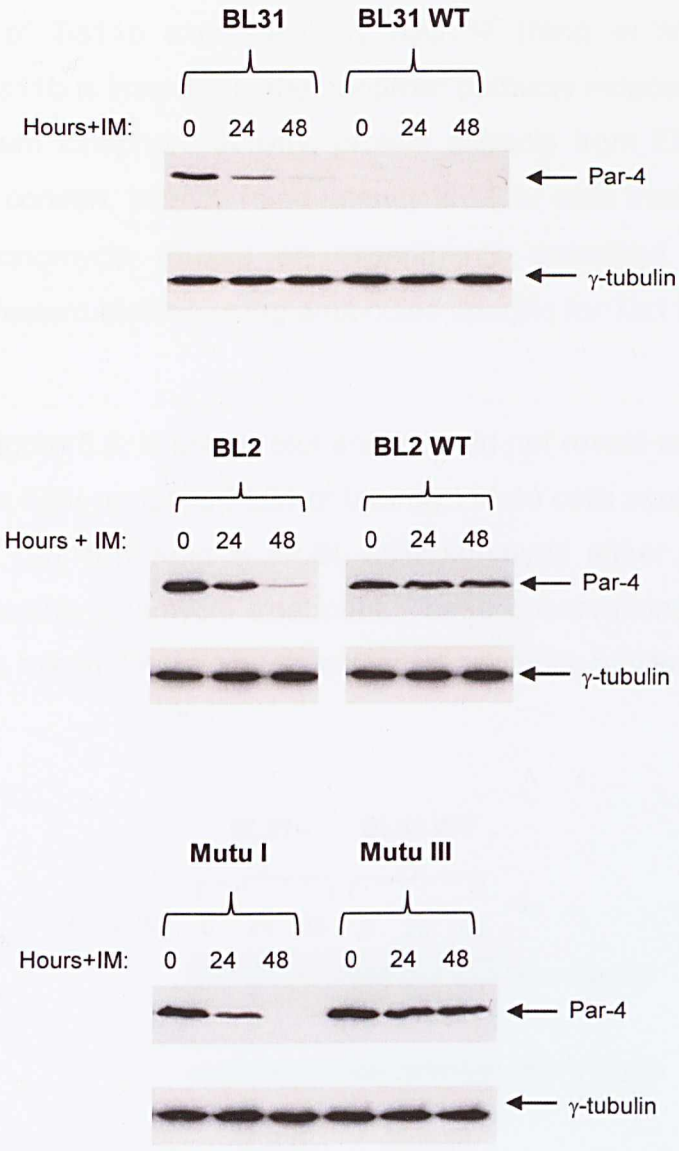


Figure 5-5 Expression of Par-4 is not induced in BL cells sensitive to ionomycin treatment. BL31 and BL2 cells, together with their wild-type (WT)-infected converts, latency I and latency III Mutu cells were treated with 1μg/ml ionomycin for up to 48 hours. Protein extracts were analysed by Western blotting using antibodies specific for Par-4. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

5.1.3 Tis11b/Berg36

Tis11b is a zinc finger containing early response gene reported to be required for apoptosis induced by the calcium ionophore A23187 in Ramos BL cells. It was

shown that Ramos cells can be prevented from undergoing apoptosis by blocking the induction of Tis11b expression by A23187 (Ning *et al.*, 1996). In order to determine if Tis11b is involved in the apoptotic pathway induced by ionomycin, which also has calcium ionophore activity, protein extracts from EBV-negative BL31, its wild-type EBV convert, latency I and latency III Mutu cells treated for up to 48 hours with 1μg/ml ionomycin (based on experiments described in Chapter 3) were analysed by Western blotting using antibodies specific for Tis11b.

As shown in Figure 5.6, Western blot analysis did not reveal any induction of Tis11b by ionomycin in EBV-negative BL31 or latency I Mutu cells sensitive to the treatment. Expression of Tis11b protein in all BL cells surveyed either remained constant or decreased following ionomycin treatment. These observations suggest that Tis11b does not play a role in the ionomycin-activated apoptotic pathway in BL cells.



Figure 5-6 Expression of Tis11b is not induced in BL cells sensitive to ionomycin treatment. BL31, its wild-type (WT) EBV convert, latency I and latency III Mutu cells were treated with 1μg/ml ionomycin for up to 48 hours. Protein extracts were analysed by Western blotting using antibodies specific for Tis11b. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

5.1.4 Bip/GRP78

Bip or GRP78 is an endoplasmic reticulum chaperone which is induced following ER stress and is a key regulator of the unfolded protein response (UPR). A previous study has shown that expression of Bip is induced following exposure of androgen-dependent prostate cancer cells to ionomycin (Miyake *et al.*, 2000). It was also demonstrated that this induction of Bip prevented cells from undergoing ionomycin-induced apoptosis.

In order to investigate if Bip is involved in the ionomycin-activated apoptotic pathway, protein extracts from EBV-negative BL31 and BL2, their wild-type EBV converts, latency I and latency III Mutu cells challenged with 1μg/ml ionomycin for up to 48 hours (based on experiments described in Chapter 3) were analysed by Western blotting using antibodies specific for Bip.

Western blot analysis revealed a substantial increase in the expression of Bip protein after 48 hours of exposure to ionomycin in EBV-negative BL31 and BL2 cells as well as latency I Mutu cells, all of which are sensitive to ionomycin (Figure 5.7). In contrast, Bip protein expression was largely unaffected in EBV-positive BL31 and BL2 cells. A slight increase was observed in latency III Mutu cells.

These observations suggest that expression of Bip is induced by ionomycin in BL cells sensitive to the treatment. This induction is not observed in EBV-positive cells resistant to ionomycin. This appears to be in contrast to the role of Bip as previously reported (Miyake *et al.*, 2000).

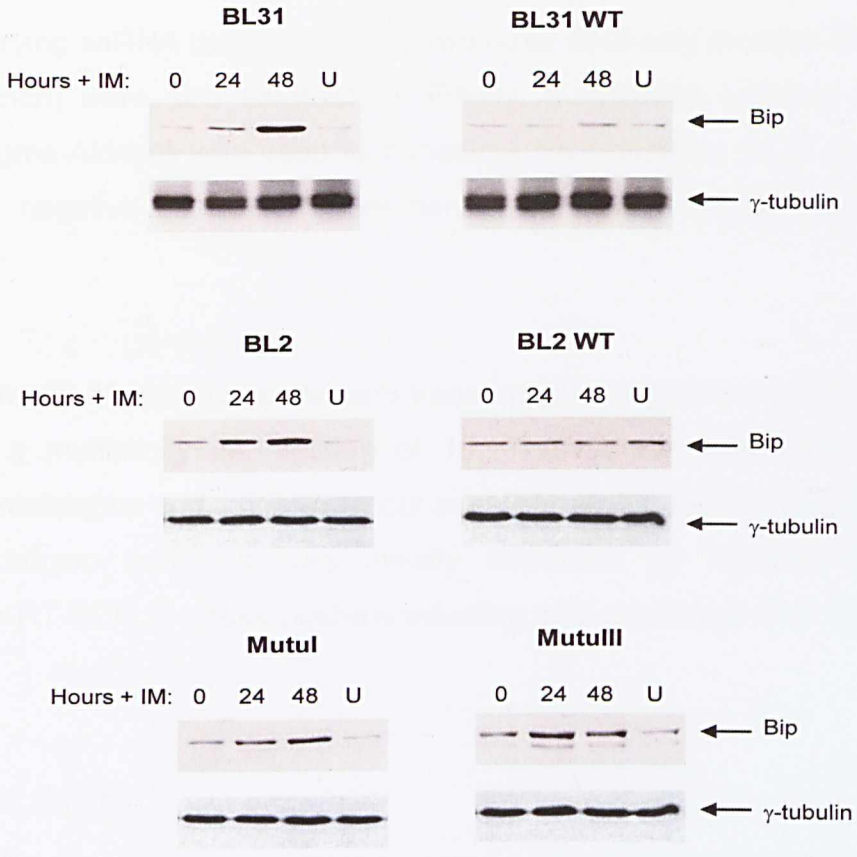


Figure 5-7 EBV inhibits induction of Bip by ionomycin in BL cells. EBV-negative BL31 and BL2, their wild-type (WT) EBV converts, latency I and latency III Mutu cells were treated with 1μg/ml ionomycin for up to 48 hours. Protein extracts were analysed by Western blotting using antibodies specific for Bip. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

5.2 Investigating the requirement for Noxa in ionomycin-induced apoptosis

Results from Section 5.1 demonstrate that the pro-apoptotic BH3-only protein Noxa is consistently upregulated by ionomycin in BL cells sensitive to the treatment. However, Noxa expression appears to be unaffected in EBV-positive BL cells resistant to ionomycin. It was therefore important to determine if Noxa directly mediates ionomycin-induced apoptosis in BL cells and if inhibition of Noxa induction was sufficient to provide protection against ionomycin. In order to study this, a panel of lentiviral vectors containing shRNA targeted against Noxa (Sigma-Aldrich) were used to transduce EBV-negative BL31 and BL2 cells (see Section 2.7). The response of transduced cells, in which Noxa expression should be knocked down, to

ionomycin can then be assessed. BL31 and BL2 cells transduced with lentiviral vectors carrying shRNA targeted against two other BH3-only proteins Bim and Puma (Sigma-Aldrich) were also established. Finally, a lentivirus carrying non-targeting shRNA (Sigma-Aldrich) was used to transduce EBV-negative BL31 and BL2 to be used as a negative control in experiments. All shRNA sequences are listed in Section 2.7.

EBV-negative BL31 and BL2 cells were transduced with lentiviral particles coding for shRNA at a multiplicity of infection of 10. Transduced cells were selected for puromycin resistance and left to grow out over 2-3 weeks. shRNA-mediated levels of gene knockdown achieved was initially assessed by Western blotting and quantitative RT-PCR 3 weeks post-transduction and monitored over longer periods in culture.

5.2.1 Noxa shRNA

Lentiviral particles expressing shRNA targeted against Noxa were used to transduce EBV-negative BL31 and BL2 cells. Cells were harvested for protein and RNA extraction at 3 weeks post-transduction. Protein extracts were separated by SDS-PAGE and analysed by Western blotting using antibodies specific for Noxa. RNA extracts were analysed by quantitative RT-PCR to determine levels of Noxa mRNA. Noxa mRNA levels were normalised to aminolevulinate, delta-, synthase 1 (ALAS1) mRNA levels. Expression of Noxa protein and mRNA in transduced cells were compared to those of parental EBV-negative BL31 and BL2 cells.

Various degrees of Noxa 'knockdown' were achieved by the five different shRNAs in both BL31 and BL2 cells as observed by Western blotting and quantitative RT-PCR (Figure 5.8). In both BL31 and BL2, the greatest level of reduction was achieved with shNoxa2 (approximately 60% at mRNA level) and shNoxa3 (approximately 50% at mRNA level). Levels of knockdown achieved with the remaining three shRNAs differed at the level of mRNA between the two BL cells lines. Western blotting for Noxa revealed substantial downregulation of Noxa protein by shRNA in both BL31 and BL2 with shNoxa2 achieving the greatest level of knockdown (Figure 5.8).

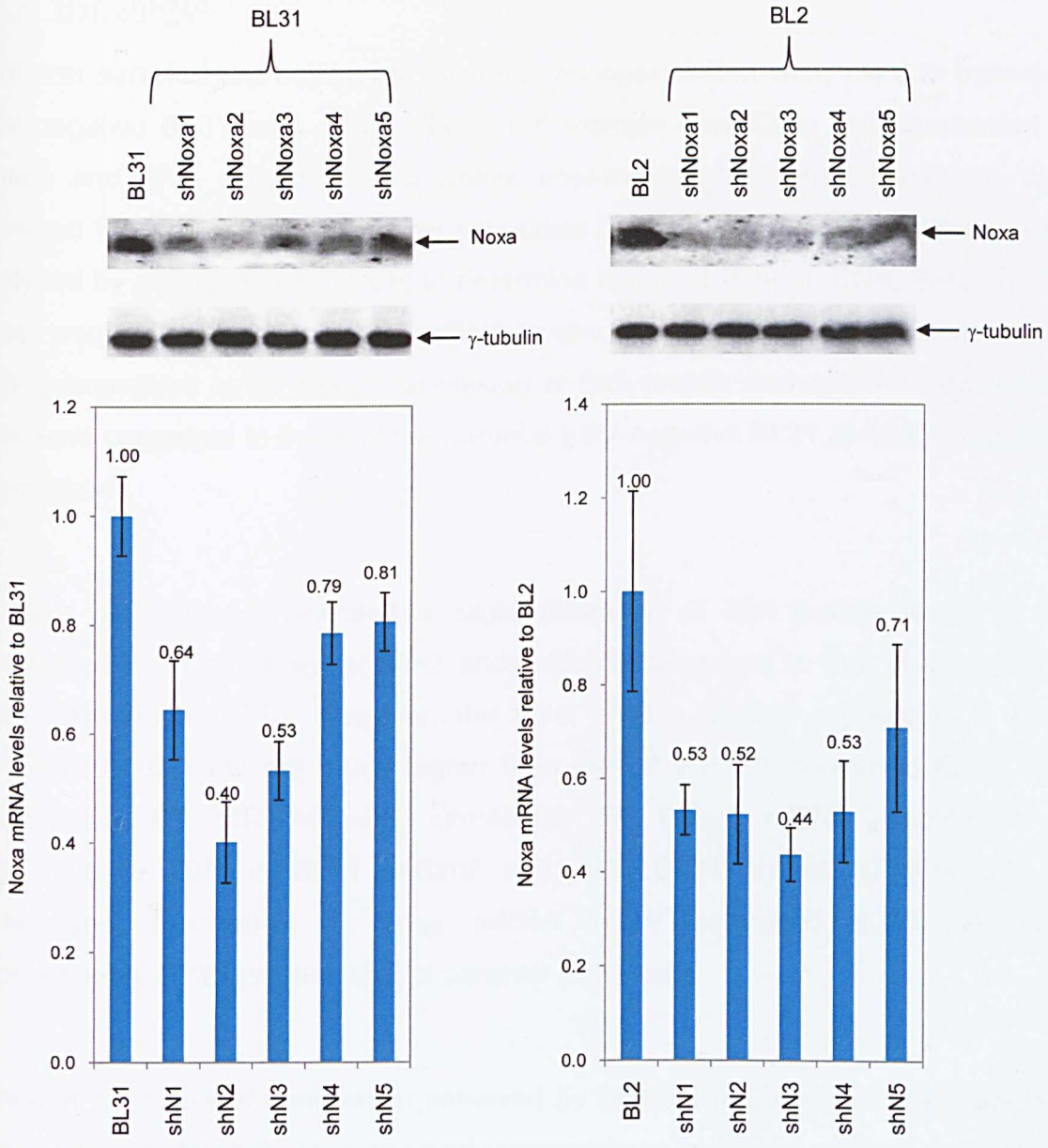


Figure 5-8 Knockdown of Noxa protein and mRNA in BL31 and BL2 cells by shRNA. Protein extracts from BL31 and BL2 cells transduced with lentiviral vectors carrying shRNA against Noxa were separated by SDS-PAGE and analysed by Western blotting using antibodies specific for Noxa. Throughout, γ-tubulin was used as a loading control. RNA was extracted from the same panel of BL31 and BL2 cells and analysed by quantitative RT-PCR to determine levels of Noxa mRNA relative to parental BL31 and BL2 cells. Noxa mRNA expression was normalised to ALAS1 mRNA levels.

5.2.2 Bim shRNA

Lentiviral particles expressing shRNA targeted against Bim were used to transduce EBV-negative BL31 cells. BL2 cells do not express Bim. Cells were harvested for protein and RNA extraction at 3 weeks post-transduction. Protein extracts were analysed by Western blotting using antibodies specific for Bim. RNA extracts were analysed by quantitative RT-PCR to determine levels of Bim_{EL} mRNA. Bim_{EL} mRNA levels were normalised to ALAS1 mRNA levels. Bim_{EL} is the most abundant isoform of Bim expressed in BL cells. Expression of Bim protein and mRNA in transduced cells were compared to those of the parental EBV-negative BL31 and its WT-infected counterpart.

Western blot analysis revealed a slight decrease of Bim protein levels in two transduced BL31 cell lines (shBim2 and shBim3) compared to that of the parental BL31 cells (Figure 5.9). However, the level of Bim protein expression in these transduced cells was still much higher than that of the WT converted BL31 cells. Quantitative RT-PCR showed expression of Bim_{EL} mRNA decreased by approximately 20% in BL31 shBim2 and 33% BL31 shBim3 (Figure 5.9). In comparison, expression of Bim_{EL} mRNA in WT-converted BL31 cells was approximately 85% less than that of parental BL31 cells.

Although the levels of knockdown achieved by Bim shRNA were not comparable to that of WT-converted BL31, it seemed reasonable to use BL31 shBim2 and shBim3 cells in subsequent experiments to rule out the involvement of Bim in the ionomycin-activated apoptotic pathway.

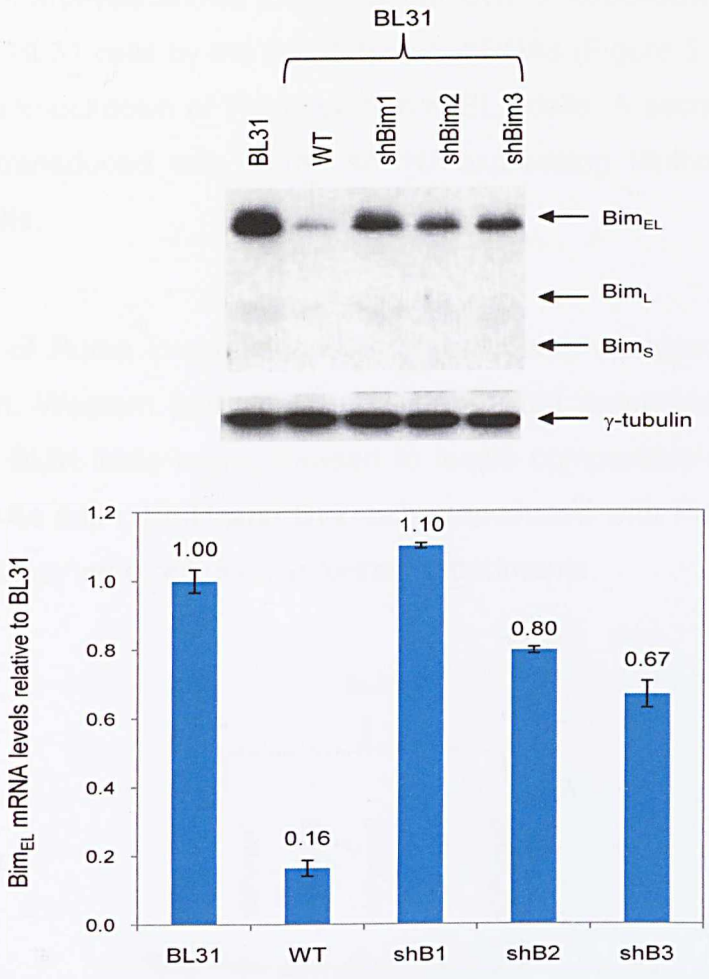


Figure 5-9 Knockdown of Bim protein and mRNA in BL31 cells by shRNA. Protein extracts from BL31 cells transduced with lentiviral vectors carrying shRNA against Bim, EBV-negative BL31 and wild-type (WT)-infected BL31 were separated by SDS-PAGE and analysed by Western blotting using antibodies specific for Bim. γ-tubulin was used as a loading control. RNA was extracted from the same panel of BL31 cells and analysed by quantitative RT-PCR to determine levels of Bim_{EL} mRNA expression relative to parental BL31 and WT-infected BL31 cells. Bim_{EL} mRNA expression was normalised to that of ALAS1 mRNA.

5.2.3 Puma shRNA

Lentiviral particles expressing shRNA targeted against Puma were used to transduce EBV-negative BL31 and BL2 cells. Cells were harvested for protein extraction at 3 weeks post-transduction. Protein extracts were analysed by Western blotting using antibodies specific for Puma. Expression of Puma protein in transduced cells was compared to those of parental EBV-negative BL31 and BL2 cells.

Western blot analysis shows that an equal level of knockdown of Puma protein was achieved in BL31 cells by the five different shRNAs (Figure 5.10). In contrast, we did not observe knockdown of Puma protein in BL2 cells. A second attempt to generate BL2 cells transduced with Puma shRNA-expressing lentiviral particles produced similar results.

Expression of Puma in transduced BL31 cells was analysed again 6 weeks post-transduction. Western blot analysis revealed that expression of Puma protein in transduced BL31 cells had increased to levels comparable to that of the parental BL31 cells. As such, BL31 and BL2 cells transduced with Puma shRNA-expressing lentiviral vectors were not used in further experiments.

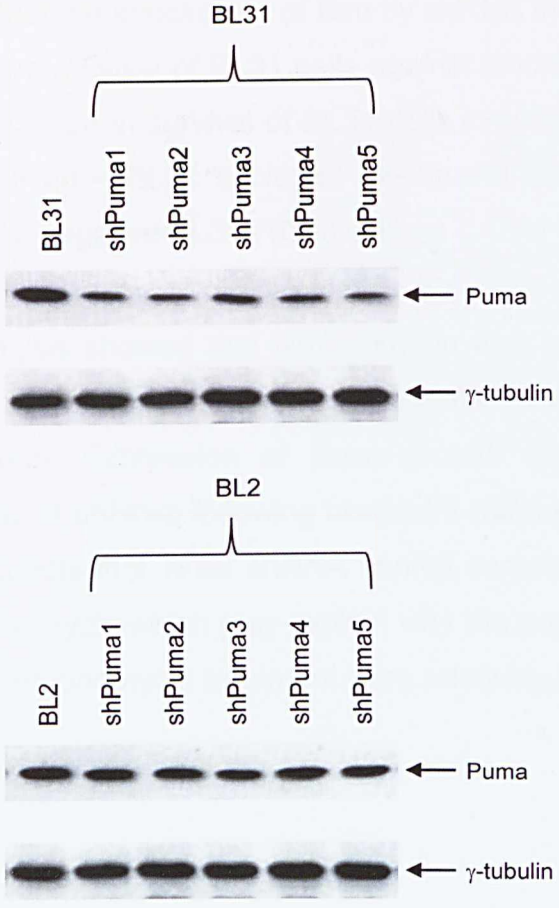


Figure 5-10 Knockdown of Puma protein in BL31 and BL2 cells by shRNA. Protein extracts from BL31 and BL2 cells transduced with lentiviral vectors carrying shRNA against Puma were analysed by Western blotting using antibodies specific for Puma. Throughout, γ-tubulin was used as a loading control.

5.2.4 Downregulation of Noxa expression by shRNA provides some protection for BL cells against ionomycin-induced apoptosis

The five BL31 shNoxa and two BL31 shBim cell lines described above were exposed to ionomycin and their response compared to that of EBV-negative and WT-converted BL31. Cells were exposed to either 1μg/ml ionomycin or DMSO (vehicle control) for up to 48 hours. Cell viability was measured after 24 hours using the CellTiter-Glo assay previously described in Section 3.1.1. Cell survival was expressed as a percentage of the vehicle-treated controls. The experiment was conducted in triplicate and the mean and standard deviation of three readings plotted as a histogram. Cells were harvested at 24 hour intervals for protein extraction and Western blot analysis.

Figure 5.11A shows that the knockdown of Bim by shRNA in BL31 shBim2 and BL31 shBim3 did not improve survival of BL31 cells against ionomycin-induced apoptosis. There was a slight increase in survival of BL31 cells in which the best level of Noxa knockdown was achieved – BL31 shNoxa2 (36%) and BL31 shNoxa3 (27%) cell lines compared to EBV-negative BL31 (10%) cells.

Western blotting analysis showed that Noxa protein was induced in EBV-negative BL31 (Fig 5.11B) and both BL31 shBim cell lines (data not shown) after 48 hours of exposure to ionomycin. Expression of Noxa protein was also found to have increased in all five BL31 shNoxa following treatment with ionomycin (Figure 5.11B). This observation suggests that Noxa shRNA cannot completely block the induction of Noxa protein by ionomycin which may explain why the population of BL31 shNoxa cells which survive after ionomycin treatment were relatively low.

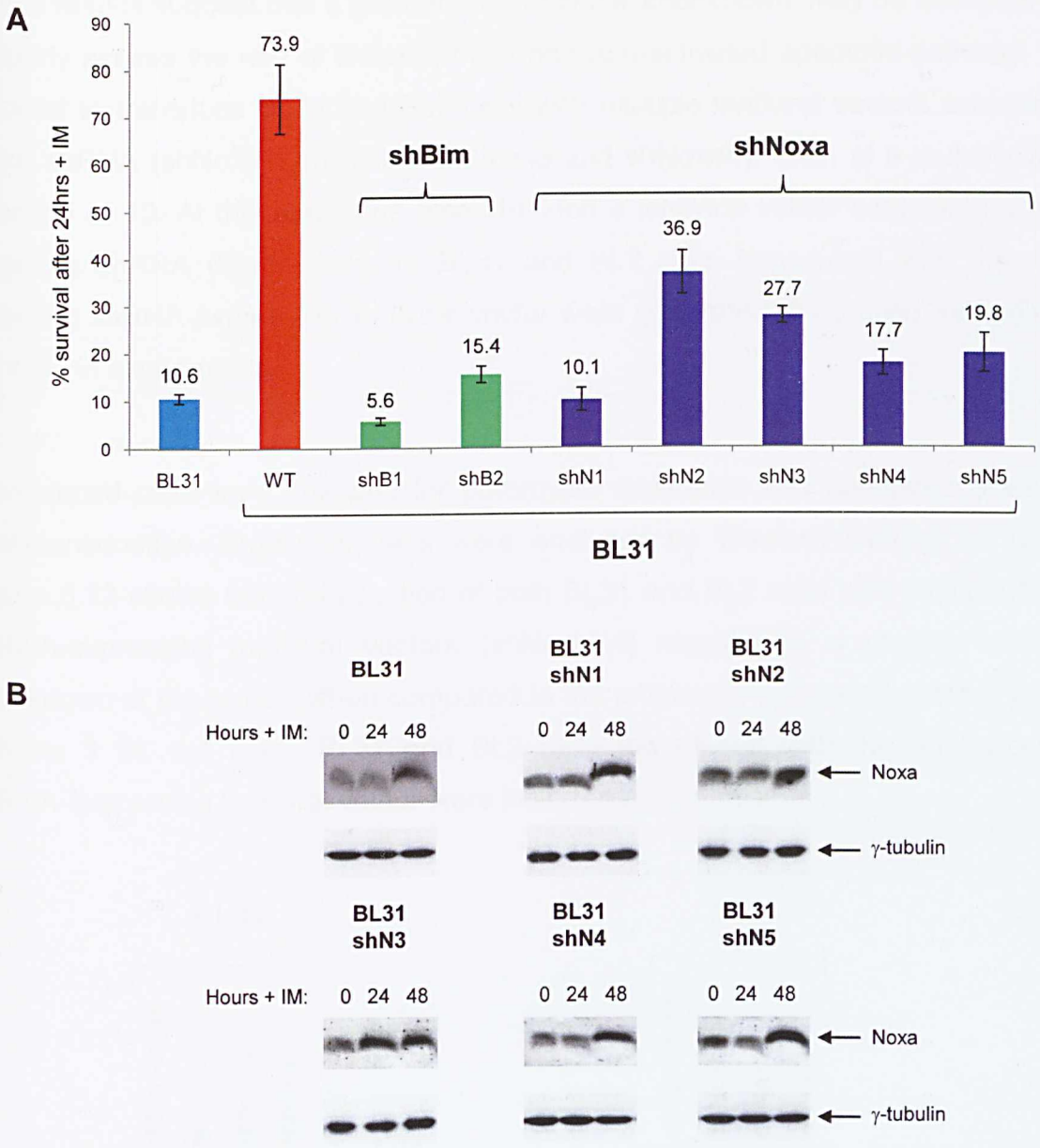


Figure 5-11 Effect of the knockdown of Noxa on ionomycin resistance. (A) BL31 cells generated by transduction with lentiviral vectors coding for shRNA directed against Bim and Noxa, parental BL31 and its wild-type (WT) EBV convert were exposed to 1μg/ml ionomycin or DMSO (vehicle control) for 24 hours. Cell survival was measured using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated controls. **(B)** EBV-negative BL31 and BL31 cells transduced with Noxa shRNA-expressing lentiviral vectors were exposed to 1μg/ml ionomycin for up to 48 hours. Protein extracts from cells were analysed by Western blotting using antibodies specific for Noxa. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

These results suggest that a greater level of Noxa 'knockdown' may be necessary to properly assess the role of Noxa in the ionomycin-activated apoptotic pathway. We decided to transduce BL31 and BL2 cells with multiple lentiviral vectors expressing Noxa shRNA (shNoxa1, shNoxa2, shNoxa3 and shNoxa4), each at a multiplicity of infection of 10. At this stage, we also obtained a lentiviral vector containing a non-targeting shRNA (Sigma-Aldrich). BL31 and BL2 cells transduced with the non-targeting shRNA-expressing lentiviral vector were generated to be used as negative controls in experiments.

Transduced cells were selected for puromycin resistance and harvested 3 weeks post-transduction. Protein extracts were analysed by Western blotting for Noxa. Figure 5.12 shows that transduction of both BL31 and BL2 cells with multiple Noxa shRNA-expressing lentiviral vectors (shNoxaX4) resulted in a greater level of knockdown of the protein when compared to the previously generated shNoxa 2 and shNoxa 3 BL cell lines. BL31 and BL2 cells transduced with the non-targeting shRNA-expressing lentiviral vector were included as controls.

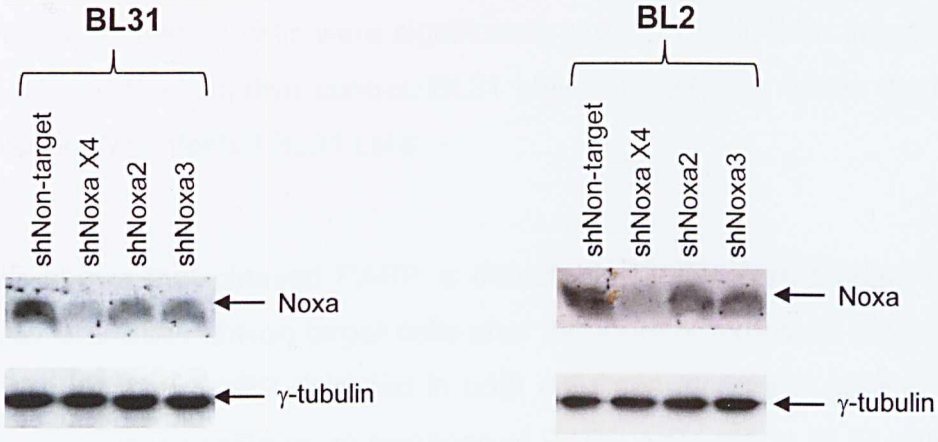


Figure 5-12 Transduction of BL cells with multiple lentiviral vectors containing shRNA directed against Noxa produces a greater level of knockdown of Noxa protein. Protein extracts from BL31 and BL2 cells transduced with single or multiple (shNoxaX4) lentiviral vectors carrying shRNA directed against Noxa were analysed by Western blotting using antibodies specific for Noxa. Protein extracts from BL31 and BL2 cells transduced using lentiviral particles carrying non-targeting shRNA (shNon-target) were included as a negative control. Throughout, γ-tubulin was used as a loading control.

BL31 and BL2 cells transduced with multiple lentiviral vectors expressing Puma and Bim (BL31 only) shRNA were also generated. However, this did not produce greater levels of knockdown of either Puma or Bim (data not shown) and the cell lines produced were not included in further experiments.

BL31 shNoxaX4 and BL31 shNon-target were exposed to ionomycin and their response compared to that of EBV-negative and WT-converted BL31. Cells were exposed to DMSO (vehicle control), 0.5μg/ml or 1μg/ml ionomycin for up to 48 hours. Cell viability was determined after 24 hours using the CellTiter-Glo assay described above. Cell survival was expressed as a percentage of the vehicle-treated controls and plotted as a histogram. Cells were harvested at 24 hour intervals for Western blotting analysis.

Figure 5.13A shows that approximately 20% of BL31 cells survive after 24 hours of exposure to 1μg/ml ionomycin. BL31 shNon-target cells are also sensitive to ionomycin treatment with survival at approximately 25%. As expected, a high proportion of WT-infected BL31 cells survive (85%) after exposure to ionomycin. Survival of BL31 shNoxaX4 cells were significantly greater (63%) than those of EBV-negative BL31 and the negative control, BL31 shNon-target, but not as high as the level observed in WT-infected BL31 cells.

Figure 5.13B shows that cleaved PARP is detectable by Western blotting in EBV-negative BL31 and BL31 shNon-target cells after 24 hours of exposure to ionomycin. The amount of cleaved PARP detected in both cell lines increases after 48 hours. Interestingly, there is very little or no evidence of PARP cleavage in BL31 shNoxaX4 cells at the 24 hour time point. Cleaved PARP is then detectable in BL31 shNoxaX4 cells after 48 hours but to a lesser extent than that seen in parental BL31 and BL31 shNon-target cells.

The experiment was repeated using EBV-negative BL2, its WT convert, BL2 shNon-target and BL2 shNoxaX4. When using the panel of BL2 cell lines, cells were

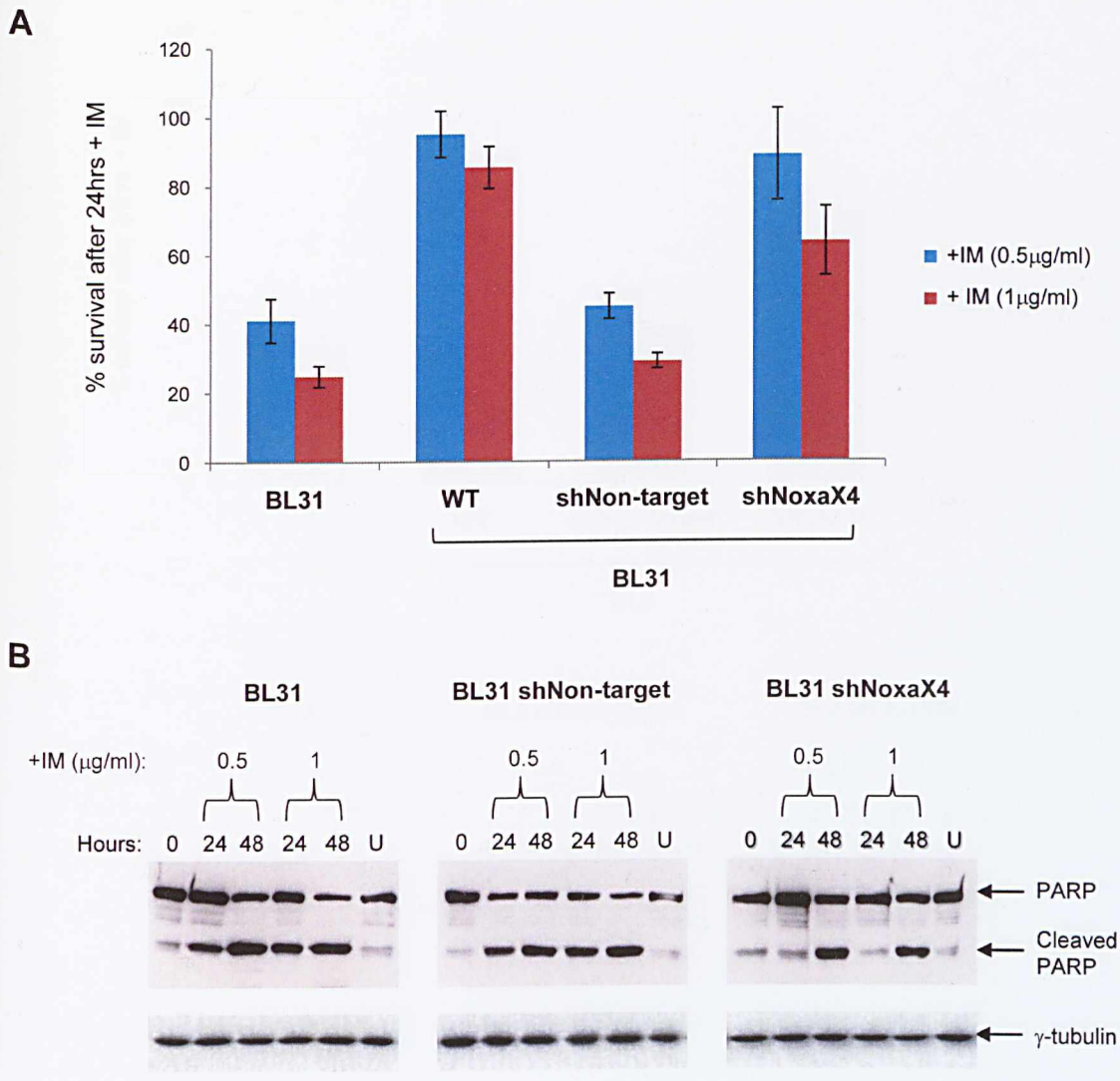


Figure 5-13 Knockdown of Noxa protein provides some protection against ionomycin-induced apoptosis in BL31 cells. (A) EBV-negative BL31, its wild-type (WT) convert and BL31 cells transduced with lentiviral particles carrying multiple Noxa shRNA (shNoxaX4) and non-targeting shRNA (shNon-target) were exposed to DMSO (vehicle control), 0.5 or 1μg/ml ionomycin for 24 hours. Cell survival was determined using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated controls. **(B)** The same panel of BL31 cells was treated with 0.5 or 1μg/ml ionomycin for up to 48 hours. Cells were harvested at 24 hour intervals. Protein extracts were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. γ-tubulin was used as a loading control. IM=ionomycin

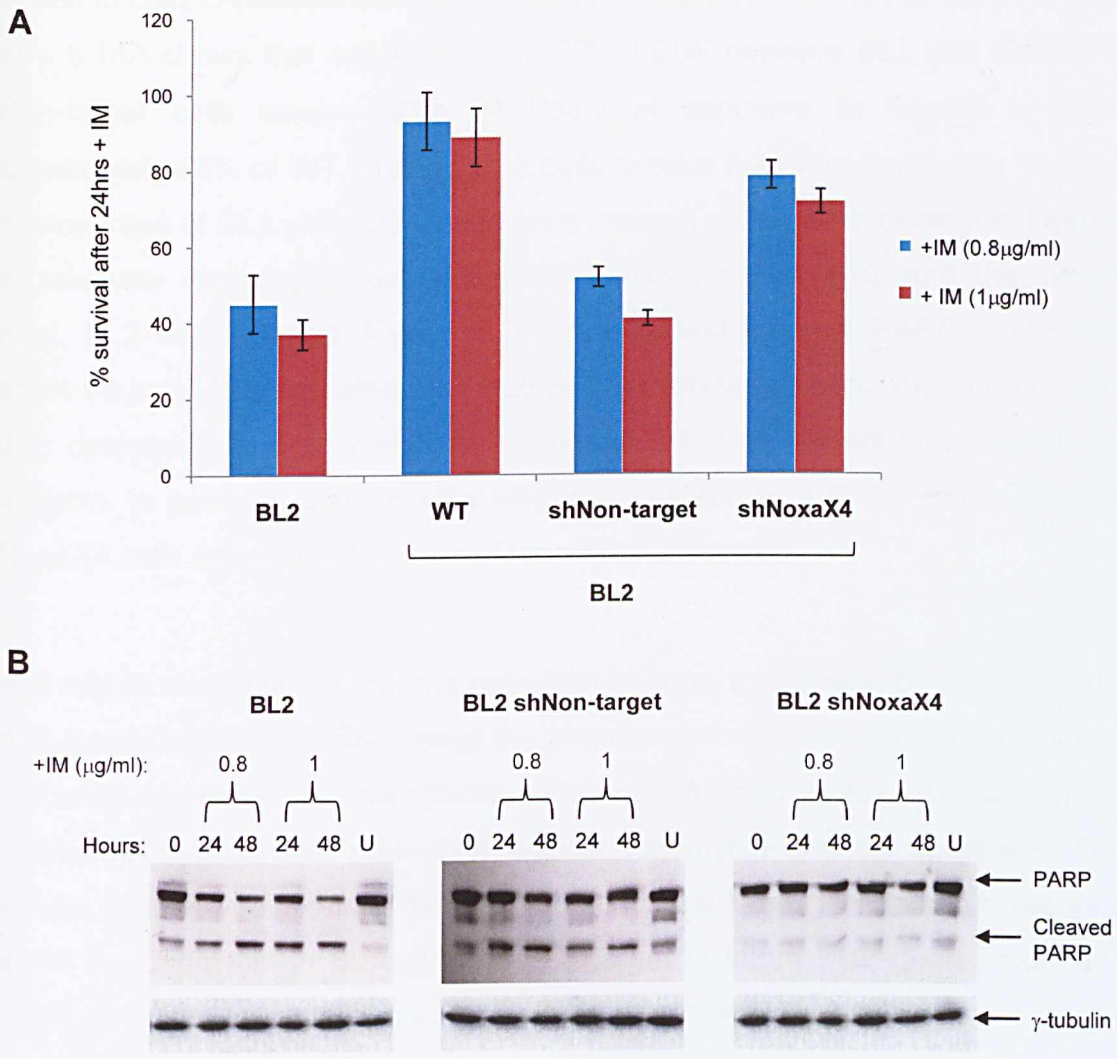


Figure 5-14 Knockdown of Noxa protein provides some protection against ionomycin-induced apoptosis in BL2 cells. (A) EBV-negative BL2, its wild-type (WT) convert and BL2 cells transduced with lentiviral particles carrying multiple Noxa shRNA (shNoxaX4) and non-targeting shRNA (shNon-target) were exposed to DMSO (vehicle control), 0.8 or 1μg/ml ionomycin for 24 hours. Cell survival was determined using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated controls. **(B)** The same panel of BL2 cells was treated with 0.8 or 1μg/ml ionomycin for up to 48 hours. Cells were harvested at 24 hour intervals. Protein extracts were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

exposed to DMSO (vehicle control), 0.8μg/ml or 1μg/ml ionomycin for up to 48 hours. Figure 5.14A shows that approximately 37% of EBV-negative BL2 and 40% of BL2 shNon-target cells survive after 24 hours of exposure to 1μg/ml ionomycin. Approximately 88% of WT infected BL2 cells survive following ionomycin treatment. The proportion of BL2 shNoxaX4 cells which remain viable after ionomycin treatment was relatively higher (approximately 70%) than parental BL2 and the negative control, BL2 shNon-target. Figure 5.14B shows that cleaved PARP is detectable after 24 hours in EBV-negative BL2 and BL2 shNon-target cells. Amount of cleaved PARP detectable in both cell lines increases following 48 hours of exposure to ionomycin. In contrast, there is very little or no evidence of PARP cleavage in BL2 shNoxaX4 cells even after 48 hours of ionomycin treatment.

These results demonstrate that the reduction of Noxa expression by shRNA in BL31 and BL2 cells significantly decreased the proportion of cells which undergo apoptosis in response to ionomycin. This indicates that the inhibition of Noxa expression alone can provide considerable protection against ionomycin-induced apoptosis. These data also suggest that Noxa is directly involved in mediating the apoptotic responses induced by ionomycin in BL cells and is a target of latent EBV gene product(s) in facilitating resistance against ionomycin-induced apoptosis.

5.3 How is Noxa regulated?

5.3.1 Noxa mRNA is upregulated by ionomycin in BL cells

Having shown that Noxa is upregulated by ionomycin at the protein level, it was necessary to determine whether Noxa was regulated at the level of transcription. EBV-negative BL31 and BL2 cells, together with their WT converts, were exposed to 1μg/ml ionomycin for up to 8 hours. Cells were harvested every 2 hours for RNA extraction. RNA from treated cells were analysed by quantitative RT-PCR to determine changes in the levels of Noxa mRNA expression as a result of treatment with ionomycin. Noxa mRNA expression was normalised to that of Ubiquitin C (UBC) mRNA. Graphs shown in Figure 5.15 shows that Noxa mRNA levels in EBV-negative BL31 cells increase following 4 hours of exposure to ionomycin. In general, Noxa

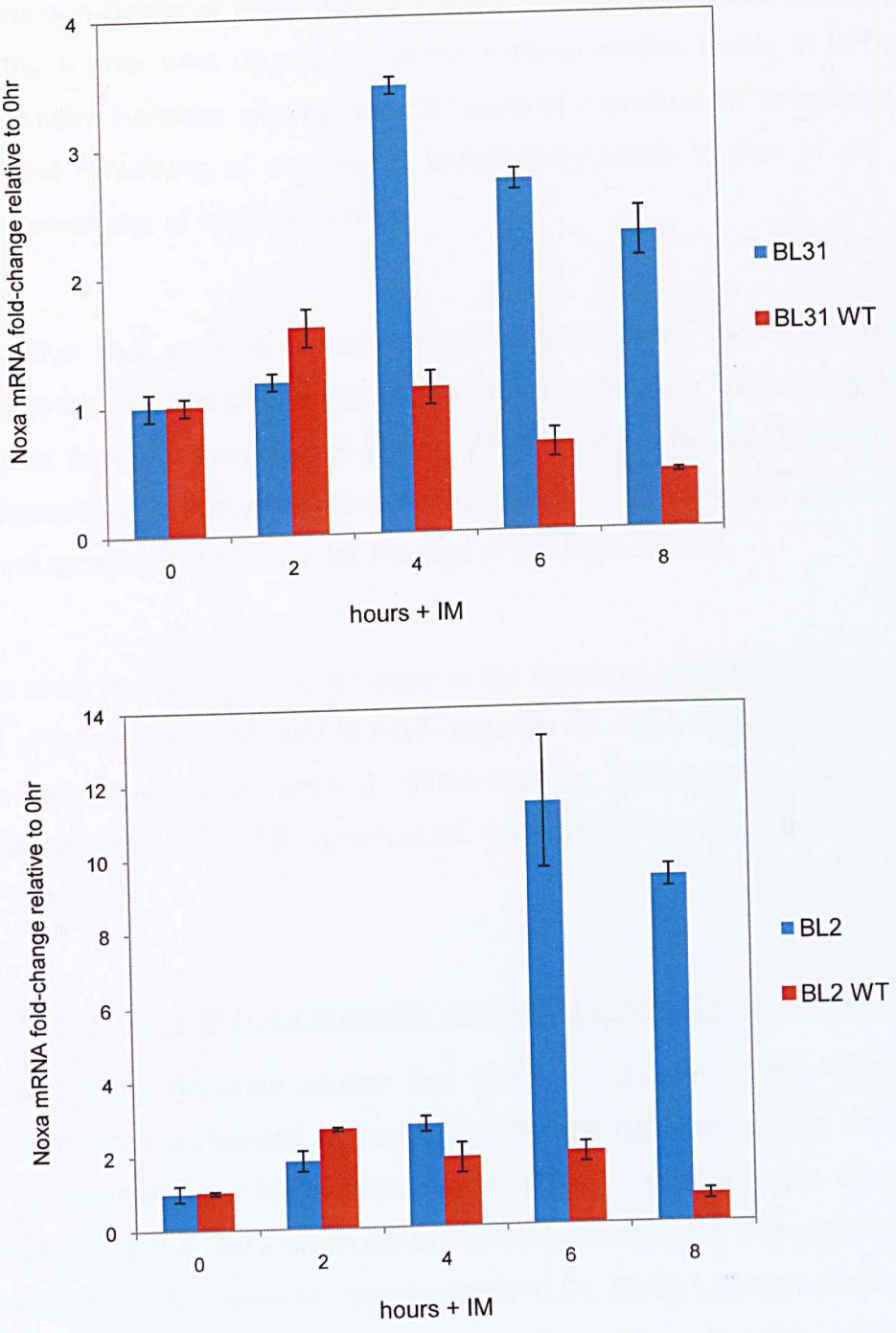


Figure 5-15 EBV suppresses the induction of Noxa mRNA by ionomycin. EBV-negative and wild-type (WT) converted BL31 and BL2 cells were exposed to 1μg/ml ionomycin for up to 8 hours. Cells were harvested at 2 hour intervals and RNA extracts prepared. RNA extracts were analysed by quantitative RT-PCR to determine levels of Noxa mRNA. Noxa mRNA expression was normalised to that of UBC mRNA. IM=ionomycin

mRNA expression peaks at the 4 hour time point before slowly decreasing towards the end of the 8 hour time course. In contrast, Noxa mRNA levels in WT-infected BL31 cells initially increase slightly after 2 hours of exposure to ionomycin before decreasing and remaining at expression levels comparable to that of the starting point for the remainder of the time course.

In EBV-negative BL2 cells, a substantial increase in Noxa mRNA expression is observed following 6 hours of exposure to ionomycin (Figure 5.15). Like WT-infected BL31, there is an initial increase of Noxa mRNA in WT-infected BL2 cells after 2 hours of exposure to ionomycin. Noxa mRNA levels in WT-infected BL2 cells then decrease and remain at low levels for the rest of the time course.

These data show that there is an increase in the levels of steady-state Noxa mRNA as a result of ionomycin treatment in EBV-negative BL cells. This may be due to an increase in Noxa gene transcription or mRNA stability. The increase of Noxa mRNA, however, is not observed in EBV-positive BL cells which are resistant to ionomycin-induced apoptosis.

5.3.2 Identifying region of Noxa promoter activated by ionomycin

Having shown in the previous section that Noxa is regulated at the transcriptional level by ionomycin, we decided to perform luciferase reporter assays to determine the effect of ionomycin on Noxa promoter activity. This would also enable us to identify the region in the Noxa promoter activated by ionomycin. We obtained several Noxa promoter-luciferase reporter constructs from Dr. Doug Leaman (Department of Biological Sciences, University of Toledo, USA). The plasmids obtained are represented in Figure 5.16.

Noxa promoter-luciferase constructs and the plasmid SV40 β-galactosidase, were co-transfected into BL31 and BL2 cells by electroporation. The cells were allowed to recover for 24 hours post-transfection. Cells were exposed to 1μg/ml ionomycin and harvested after a further 6 hours. A luciferase assay was then performed to

determine the activity of the exogenous Noxa promoter. A β-galactosidase assay was also performed as a control for transfection efficiency.

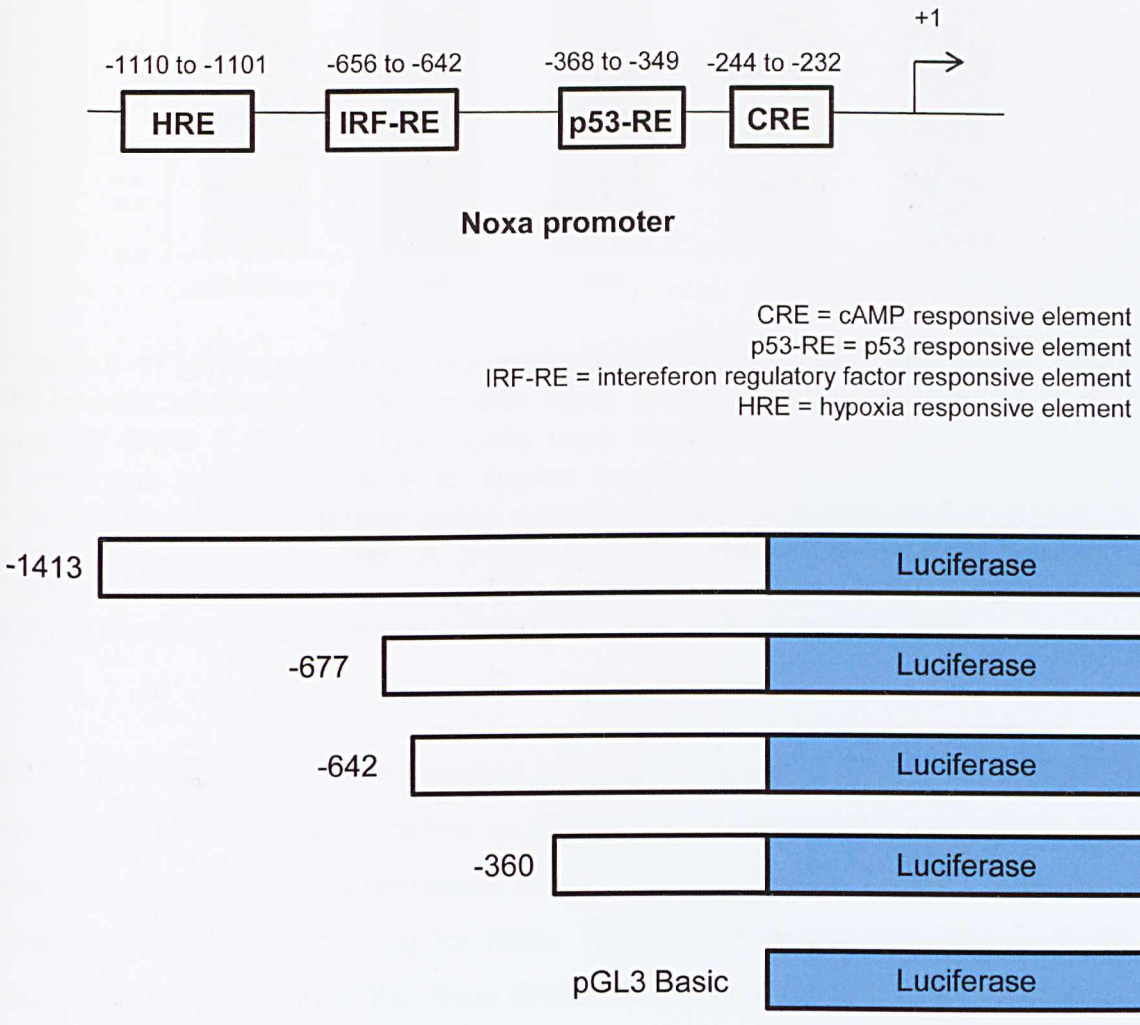


Figure 5-16 Schematic representation of Noxa promoter-luciferase reporter constructs used.

It proved very difficult to transfect EBV-negative BL31 and BL2 cells. Values obtained from β-galactosidase assays were very low and comparable to background. Experiments were repeated using the cell lines BJAB and DG75 which are regularly used in the lab for reporter gene assays. Transfection efficiency was much improved but no activation of the exogenous Noxa promoter was observed with any of the constructs used (Figure 5.17).

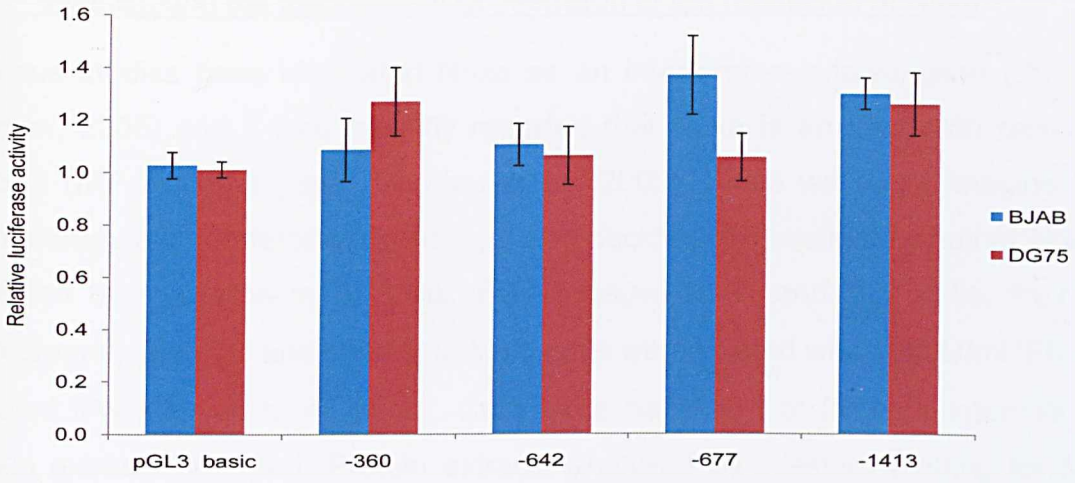


Figure 5-17 Ionomycin does not activate exogenous Noxa promoter. BJAB and DG75 cells were co-transfected with Noxa promoter-luciferase constructs and the plasmid SV40 β-galactosidase. Cells were allowed to recover for 24 hours post-transfection before exposure to 1μg/ml ionomycin. Cells were harvested after a further 6 hours. A luciferase assay was performed to determine the activity of the exogenous Noxa promoter. A β-galactosidase assay was also performed as a control for transfection efficiency. The histogram shown is a ratio of the luciferase activity measured in ionomycin-treated cells relative to untreated cells.

BJAB and DG75 cells were exposed to 1μg/ml ionomycin for 48 hours. Both BJAB and DG75 cells are not sensitive to ionomycin-induced apoptosis. Protein extracts were prepared from cells harvested every 24 hours. Protein extracts prepared were analysed by Western blotting for Noxa. Figure 5.18 shows that ionomycin did not induce an upregulation of the Noxa protein in these cells. This in turn suggests that ionomycin does not activate the endogenous Noxa promoter in these cells.

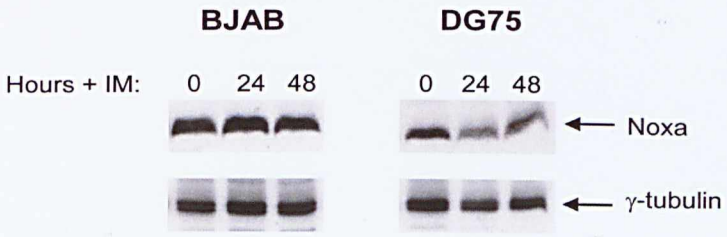


Figure 5-18 Ionomycin does not induce expression of Noxa in BJAB and DG75 cells. Cells were treated with 1μg/ml ionomycin for up to 48 hours and harvested at 24 hour intervals. Protein extracts from treated cells were separated by SDS-PAGE and analysed by Western blotting for Noxa. Throughout, γ-tubulin was used as a loading control. IM=ionomycin

5.3.3 Investigating the involvement of interferon in the regulation of Noxa

Previous studies have implicated Noxa as an interferon-regulated gene (Sun and Leaman, 2005) and it was recently reported that Noxa is an interferon regulating factor-3 (IRF-3) target gene (Goubau *et al.*, 2009). Since we could imagine EBV might deregulate interferon responses, it was decided to investigate whether Noxa is regulated by interferon in BL cells. EBV-negative BL31 and BL2 cells, their WT counterparts, latency I and latency III Mutu cells were treated with 1000U/ml IFN-α or 150U/ml IFN-γ for up to 48 hours. Cells were harvested at 24 hour intervals and protein extracts prepared. Protein extracts analysed by Western blotting for Noxa expression. Protein extracts from untreated cells harvested at the same time points were included for comparison.

As shown in Figure 5.19, Noxa protein expression is largely unaffected by stimulation with IFN-α or IFN-γ in both EBV-negative and -positive BL31 cells. Western blot analysis also shows that Noxa expression in both EBV-negative and -positive BL2 cells either remained constant or decreased following 48 hours of stimulation with IFN-α or IFN-γ. Similarly, levels of Noxa protein in latency I and latency III Mutu cells generally decreased after 48 hours of treatment with IFN-α or IFN-γ.

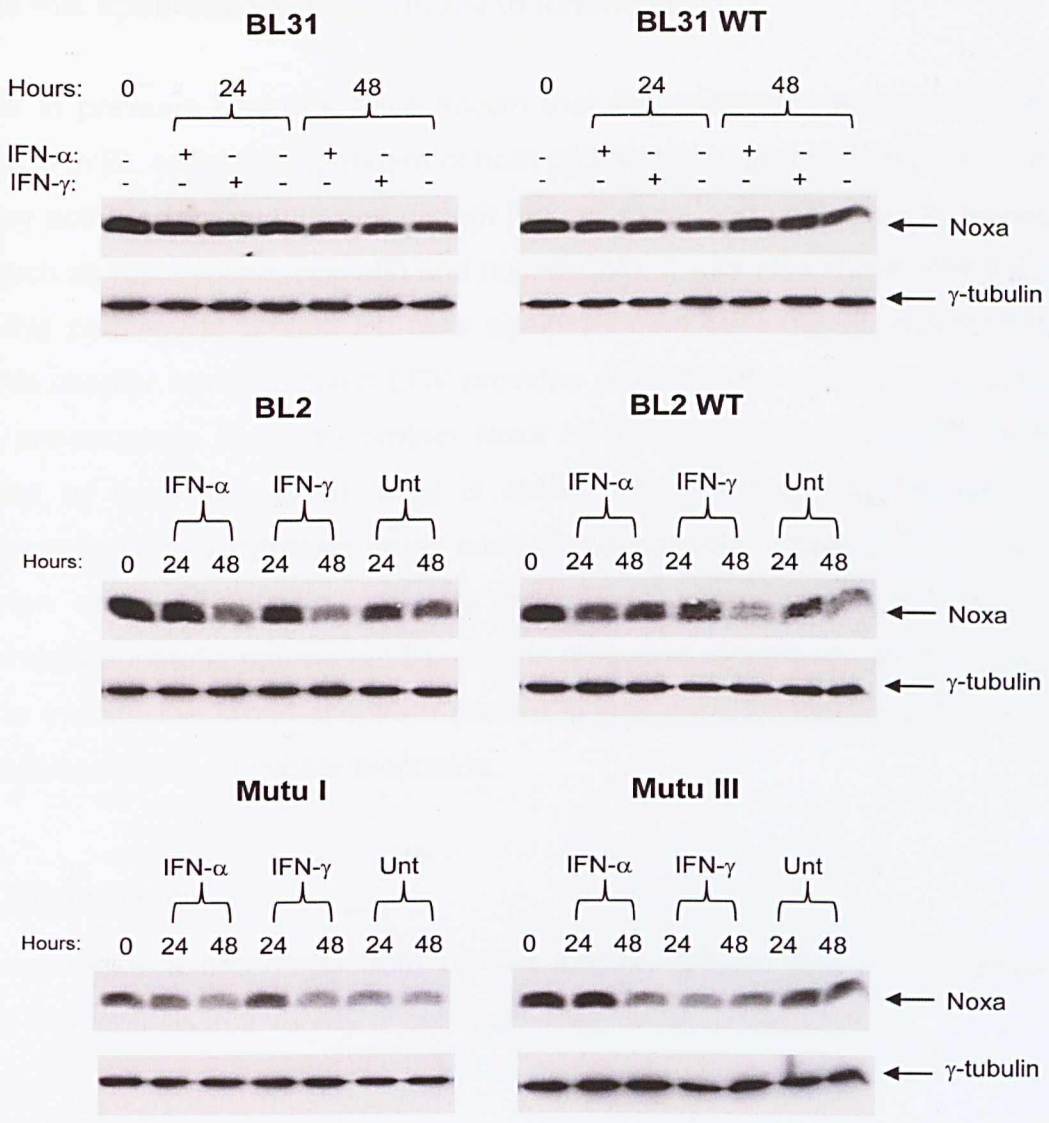


Figure 5-19 IFN-α and IFN–γ do not induce Noxa expression in BL cells. EBV-negative BL31 and BL2, their wild-type (WT)-infected counterparts, latency I and latency III Mutu cells were stimulated with 1000U/ml recombinant IFN-α or 150U/ml recombinant IFN-γ for up to 48 hours. Treated and untreated cells were harvested at 24 hour intervals for protein extraction. Protein extracts were analysed by Western blotting using antibodies specific for Noxa. Throughout, γ-tubulin was used as a loading control.

5.4 Is this apoptotic pathway unique to ionomycin?

Results in previous chapters have shown that the apoptotic pathway induced by ionomycin in BL cells is independent of both p53 and Bim, and quite distinct from the pathway activated by agents that disrupt the cell cycle and can result in damage to DNA such as roscovotine, cisplatin and nocodazole. It was also shown that EBV can block this pathway to protect BL cells against ionomycin-induced apoptosis. Data from this chapter have indicated EBV provides protection by inhibiting the induction of the pro-apoptotic BH3-only protein Noxa by ionomycin. The apoptotic pathway activated by ionomycin in BL cells is clearly different from the Bim-dependent pathway activated by cytotoxic drugs such as nocodazole, roscovitine and cisplatin (Anderton *et al.*, 2008). In order to investigate whether the p53- and Bim-independent apoptotic pathway in BL cells is unique to ionomycin, we extended this study to include the broad-spectrum protein kinase inhibitor staurosporine and the DNA topoisomerase II inhibitor etoposide.

5.4.1 Staurosporine

Staurosporine is a broad-spectrum protein kinase inhibitor and is a widely used inducer of apoptosis in a variety of cell types (see Section 1.4.2).

In order to determine whether staurosporine activates the same apoptotic pathway in BL cells as ionomcyin, we initially assessed the response of EBV-negative BL2 and its WT-infected counterpart to staurosporine. Cells were exposed to either 0.25μM staurosporine or DMSO (vehicle control) for up to 24 hrs. Cell viability was quantified after 24 hrs using the CellTiter-Glo assay previously described in Section 3.1.1. Cell survival was expressed as a percentage of vehicle-treated controls. The experiment was conducted in triplicate and the mean and standard deviation of three readings plotted as a histogram. The histogram shown in Figure 5.20A is representative of the outcome of two independent experiments. Cells were also harvested at the 10 and 24 hr time points for protein extraction and Western blot analysis.

Figure 5.20A shows that almost all EBV-negative BL2 cells do not survive following 24 hrs of exposure to staurosporine. In contrast, approximately 40% of WT-infected BL2 cells remain viable after 24 hrs. Western blot analysis revealed that a substantial amount of cleaved PARP is detectable after 10 hrs in EBV-negative BL2 cells (Figure 5.20B). PARP cleavage appears to be complete after 24 hrs indicating that BL2 cells underwent apoptosis in response to staurosporine treatment. Cleaved PARP was also detectable in WT-infected BL2 cells after 10 and 24 hrs of exposure to staurosporine but at lower levels than those observed in EBV-negative BL2. These data indicate that EBV infection can protect BL2 cells against staurosporine-induced apoptosis. EBV-mediated resistance to staurosporine is independent of Bim since BL2 cells do not express Bim.

Protein extracts were also analysed by Western blotting for Noxa to determine if the apoptotic pathway induced by staurosporine, like ionomycin, is associated with Noxa. A significant induction of Noxa protein is observed in EBV-negative BL2 after 24 hrs of exposure to staurosporine (Figure 5.20C). Conversely, Western blot analysis revealed that Noxa protein levels decreased slightly in response to staurosporine challenge in WT-infected BL2. Since BL2 cells carry WT p53, Western blotting analysis for p53 was performed to investigate if the induction of Noxa is a result of p53 activation. p53 does not appear to be induced in either EBV-negative or -positive BL2 cell lines following exposure to staurosporine (Figure 5.20C). These data suggest that Noxa is induced by staurosporine in a p53-independent manner and EBV can inhibit this induction of Noxa.

Experiments described above were repeated using latency I and III Mutu cells. Figure 5.21A shows that while latency I Mutu cells are sensitive to staurosporine treatment, approximately 60% of latency III Mutu cells survive after 24 hrs. Western blot analysis for PARP shows that cleaved PARP is detectable in latency I Mutu cells after 10 hrs of exposure to staurosporine. PARP cleavage appears to be almost complete by 24 hrs (Figure 5.21B). In contrast, there is no evidence of PARP cleavage in latency III Mutu cells even after 24 hrs. Western blot analysis for Noxa revealed that the protein is siginificantly upregulated in latency I Mutu cells following

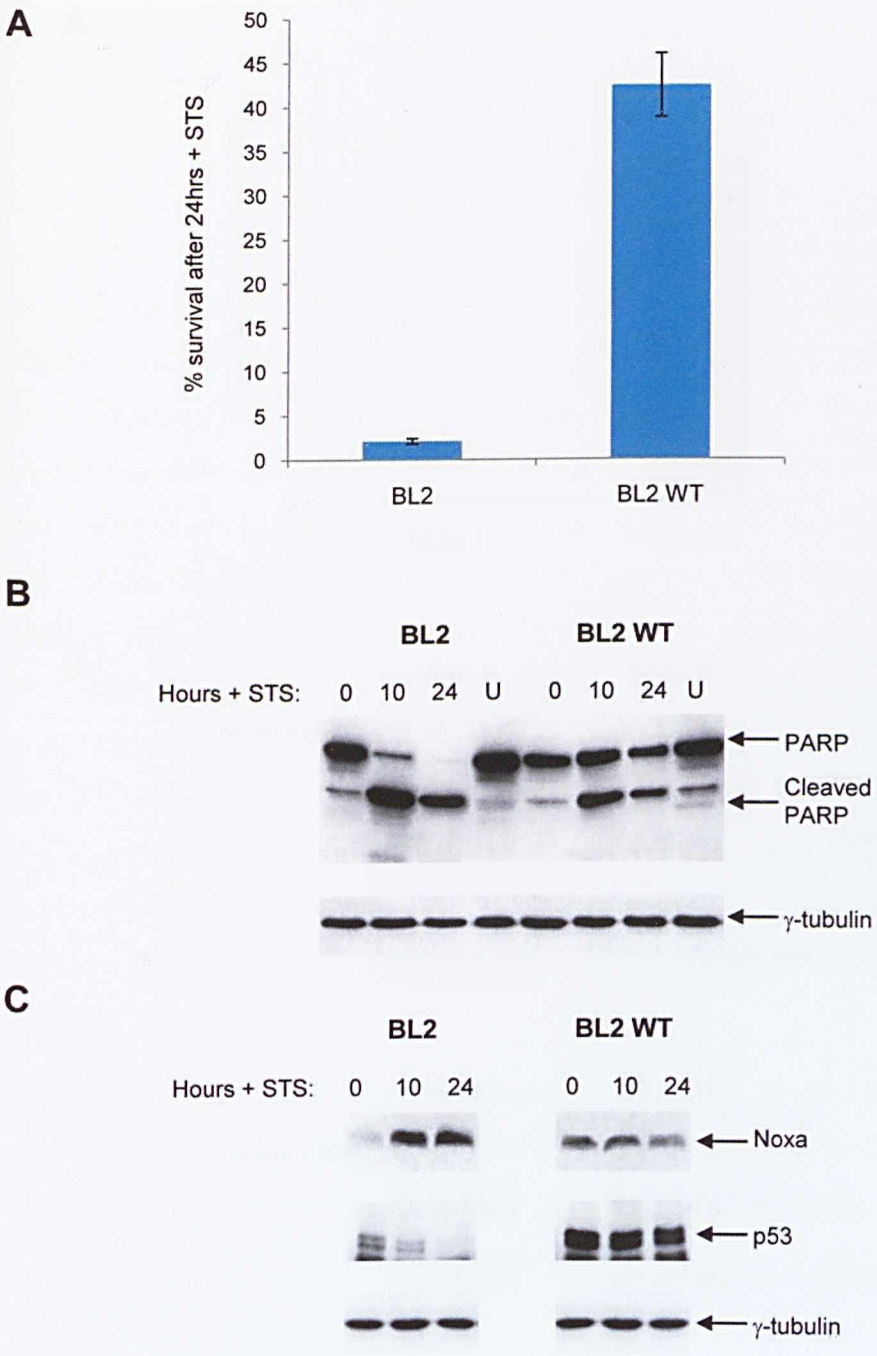


Figure 5-20 EBV protects BL2 cells from staurosporine-induced apoptosis. EBV-negative and wild-type (WT)-converted BL2 cells were treated with 0.25μM staurosporine or DMSO (vehicle control) for up to 24 hours. **(A)** Cell survival was determined after 24 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at the 10 and 24 hour time points. Protein extracts from treated cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. **(C)** Protein extracts were also analysed by Western blotting using antibodies specific for Noxa and p53. Throughout, γ-tubulin was used as a loading control. STS=staurosporine

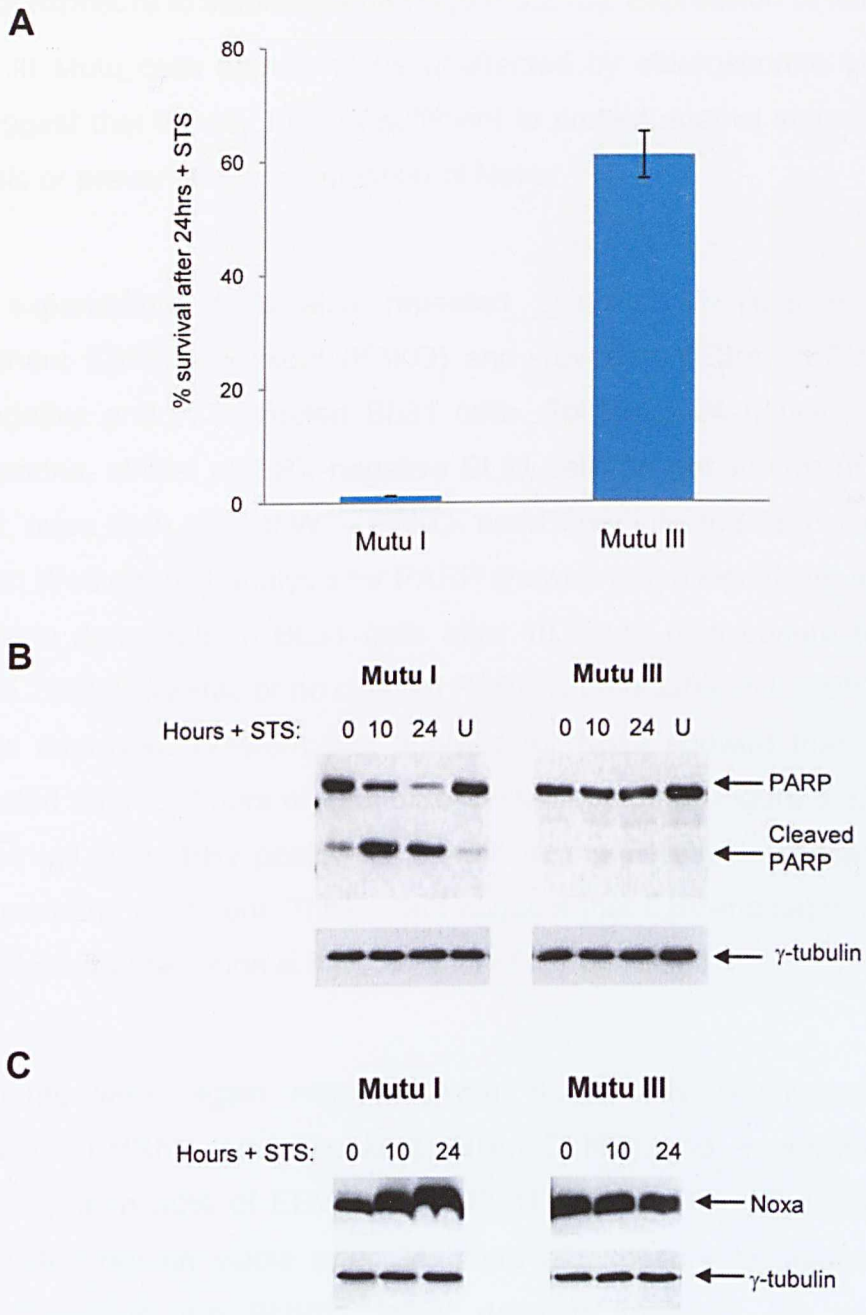


Figure 5-21 Latency I is not sufficient to protect against staurosporine-induced apoptosis. Latency I and latency III Mutu cells were exposed to 0.25μM staurosporine or DMSO (vehicle control) for up to 24 hours. **(A)** Cell survival was determined after 24 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at the 10 and 24 hour time points. Protein extracts from cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. **(C)** Protein extracts were also analysed by Western blotting using antibodies specific for Noxa. Throughout, γ-tubulin was used as a loading control. STS=staurosporine

24 hrs of exposure to staurosporine (Figure 5.21C). Expression of Noxa protein in latency III Mutu cells appear to be unaffected by staurosporine treatment. These data suggest that latency I is not sufficient to protect against staurosporine-induced apoptosis or prevent the accumulation of Noxa.

These experiments were also repeated using BL31 cells established using recombinant EBNA3-knockout (E3KO) and -revertant (E3rev) EBVs together with EBV-negative and WT infected BL31 cells. Following 24 hours of treatment with staurosporine, almost all EBV-negative BL31 cells do not survive (Figure 5.22A). In contrast, more than 45% of WT-, E3KO- and E3rev-infected BL31 cells survive after 24 hours. Western blot analysis for PARP showed that a significant amount of PARP cleavage is detectable in BL31 cells after 10 hours of exposure to staurosporine (Figure 5.22B). Very little or no cleaved PARP is detectable in the EBV-positive BL31 cell lines surveyed. Western blot analysis for Noxa showed that Noxa protein is upregulated after 24 hours of exposure to staurosporine (Figure 5.22C). Expression of Noxa in all of the EBV-positive BL31 cell lines generally decreased after 24 hours of staurosporine treatment. These data suggest that EBV-mediated protection of BL cells against staurosporine is independent of the EBNA3s.

Experiments were again repeated with BL31 cells newly established using recombinant BHRF1 locus-knockout (BHLOC KO) and -revertant (BHLOC rev) EBVs. Less than 30% of EBV-positive BL31 cells in which the BHRF1 locus has been deleted remain viable after 24 hours of exposure to staurosporine (Figure 5.23A). Reverting the BHRF1 locus deletion improves the survival of cells substantially with approximately 60% of BL31 BHLOC rev cells still viable after 24 hours. Western blotting analysis shows evidence of PARP cleavage in both BL31 BHLOC KO cell lines after 10 hours of staurosporine challenge (Figure 5.23B). In contrast, cleaved PARP is undetectable even after 24 hours in BL31 BHLOC rev cells. While deletion of the BHRF1 locus sensitizes cells to staurosporine-induced apoptosis, there is no evidence of Noxa induction by staurosporine in both BL31 BHLOC KO cell lines (Figure 5.23C). Like with ionomycin, this suggests that the apoptotic pathway induced by staurosporine also involves other pro-apoptotic factors besides Noxa.

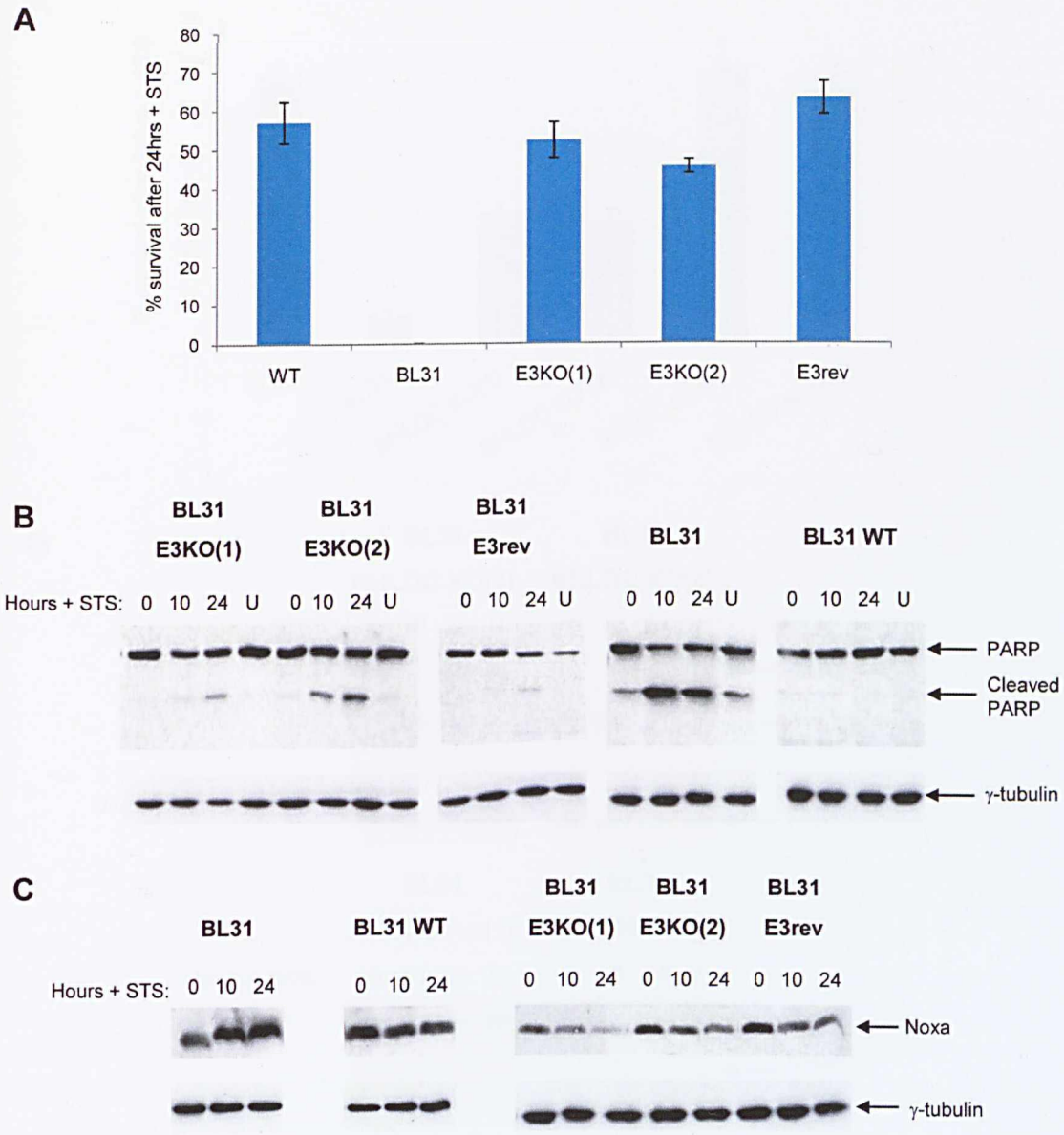


Figure 5-22 EBV-mediated protection of BL31 cells from staurosporine-induced apoptosis is independent of the EBNA3 locus. EBV-negative BL31, its wild-type (WT) convert and BL31 established with recombinant EBNA3-knockout (E3KO) and revertant (E3rev) EBVs were treated with 0.25μM staurosporine or DMSO (vehicle control) for up to 24 hours. **(A)** Cell survival was determined after 24 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at the 10 and 24 hour time points. Protein extracts from treated cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. **(C)** Protein extracts were also analysed by Western blotting using antibodies specific for Noxa and p53. Throughout, γ-tubulin was used as a loading control. STS=staurosporine

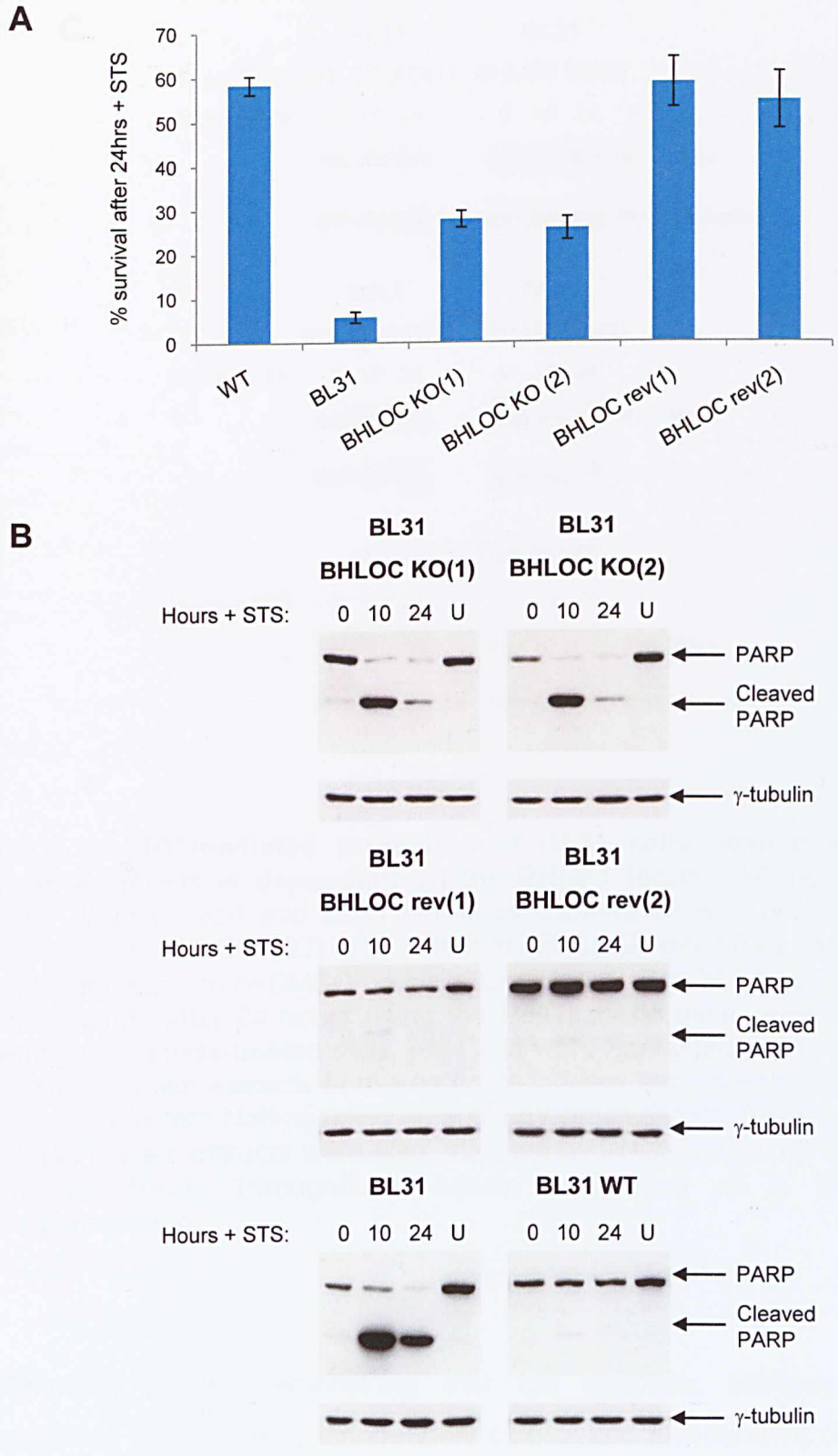


Figure 5-23 EBV-mediated protection of BL31 cells against staurosporine-induced apoptosis is dependent on the BHRF1 locus. (continued overleaf)

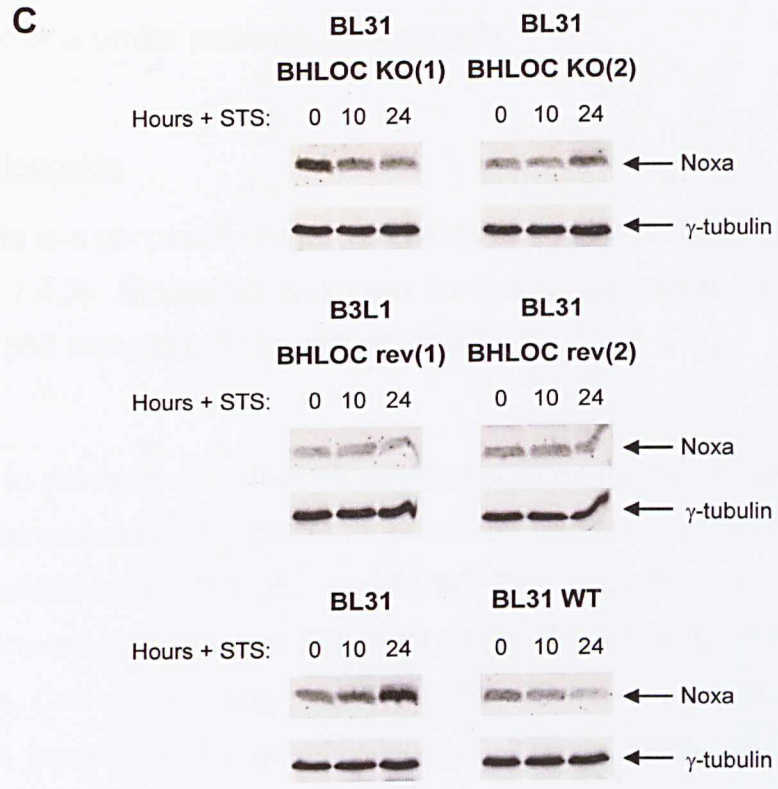


Figure 5.23 EBV-mediated protection of BL31 cells against staurosporine-induced apoptosis is dependent on the BHRF1 locus. EBV-negative BL31, its wild-type (WT) convert and BL31 cell lines established with recombinant BHRF1 locus-knockout (BHLOC KO) and revertant (BHLOC rev) EBVs were treated with 0.25μM staurosporine or DMSO (vehicle control) for up to 24 hours. **(A)** Cell survival was determined after 24 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at the 10 and 24 hour time points. Protein extracts from treated cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. **(C)** Protein extracts were also analysed by Western blotting using antibodies specific for Noxa. Throughout, γ-tubulin was used as a loading control. STS=staurosporine

In summary, results demonstrate that the apoptotic pathway activated by staurosporine in BL cells is independent of p53 and independent of EBNA3A and EBNA3C (and hence Bim). EBV-mediated resistance to staurosporine-induced apoptosis is at least in part dependent on the BHRF1 locus. Staurosporine treatment also appears to be associated with the induction of Noxa. All these

observations suggest that staurosporine induces apoptosis in BL cells by activating the same or a similar pathway as ionomycin.

5.4.2 Etoposide

Etoposide is a genotoxic drug which binds to and inhibits DNA topoisomerase II (see Section 1.4.3). Etoposide is known to induce apoptosis through DNA damage-induced p53 activation (Karpinich *et al.*, 2002).

In order to determine whether etoposide activates the same apoptotic pathway in BL cells as ionomycin or groups with roscovitine, cisplatin and nocodazole, the response of BL2, which carries WT p53, and its WT EBV convert to etoposide was examined. Cells were exposed to either 800ng/ml etoposide or DMSO (vehicle control) for up to 72 hours. Cell viability was measured after 72 hours using the CellTiter-Glo assay which is based on the quantification of ATP in viable cells. Cell survival was expressed as a percentage of vehicle-treated controls. The experiment was conducted in triplicate and the histogram shows the mean and standard deviation of the three readings. The histogram shown in Figure 5.24A is representative of the outcome of two independent experiments. Cells were also harvested at 24 hour intervals for protein extraction and Western blotting analysis.

Figure 5.24A shows that less than 30% of BL2 cells survived after 72 hours of treatment with etoposide. The survival of WT infected BL2 cells was comparable (approximately 33%) to that of EBV-negative BL2 cells. Figure 5.24B shows that significant amount of cleaved PARP is detectable in both EBV-negative and -positive BL2 cells after 24 hours of treatment with etoposide. PARP cleavage appears to be complete in both cell lines by the end of the time course. These data suggest that EBV does not protect BL2 cells against etoposide-induced apoptosis.

Protein extracts were also analysed by Western blotting for expression of p53, Noxa and Puma. As shown in Figure 5.24C, p53 is upregulated in both BL2 and BL2 WT after 24 hours of exposure to etoposide. Interestingly, Western blot analysis showed that neither Noxa nor Puma (another p53-inducible gene) was upregulated following

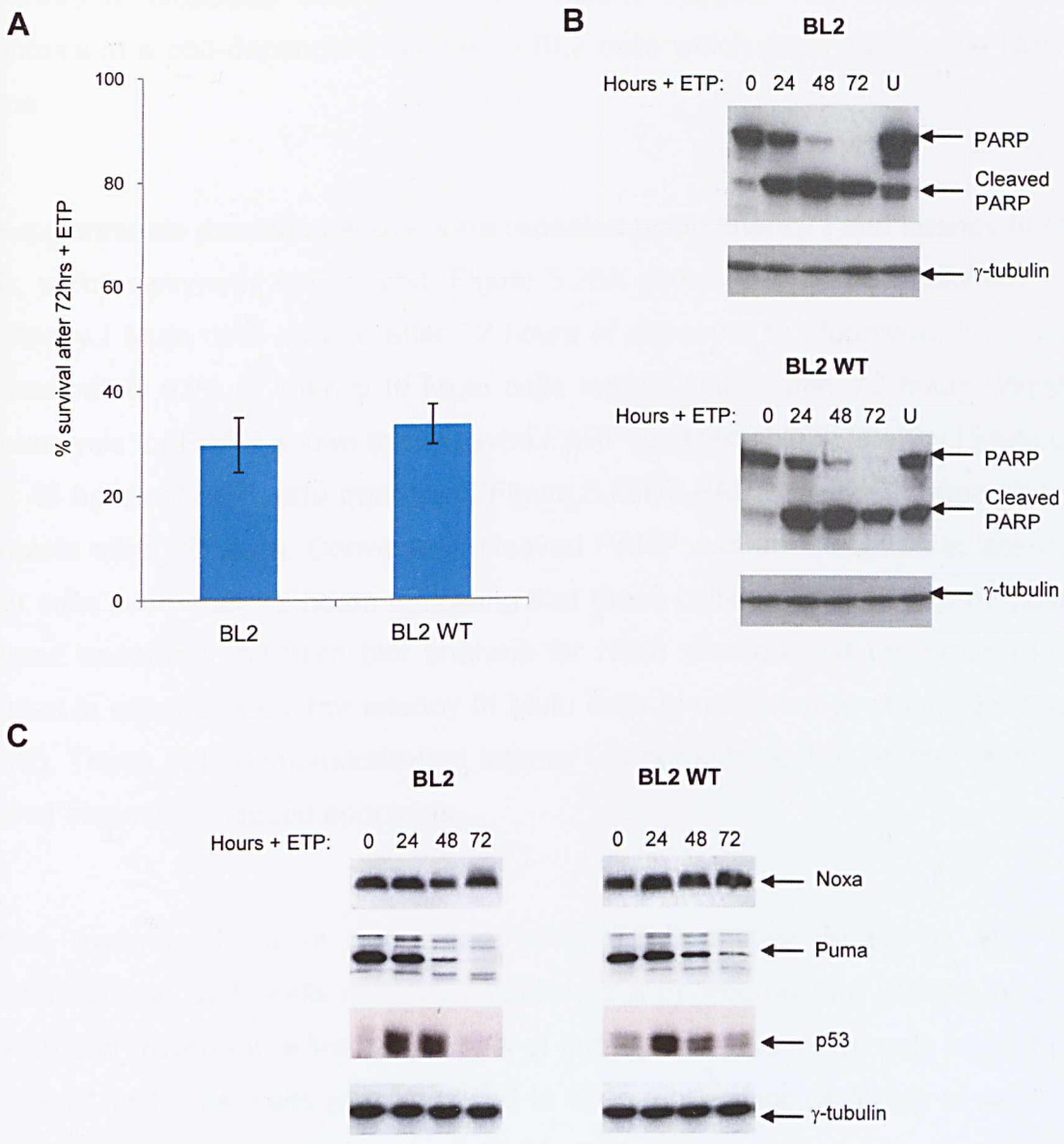


Figure 5-24 EBV does not protect BL2 cells from etoposide-induced apoptosis. BL2 and its wild-type (WT)-infected counterpart were exposed to 800ng/ml etoposide or DMSO (vehicle control) for up to 72 hours. **(A)** Cell survival was determined after 72 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at 24 hour intervals. Protein extracts from treated cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. **(C)** Protein extracts were also analysed by Western blotting using antibodies specific for Noxa, Puma and p53. Throughout, γ-tubulin was used as a loading control. ETP=etoposide

72 hours of etoposide treatment. These results suggest that etoposide induces apoptosis in a p53-dependent manner in BL2 cells which does not involve Noxa or Puma.

The experiments described above were repeated using latency I and latency III Mutu cells, which carry only mutant p53. Figure 5.25A shows that only approximately 5% of latency I Mutu cells survive after 72 hours of exposure to etoposide. In contrast, approximately 83% of latency III Mutu cells remain viable after 72 hours. Western blot analysis for PARP shows that cleaved PARP is detectable in latency I Mutu cells after 48 hours of etoposide treatment (Figure 5.25B). PARP cleavage appears to be complete after 72 hours. Conversely, cleaved PARP was undetectable in latency III Mutu cells even after 72 hours indicating that these cells are resistant to etoposide-induced apoptosis. Western blot analysis for Noxa showed that the protein is not induced in either latency I or latency III Mutu cells in response to etoposide (Figure 5.25C). These data demonstrate that latency I is not sufficient to protect Mutu cells against etoposide-induced apoptosis.

Similar experiments were conducted using EBV-negative BL31, its WT EBV counterpart and BL31 cells recently established with recombinant EBNA3-knockout (E3KO) and -revertant (E3rev) EBVs. All of these, like Mutu, carry only mutant p53. With BL31 cell lines, cells were exposed to 500ng/ml etoposide for up to 48 hours. Figure 5.26A shows that almost all BL31 cells fail to survive after 48 hours of exposure to etoposide. In contrast, over 80% of both WT- and E3rev-infected BL31 cells survive after 48 hours. Interestingly, less than 30% of both BL31 E3KO cell lines survive after 48 hours. Western blot analysis for PARP revealed a significant amount of cleaved PARP was detectable in EBV-negative BL31 cells after 24 hours of exposure to etoposide (Figure 5.26B). PARP cleavage appears to be complete after 48 hours. Cleaved PARP was also detectable in both BL31 E3KO cell lines after 24 hours of etoposide treatment. Conversely, cleaved PARP was undetectable in both the WT-infected and EBNA3-revertant BL31 cell lines even after 48 hours. Western blot analysis for Noxa showed that Noxa protein was not induced in response to etoposide in any of the BL31 cell lines surveyed (Figure 5.26C). These data suggest that EBV protects BL31 cells against etoposide-induced apoptosis.

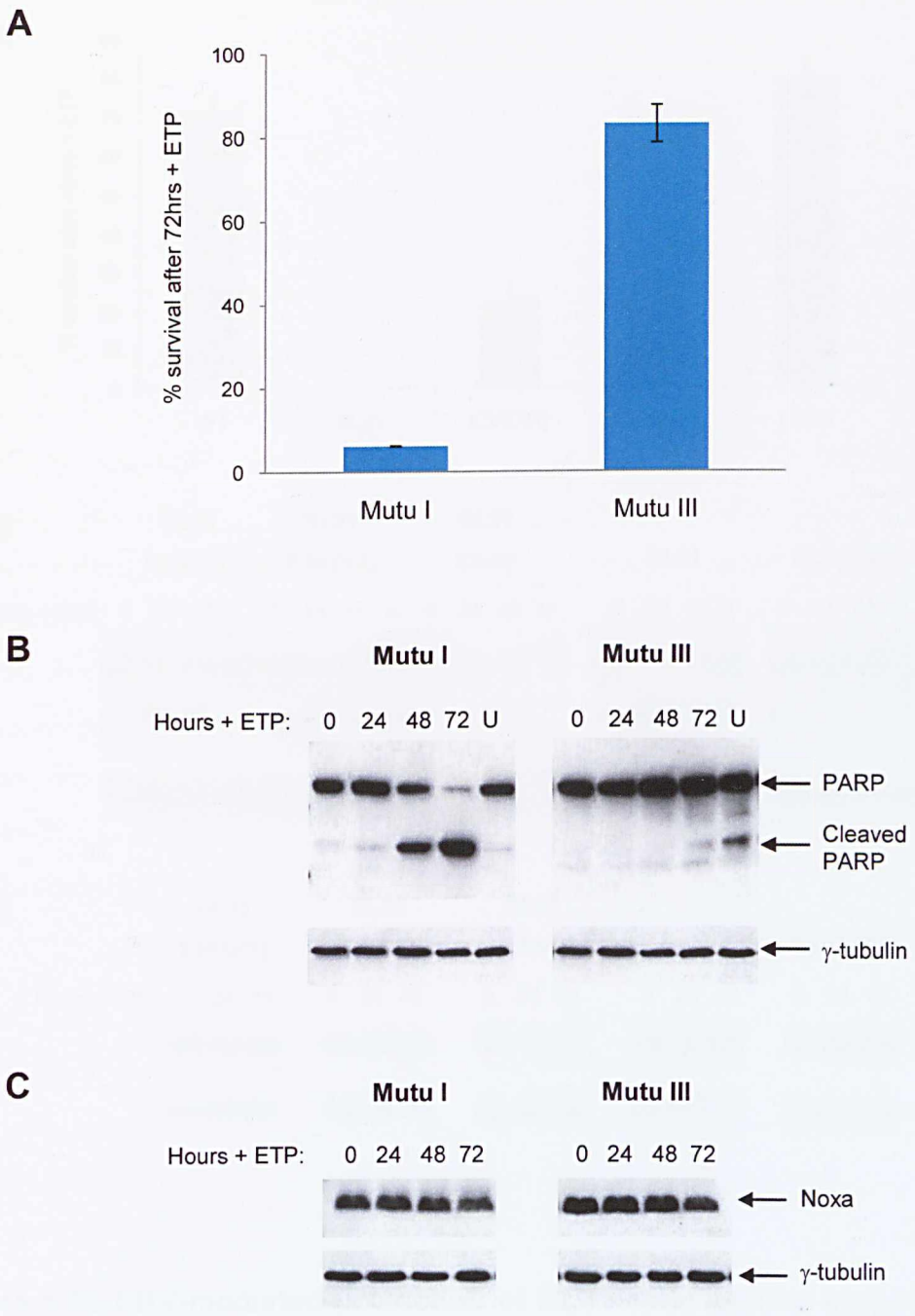


Figure 5-25 Latency I is not sufficient to protect against etoposide-induced apoptosis. Latency I and latency III Mutu cells were exposed to 800ng/ml etoposide or DMSO (vehicle control) for up to 72 hours. **(A)** Cell survival was determined after 72 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at 24 hour intervals. Protein extracts from cells were separated by SDS-PAGE and analysed by Western blotting using an antibody which detects full-length and cleaved PARP. **(C)** Protein extracts were also analysed by Western blotting using antibodies specific for Noxa. Throughout, γ-tubulin was used as a loading control. ETP=etoposide

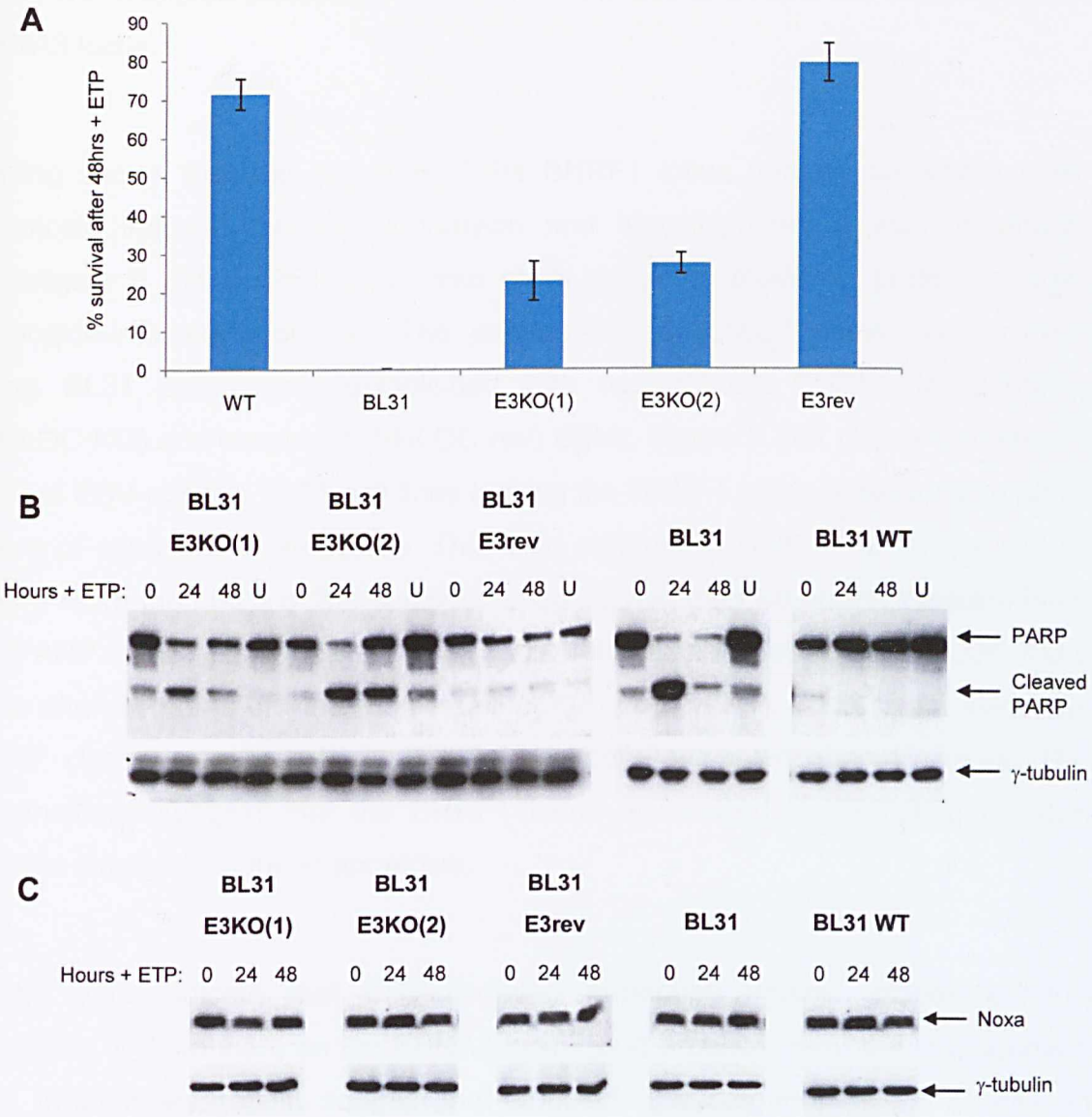


Figure 5-26 EBV-mediated protection of BL31 cells against etoposide-induced apoptosis is dependent on the EBNA3 locus. EBV-negative BL31, its wild-tyep (WT) convert and BL31 recently established with recombinant EBNA3-knockout (E3KO) and revertant (E3rev) EBV were exposed to 500ng/ml etoposide or DMSO (vehicle control) for up to 48 hours. **(A)** Cell survival was determined after 48 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at 24 hour intervals. Protein extracts were separated by SDS-PAGE and analysed by Western blotting using antibodies which detect full-length and cleaved PARP. **(C)** Protein extracts were also analysed by Western blotting using antibodies specific for Noxa. Throughout, γ-tubulin was used as a loading control. ETP=etoposide

This EBV-mediated protection is independent of p53 and Noxa but dependent on the EBNA3 locus.

Having shown that the absence of the BHRF1 locus partially sensitizes cells to apoptosis induced by both ionomycin and staurosporine, it was important to investigate if the BHRF1 locus also plays a role in providing protection against etoposide-induced apoptosis. The experiments described above were repeated using BL31 cells newly established with recombinant BHRF1 locus-knockout (BHLOC KO) and -revertant (BHLOC rev) EBVs. Figure 5.27A shows that less than 35% of EBV-positive BL31 cell lines lacking the BHRF1 locus remain viable after 48 hours of exposure to etoposide. This is in contrast to BL31 BHLOC rev cell lines where more than 65% of the cell population survive after 48 hours. Western blotting for PARP showed that cleaved PARP was detectable in both BL31 BHLOC KO cell lines after 24 hours of etoposide treatment (Figure 5.27B). There is no evidence of PARP cleavage in both BL31 BHLOC rev cell lines even after 48 hours. These observations suggest that the BHRF1 locus is involved in mediating resistance against etoposide-induced apoptosis.

These data indicate that EBV-mediated protection against etoposide-induced apoptosis is dependent on the EBNA3 locus (and probably Bim) but independent of p53. In summary, results suggest that etoposide induces apoptosis in BL cells in a similar manner to that of the other genotoxic drugs (nocodazole, roscovitine and cisplatin) previously studied in the lab. These results also confirm that EBV cannot protect against genotoxic drugs if wild-type p53 is present (Allday *et al.*, 1995a; O'Nions and Allday, 2003). Interestingly, the BHRF1 locus also appears to play a role in EBV-mediated resistance to etoposide (like ionomycin and staurosporine).

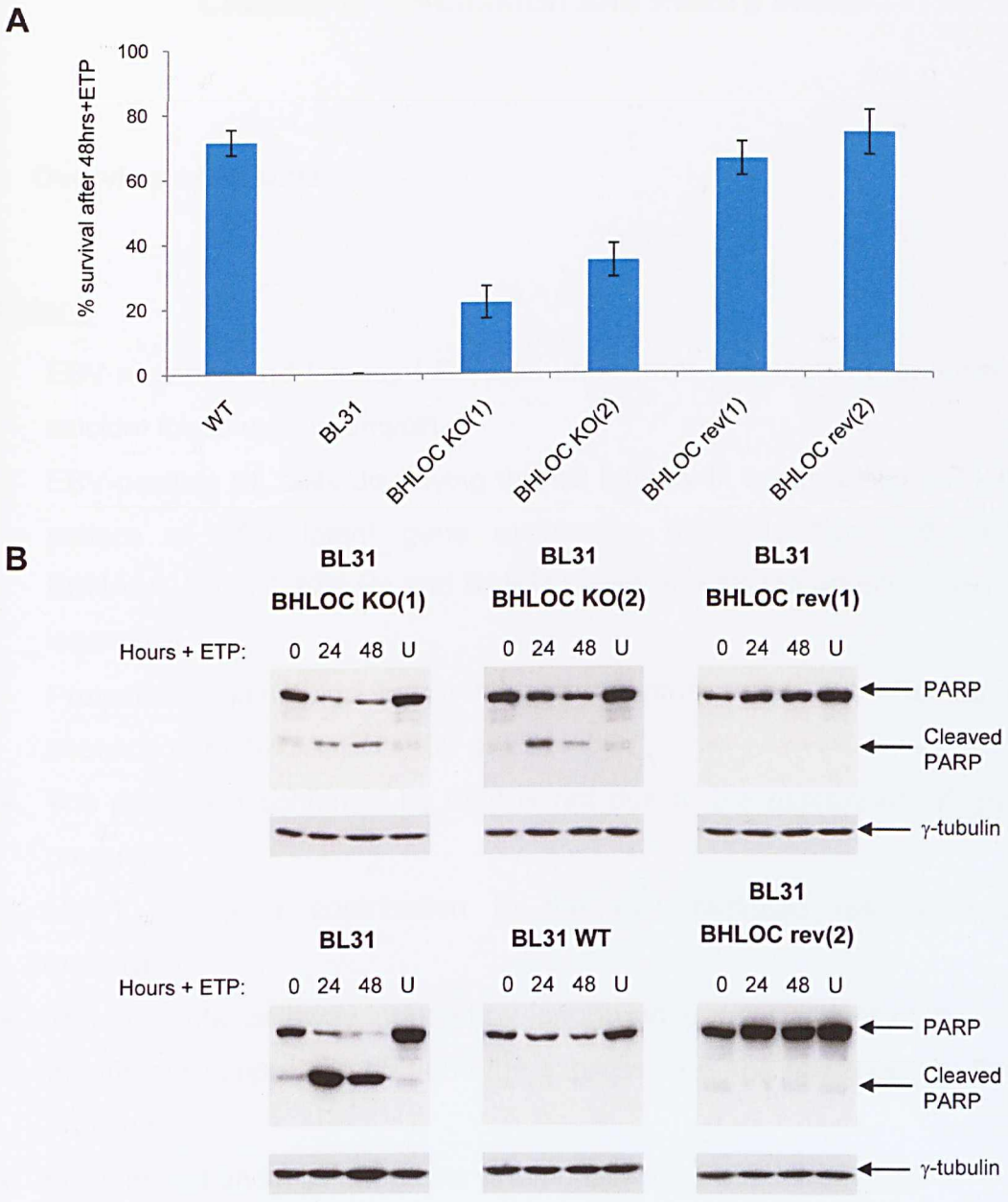


Figure 5-27 EBV-mediated protection of BL31 cells against etoposide-induced apoptosis is dependent on the BHRF1 locus. EBV-negative BL31, its wild-type (WT) convert and BL31 recently established with recombinant BHRF1 locus-knockout (BHLOC KO) and revertant (BHLOC rev) EBVs were exposed to 500ng/ml etoposide or DMSO (vehicle control) for up to 48 hours. **(A)** Cell survival was determined after 48 hours using the CellTiter-Glo assay and expressed as a percentage of vehicle-treated cells. **(B)** Cells were harvested at 24 hour intervals. Protein extracts were separated by SDS-PAGE and analysed by Western blotting using antibodies which detect full-length and cleaved PARP. γ-tubulin was used as a loading control. ETP=etoposide

Chapter 6 Discussion and Future Work

6.1 Overview of Results

Chapter 3

- EBV-negative and latency I BL cells underwent apoptosis in response to the calcium ionophore ionomycin.
- EBV-positive BL cells displaying the full latency III or a restricted P3HR1-like pattern of EBV latent gene expression (EBNA1, truncated EBNA-LP, EBNA3A, 3B, 3C, EBERs and BARTs) were resistant to apoptosis induced by ionomycin.
- Protection against ionomycin-induced apoptosis was not affected by the absence of EBNA3A, EBNA3B or EBNA3C.
- The protection conferred by EBV is not due to the expression of lytic gene products.
- LMP1 makes a contribution to the EBV-mediated resistance against ionomycin.
- The apoptotic pathway induced by ionomycin is independent of the status of the tumour suppressor p53 and the expression of the pro-apoptotic BH3-only protein Bim.
- LCLs do not undergo apoptosis when challenged with ionomycin.

Chapter 4

- Recombinant and revertant EBVs in which the EBNA3 locus is deleted and reinserted have been constructed.
- Deletion of the entire EBNA3 locus does not affect the EBV-mediated protection against ionomycin in BL cells.
- EBV-positive BL cells lacking EBER1 or EBER2 expression were similarly resistant to apoptosis induced by ionomycin.

- Recombinant and revertant EBVs in which the BHRF1 locus is deleted and reinstated have been constructed.
- Deletion of the BHRF1 locus partially abrogates resistance to ionomycin-induced apoptosis.

Chapter 5

- Accumulation of the pro-apoptotic protein Noxa was observed in BL cells sensitive to ionomycin. In contrast, upregulation of Noxa protein was not observed in the panel of EBV-positive BL cells resistant to ionomycin-induced apoptosis.
- Expression of LMP1 alone is insufficient for preventing the accumulation of Noxa protein.
- Although deletion of the BHRF1 locus partially sensitizes cells to ionomycin-induced apoptosis, expression of Noxa was unaffected by ionomycin treatment.
- Induction of Noxa was observed at the level of transcription.
- Inhibition of Noxa expression by shRNA enhanced the survival of EBV-negative BL cells challenged with ionomycin.
- Staurosporine, like ionomycin, activates a p53- and Bim-independent apoptotic pathway in BL cells.
- Etoposide-induced apoptosis in BL cells is independent on p53 status but is dependent on the EBNA3 locus (and hence Bim).
- The BHRF1 locus contributes to the EBV-mediated protection of BL cells against apoptosis induced by both staurosporine and etoposide.

6.2 Latent EBV confers resistance against ionomycin-induced apoptosis in Burkitt's lymphoma and human B cells

Many studies have demonstrated that latent EBV confers resistance against apoptosis induced by a variety of stimuli including genotoxic agents such as nocodazole and cisplatin, calcium ionophores, cross-linking of BCR, cytokine deprivation and γ-irratidation (Gregory *et al.*, 1991; Kelly *et al.*, 2005; Leao *et al.*,

2007; Anderton *et al.*, 2008) in BL cells. Previous work in the lab has shown that the resistance conferred by EBV against cell death induced by agents that cause damage to DNA (the mitotic spindle poison nocodazole, the cyclin-dependent kinase inhibitor roscovitine and the DNA cross-linking agent cisplatin) is dependent on the co-expression of EBNA3A and EBNA3C (Anderton *et al.*, 2008). This protection correlates with the downregulation of the BH3-only pro-apoptotic protein Bim by EBNA3A and EBNA3C but is independent of the tumour suppressor p53. In the absence of Bim (eg. in BL2 cells), latent EBV does not provide any survival advantage against apoptosis induced by these genotoxic drugs. These findings appeared to be inconsistent with the observations made by Kelly and colleagues that EBV can protect BL2 cells (which carry WT p53 but are Bim-negative) and BL31 cells (which carry mutant p53 but are Bim-positive) to a similar extent against apoptosis induced by the calcium ionophore ionomycin and anti-IgM (Kelly *et al.*, 2005). We hypothesised that ionomycin and anti-IgM activate a second distinct apoptotic pathway in BL cells which is also independent of p53 status but not associated with Bim.

Initial work described in this thesis was performed to characterise the Bim- and p53-independent apoptotic pathway by assessing the response of BL cells expressing full latency III or restricted sets of latent genes using the calcium ionophore ionomycin as the inducer of apoptosis. From data presented in Chapter 3, we demonstrate that EBV-negative BL41 and BL31 (which are both mutant for p53) as well as BL2 (which carry WT p53) are sensitive to ionomycin treatment. Following a 48 hour exposure to ionomycin, a substantial decrease in viable cell counts was consistently observed. This was accompanied by proteolytic cleavage of the caspase substrate PARP indicating that cells were undergoing apoptosis in response to ionomycin. *In vitro* converts of these BL cell lines, such as BL41/B95.8, BL41/P3HR1, and BL cells lines recently established with recombinant EBNA2-knockout (E2KO) EBVs (BL31+E2KO-IG and BL2+E2KO) are resistant to ionomycin treatment. In most cases, viable cell counts actually increased after 48 hours of exposure to ionomycin indicating that some cells continued proliferating even in the presence of ionomycin. This was consistent with the observation that full-length PARP remained intact even after 48 hours of exposure to ionomycin. Recently isolated BL cell lines, Oku-BL and Sal-BL,

which display a P3HR1-like pattern of latent gene expression (EBNA1, EBNA3A, EBNA3B, EBNA3C, truncated EBNA-LP, EBERs and BARTs) (Kelly *et al.*, 2002) and carry WT p53 (Anderton *et al.*, 2008) were similarly protected from apoptosis, confirming observations made by Kelly and colleagues (Kelly *et al.*, 2005). These data collectively demonstrate that EBV confers resistance against ionomycin-induced apoptosis in BL cells. This EBV-mediated protection is independent of p53 given that EBV protects cells carrying both mutant (BL31 and BL41) and WT (BL2, Oku-BL, Sal-BL) p53. This protection is also independent of Bim status since EBV provides a similar degree of protection to both Bim-negative (BL2) and Bim-positive (BL31 and BL41) cells. These data also implied that EBNA2, the LMPs and full-length EBNA-LP are not required for protection against ionomycin-induced apoptosis. Given that the expression of truncated EBNA-LP was very low or undetectable in Oku-BL compared to Sal-BL (Kelly *et al.*, 2005) and the fact that this difference in expression levels did not diminish the resistance of Oku-BL to ionomycin, it is unlikely that truncated EBNA-LP plays a significant role in mediating protection from apoptosis.

Although results so far indicate that LMP1 is not required in the ionomycin resistance phenotype, we decided to investigate further its involvement because LMP1 is widely reported to possess anti-apoptotic properties. LMP1 has been shown to promote cell survival and proliferation by upregulating the expression of anti-apoptotic proteins including Bcl-2 (Henderson *et al.*, 1991), Mcl-1 (Wang *et al.*, 1996) and A20 (Fries *et al.*, 1996), and acting as a viral mimic for CD40 signaling in B cells (Kilger *et al.*, 1998). Investigation into the role of LMP1 was performed by using EBV-negative BL41 cells in which LMP1 is constitutively expressed from a stably maintained plasmid at levels comparable to those seen in B95.8-infected BL41 cells (Cuomo *et al.*, 1992; Wade and Allday, 2000). The expression of LMP1 alone enhances the survival of BL41 cells challenged with ionomycin albeit not to the same degree as seen when the full latency III or P3HR1-like set of latent genes are expressed. It is clear from these data that while LMP1 is able to provide some protection against ionomycin, it is not the only EBV factor involved in this phenotype. Previous studies conducted in the same system have shown that LMP1 has no protective effect on cells exposed to cisplatin (Wade and Allday, 2000) but can improve the survival of

cells undergoing apoptosis due to cytokine deprivation (Henderson *et al.*, 1991). It has been suggested that this is due to the elevated levels of Bcl-2 in LMP1-expressing cells. While expression of Bcl-2 protein does appear to be upregulated by LMP1 in this system (data not shown), LMP1 expression does not always correlate with increased Bcl-2 expression in other BL cells surveyed (our unpublished observations).

Similar experiments were performed using a BL cell line exhibiting the restricted latency I form of infection, Mutu I, to narrow down candidate gene(s) involved in the EBV-mediated rescue from ionomycin-induced apoptosis. These experiments showed that the type I pattern of latent gene expression (EBNA1, EBERs, and BARTs) is insufficient for resistance against ionomycin. In contrast, Mutu cells that had drifted in culture to produce the full latency III pattern of EBV gene expression, Mutu III, were protected from apoptosis induced by ionomycin. These observations indicate that EBNA1, EBERs and BARTs are not involved in conferring protection against ionomycin-induced apoptosis. However, it is interesting to note that in comparison to EBV-negative BLs, latency I infection can provide some survival advantage at lower doses of ionomycin (0.25 μg/ml compared to 1μg/ml) (Kelly *et al.*, 2006).

At this point, the most obvious candidates for mediating the protection from ionomycin-induced apoptosis were EBNA3A, EBNA3B and EBNA3C. Experiments performed using Bim-positive, p53 mutant BL31 cells established with recombinant EBVs in which the individual EBNA3 genes were deleted (Anderton *et al.*, 2008) are resistant to ionomycin. These data demonstrate that in contrast to the EBNA3A- and EBNA3C-mediated resistance against genotoxic agents, the protection from ionomycin-induced apoptosis is not affected by the loss of EBNA3A, EBNA3B or EBNA3C. Further experiments conducted using Bim-negative, WT p53-carrying BL2 cells converted using the same recombinant EBNA3A, EBNA3B or EBNA3C-knockout EBVs produced similar results. These data confirm that EBNA3A, EBNA3B and EBNA3C individually are not responsible for the EBV-mediated protection of BL cells against ionomycin-induced apoptosis.

Ionomycin is known to induce the EBV lytic cycle in some latently infected B cells (Faggioni *et al.*, 1986). Although several activators of the EBV lytic program can concomitantly induce apoptosis, lytic gene expression has been shown to protect cells containing replicating virus from cell death (Inman *et al.*, 2001). Two lytic gene products reported to exhibit anti-apoptotic activity are the viral Bcl-2 homologues, BHRF1 (Henderson *et al.*, 1993) and BALF1 (Marshall *et al.*, 1999). Using the human serum EE (from a patient with chronic infectious mononucleosis with a high titre of antibodies for EBV lytic antigens) to detect early lytic antigens, we demonstrate that there is no evidence of lytic cycle reactivation by ionomycin in the BL cells resistant to the treatment used in this study. These observations were supported by further analyses using a monoclonal antibody against BHRF1. Antibodies for BALF1 are not available. Hence, we can conclude that anti-apoptotic effects mediated by proteins expressed only in the EBV lytic cycle are not responsible for resistance to ionomycin.

As the work described so far has been carried out using BL cells, it was also important to examine the response of EBV-transformed B cells. It has previously been demonstrated that LCLs undergo apoptosis in response to genotoxic agents (Allday *et al.*, 1995a; Wade and Allday, 2000; O'Nions and Allday, 2003). This was attributed to the presence of WT p53 in LCLs and the inability of EBV to prevent LCLs from undergoing p53-mediated apoptosis (Allday *et al.*, 1995b). It was therefore interesting to observe that LCLs are resistant to apoptosis induced by ionomycin. These findings support our hypothesis that the apoptotic pathway activated by ionomycin is indeed distinct from the pathway activated by genotoxic drugs.

6.3 The effect of EBV latent gene products on protection against ionomycin-induced apoptosis – which EBV gene(s) is responsible?

In our search for the EBV latent gene(s) responsible for ionomycin resistance, the response of EBV-positive BL cells expressing restricted sets of latent viral proteins were examined as described in Chapter 3. These results suggest that genes

associated with latency I (EBNA1, EBERs and BARTs) can be excluded because cells exhibiting type I latency are not protected against ionomycin-induced apoptosis. EBNA2, full-length EBNA-LP, LMP2A and LMP2B can also be eliminated from our search because BL cells lacking the expression of these EBV latent proteins were protected from ionomycin-induced apoptosis to a similar extent as those displaying the full latency III phenotype. Partial protection against ionomycin-induced apoptosis was observed in BL cells transfected with LMP1-expression vectors indicating that while LMP1 can confer some protection against ionomycin, it is not the only EBV latent gene involved in suppressing the ionomycin-activated apoptotic pathway.

Although results have indicated that EBNA3A, EBNA3B and EBNA3C individually are not required for protection of BL cells against ionomycin-induced apoptosis, we could not rule out the possibility of functional redundancy between the EBNA3 proteins since they share limited homology. It was important to examine further the role of the EBNA3s given that EBNA3A and EBNA3C are known mediators of anti-apoptotic activity (Anderton *et al.*, 2008). In order to investigate the contribution of the EBNA3s collectively, a recombinant EBV harbouring a deletion of the entire EBNA3 locus was constructed (E3KO). A revertant EBV (E3rev) in which the EBNA3 locus was reinstated was also generated as a control for second site mutations that may have taken place during the recombination process. These recombinant E3KO and E3rev EBVs were used to establish stable BL31 and BL2 cell lines in order to investigate the contribution of the EBNA3 proteins collectively in the rescue of BL cells from ionomycin-induced apoptosis. Results indicate that there is no functional redundancy between the EBNA3 proteins since the loss of all three EBNA3 proteins did not abrogate resistance to ionomycin-induced apoptosis. These observations confirm that the EBNA3 locus is not required for the ionomycin resistance phenotype.

There have been reports that the non-coding viral RNAs, EBER1 and EBER2, perform anti-apoptotic functions (Iwakiri and Takada, 2010). The EBERs had previously been eliminated from our search for the ionomycin resistance gene(s) since the presence of the EBERs in latency I Mutu cells was insufficient for protection against ionomycin. However, since the levels of EBERs are known to be

highly variable between different BL cells (Paul Farrell – personal communication) and this result was based on just one BL cell line, we decided to investigate further the possible involvement of EBER1 and EBER2. Recombinant EBER1 KO and EBER2 KO viruses, constructed from the pHB9 EBV-BAC (by Paul Farrell and Goran Gregorovic), were used to establish stable BL31 and BL2 cell lines. This enabled us to assess the contribution of EBER1 or EBER2 to the resistance against ionomycin-induced apoptosis in the same viral genetic background in which our panel of recombinant EBVs was constructed. The loss of either EBER1 or EBER2 in converted BL31 and BL2 cells did not sensitize these cells to ionomycin-induced apoptosis. These observations indicate that EBER1 and EBER2 are unnecessary for EBV-mediated rescue against ionomycin.

We have previously discussed the possible contribution of the viral Bcl-2 homologue BHRF1 in its capacity as a lytic protein. BHRF1 is thought to be essential for the initial evasion of apoptosis *in vivo* which allows EBV to establish a latent infection and cause cellular transformation. Expression of BHRF1 becomes undetectable once latent infection is established (Altmann and Hammerschmidt, 2005). Recent work has shown that BHRF1 may not be a strictly lytic protein as it has been detected by Western blotting during latency in Wp-restricted BL cells (Kelly *et al.*, 2009). It is thought that the BHRF1 protein is inappropriately expressed in Wp-restricted cells as the deletion spanning the EBNA2 region found in these cells brings the BHRF1 gene in closer proximity to Wp. By using a highly sensitive T cell recognition assay, Kelly and colleagues also demonstrate that BHRF1 is expressed at very low levels in LCLs where Cp is the dominant promoter but Wp remains constitutively active at low levels (Kelly *et al.*, 2009). Studies performed using latency I BL cells transfected with a BHRF1-expression plasmid have shown that BHRF1 can provide survival advantage against apoptosis induced by cytokine deprivation, ionomycin and anti-IgM (Henderson *et al.*, 1993; Kelly *et al.*, 2009). Since both these studies were conducted in BHRF1 gene transfectants of BL cells in the absence of most other viral latent genes, this prompted us to address the contribution of the BHRF1 protein to ionomycin resistance in the context of the virus. Hence, a recombinant EBV-BAC in which the BHRF1 ORF has been deleted and its revertant were constructed. However, stable HEK293 producer cell lines harbouring the

BHRF1 ORF-KO BAC grew poorly and generated very low titres of infectious virus. We were unable to establish stable BL cell lines using recombinant BHRF1 ORF-KO EBV for further investigation. It is possible that the deletion of the BHRF1 ORF may have affected the function of *oriLyt*. A recent report has suggested that several regions within the BHRF1 gene contributed to efficient *oriLyt* function (Rennekamp and Lieberman, 2011).

The three EBV miRNAs encoded within the BHRF1 locus which have been detected in BL cells exhibiting latency III as well as LCLs (Cai *et al.*, 2006) are potential candidates in our search for the ionomycin resistant gene(s). Predicted targets of the BHRF1 miRNAs include regulators of cell proliferation and apoptosis. For example, predicted targets of miR-BHRF1-1 include the tumour suppressor p53 and the transcription factor E2F1 (or retinoblastoma binding protein 3) (Pfeffer *et al.*, 2004) while miR-BHRF1-3 is predicted to target the pro-apoptotic BH3-only factor Bid (Lagana *et al.*, 2010). A recent study by Seto and colleagues has demonstrated that the BHRF1 miRNAs can inhibit apoptosis and promote cell cycle progression and proliferation during the early stages of B cell infection (Seto *et al.*, 2010). miR-BHRF1-1 is located within the 5'UTR of the BHRF1 gene while miR-BHRF1-2 and miR-BHRF1-3 are located within the 3'UTR. Due to concerns that deletion of the BHRF1 miRNAs may affect normal expression of the BHRF1 protein, a recombinant EBV-BAC which harbours a deletion of the entire BHRF1 locus and its revertant were generated for initial investigations into the contribution of BHRF1 and the BHRF1 miRNAs. HEK293 producer cell lines harbouring the BHRF1 locus-KO BAC generally generated lower titres of infectious virus than those harbouring its revertant. In comparison to the revertant, BL cells infected with the recombinant BHRF1 locus KO virus grew poorly early after infection but improved after several passages in culture. We successfully established BL31 cell lines using the recombinant BHRF1 locus KO virus but not BL2. Deletion of the BHRF1 locus sensitized converted BL31 cells to ionomycin indicating that the BHRF1 locus is involved in mediating the resistance to ionomycin. Having shown that the BHRF1 locus affects ionomycin resistance, it is important to tease out if this is due to the BHRF1 protein or miRNAs.

The loss of protection due to deletion of the BHRF1 locus is only partial, implying that other EBV factors are also necessary for ionomycin resistance. Since results from Chapter 3 indicate that LMP1 may contribute to this phenotype, we hypothesised that the resistance against ionomycin is dependent on both LMP1 and the BHRF1 locus. In order to investigate this, we constructed a recombinant EBV-BAC harbouring compound deletion of the EBNA2 ORF (which cannot express LMP1) and the BHRF1 locus (data not shown). Unfortunately, we were unable to progress further with this work due to time constraints.

It has been discussed in Section 6.2 that it is unlikely truncated EBNA-LP is involved in the EBV-mediated resistance to ionomycin. However, since truncated (but not full-length) EBNA-LP has been associated with protection of BL cells against apoptosis through the inhibition of protein phosphatase 2A (Garibal *et al.*, 2007) (see Section 1.3), it would be prudent to assess the contribution of EBNA-LP by generating a recombinant virus in which EBNA-LP is deleted .

Other candidates in our search for the ionomycin resistant gene(s) are the BART miRNAs which have been reported to be expressed in BL cells albeit at lower levels that those observed in NPC cells (Cai *et al.*, 2006). There is some experimental evidence that BART miRNAs can target cellular pro-apoptotic factors to promote cell survival. It has demonstrated that miR-BART5 suppresses expression of the BH3-only pro-apoptotic protein Puma to rescue NPC cells against apoptosis induced by the genotoxic agents adriamycin and etoposide (Choy *et al.*, 2008). However, miR-BART5 is not expressed in most of the EBV-positive BL cells included in this study. The 12kb deletion spanning the BART region in B95.8 (the strain from which pHB9 EBV-BAC was constructed) has eliminated 17 of the 22 BART miRNAs, leaving only miR-BART1, 2, 3, 4 and 15. Predicted targets of BART miRNAs present in B95.8 include several cellular pro-apoptotic factors. For example, caspase-10 and caspase-3 are predicted targets of miR-BART3 and miR-BART15 respectively (Lagana *et al.*, 2010). The BART miRNAs may also regulate apoptosis by modulating viral targets. For example, miR-BART1 has been shown to inhibit the growth-inhibitory effects resulting from overexpression of LMP1 in epithelial cells (Lo *et al.*, 2007).

6.4 Cellular factors involved in EBV-mediated protection against ionomycin-induced apoptosis

Previous experiments using the genotoxic drugs cisplatin, roscovitine and nocodazole have revealed an apoptotic pathway which is dependent on the pro-apoptotic BH3-only protein Bim (Leao *et al.*, 2007; Anderton *et al.*, 2008). This pathway is also dependent on EBNA3A and EBNA3C as the absence of either latent protein results in an upregulation of Bim and consequently renders BL cells sensitive to apoptosis induced by the genotoxic agents mentioned above. This Bim-dependent apoptotic pathway was shown to be independent of the tumour suppressor p53. Using ionomycin as the inducer of apoptosis, we have demonstrated that EBV-mediated protection is independent of Bim since EBV infection can rescue Bim-negative BL2 cells from ionomycin-induced apoptosis. In addition, EBV-positive BL31 cells lacking EBNA3A and/or EBNA3C are resistant to ionomycin treatment even though repression of Bim is relieved in these cells. Protection conferred by EBV against ionomycin is observed in BL cells carrying WT (BL2, Oku-BL and Sal-BL) and mutant (BL41 and BL31) p53 (Allday *et al.*, 1995b; Anderton *et al.*, 2008) indicating that this apoptotic pathway is not dependent on the p53 status of BL cells. Hence, we can conclude that the apoptotic pathway induced by the calcium ionophore ionomycin is both Bim- and p53-independent.

In order to identify cellular factors associated with the apoptotic pathway activated by ionomycin, we initially surveyed the expression of pro-apoptotic BH3-only members of the Bcl-2 family (including Bad, Bid and Puma) by Western blotting in cells exposed to ionomycin since BH3-only proteins are critical mediators of the intrinsic apoptotic pathway. With the exception of Noxa, Western blot analyses for all other BH3-only proteins surveyed did not reveal any trends consistent with survival data. Results from Section 5.1 have shown that in response to ionomycin, Noxa protein is consistently upregulated in EBV-negative and latency I BL cells sensitive to the treatment. In contrast, upregulation of Noxa protein was not observed in any EBV-positive BL cells resistant to ionomycin-induced apoptosis. These data suggest that apoptosis triggered by ionomycin involves the induction of Noxa and that EBV rescues BL cells from apoptosis by inhibiting the induction of Noxa.

Although we have shown that LMP1 can provide partial protection against ionomycin-induced apoptosis, the expression of LMP1 alone was insufficient for preventing the induction of Noxa protein. It was also surprising to observe that Noxa protein was not upregulated in EBV-positive BL cells lacking the BHRF1 locus which are partially sensitive to ionomycin treatment. This implies that ionomycin must also activate other pro-apoptotic factors besides Noxa.

Having shown that sensitivity to ionomycin is consistent with upregulation of Noxa, it was important to determine if Noxa is involved in mediating ionomycin-induced apoptosis. To address this issue, we used lentiviral vectors expressing Noxa shRNA to establish stable EBV-negative BL cell lines in which Noxa expression is knocked down. Reducing Noxa expression by shRNA enhanced the survival of EBV-negative BL31 and BL2 cells challenged with ionomycin. This confirms that ionomycin-induced apoptosis is mediated by Noxa and inhibition of Noxa induction, as seen in EBV-positive BL cells resistant to ionomycin treatment, promotes cell survival.

Having confirmed the role of Noxa in the ionomycin-activated apoptotic pathway, we were keen to understand how Noxa is regulated in these cells in order to identify ionomycin-responsive modulators of Noxa induction and elucidate the mechanism(s) employed by EBV to inhibit Noxa induction and protect cells from ionomycin-induced apoptosis. Noxa was originally identified as a p53 target gene (Oda *et al.*, 2000a). Recent studies have also demonstrated that Noxa can be activated in a p53-independent manner by E1A (Flinterman *et al.*, 2005), p73 (Martin *et al.*, 2009) and interferon via IRF-1 or IRF-3 (Lallemand *et al.*, 2007). By using quantitative real-time PCR, we established that Noxa was upregulated at the level of transcription in EBV-negative BL31 and EBV-negative BL2 cells, both of which are sensitive to ionomycin. The upregulation of Noxa mRNA observed in BL2 was significantly greater than that observed in BL31. This may be due to the presence of WT p53 in BL2. In contrast, upregulation of Noxa mRNA levels was not observed in WT-infected BL cells resistant to ionomycin.

We had hoped to recapitulate these results by performing a luciferase assay to determine the effect of ionomycin on Noxa promoter activity. If these experiments had shown that ionomycin activated the Noxa promoter, we will then be able to systematically delete regions of the Noxa promoter (eg. p53-response elements, IRF-response elements) and re-test for activity in order to identify regions of the Noxa promoter activated by ionomycin. However, we did not observe any increase in Noxa promoter activity by ionomycin in these experiments. This may be due to the fact that these experiments were carried out using BL cells (DG75 and BJAB) which are not sensitive to ionomycin treatment. Consistent with that, upregulation of Noxa protein is not observed in these cells. Alternatively, the Noxa promoter-luciferase reporter constructs used may not contain the promoter region activated by ionomycin. We were unable to perform these experiments using BL31 and BL2 cells due to the very poor transfection efficiencies obtained when using these cell lines.

A microarray experiment can be performed to identify factors involved in the regulation of Noxa and other components of the ionomycin-activated apoptotic pathway.

6.5 Apoptotic pathways activated by staurosporine and etoposide in BL cells

In BL cells, genotoxic agents activate a Bim-dependent apoptotic pathway while the calcium ionophore ionomycin activates an apoptotic pathway associated with Noxa. Latent EBV can suppress both apoptotic pathways to promote cell survival. We hypothesised that other genotoxins also induce the Bim-dependent apoptotic pathway while non-genotoxic agents induce the Noxa-associated apoptotic pathway in BL cells. In order to investigate this hypothesis, the responses of BL cells to apoptosis induced by the protein kinase inhibitor staurosporine and the topoisomerase II inhibitor etoposide were examined. While it is likely that anti-IgM activates the same pathway as ionomycin, we decided not to include anti-IgM in this study due to the high cost of the antibody used to induce apoptosis.

When challenged with staurosporine, EBV-negative and latency I BL cells rapidly undergo apoptosis. Infection with WT EBV provides a survival advantage over uninfected cells regardless of Bim and p53 status. Deletion of the EBNA3 locus did not affect resistance to staurosporine-induced apoptosis indicating that the EBNA3 proteins are not necessary for this protection. EBV-mediated staurosporine resistance is however, at least partially, dependent on the BHRF1 locus. Upregulation of Noxa protein was observed in EBV-negative and latency I BL cells sensitive to staurosporine but not in EBV-positive BL cells resistant to the treatment. Although deletion of the BHRF1 locus partially sensitises BL cells to staurosporine-induced apoptosis, we did not detect any upregulation of Noxa protein. These observations indicate that staurosporine, like ionomycin, activates the Noxa-associated apoptotic pathway in BL cells.

Etoposide is a DNA topoiosmerase II inhibitor and induces apoptosis via a different mechanism from the other genotoxic agents previously investigated. EBV-negative and latency I BL cells are sensitive to etoposide and undergo apoptosis in response to the treatment. The full latency III pattern of gene expression rescued Bim-positive BL31 and Mutu cells from etoposide-induced apoptosis. In contrast, latent EBV did not confer resistance against etoposide in Bim-negative BL2 cells. Apoptosis triggered by etoposide in BL2 cells appears to be mediated by p53 which is consistent with previous studies showing that EBV does not confer protection against p53-dependent apoptosis (Allday *et al.*, 1995b). These results suggest that EBV-mediated protection against etoposide is dependent on Bim. This is supported by the observation that deletion of the EBNA3 locus, which results in the loss of Bim downregulation by EBNA3A and EBNA3C, sensitizes BL31 cells to etoposide-induced apoptosis. It was surprising to discover that deletion of the BHRF1 locus partially diminishes resistance to etoposide even though EBV-mediated downregulation of Bim is unaffected in these cells. Apoptosis induced by etoposide does not appear to be mediated by Noxa since induction of Noxa was not observed in BL cells sensitive to etoposide. Although it was shown that p53 is upregulated by etoposide in BL2 cells, it was surprising to observe that neither Puma nor Noxa, the two BH3-only proteins associated with p53-mediated apoptosis, appear to be involved in mediating apoptosis. These observations suggest that etoposide, like the

set of genotoxic agents previously investigated, induce the Bim-dependent apoptotic pathway. However, the discovery that the BHRF1 locus can also contribute to etoposide resistance without affecting Bim suggests that there are other pro-apoptotic factors involved in mediating etoposide-induced apoptosis which can also be suppressed by EBV. Further investigation into the role of the BHRF1 locus in the resistance to cisplatin, nocodazole and roscovitine is important for confirming these observations and obtaining a better understanding of the mechanism by which the EBV suppresses apoptosis through the BHRF1 locus.

We can conclude that agents which induce genotoxic stress primarily activate the Bim-dependent apoptotic pathway while non-genotoxins induce apoptosis via the Noxa-associated pathway. While we have highlighted the differences between the apoptotic pathways induced by these two sets of apoptotic inducers, they do share one common aspect: the participation of the BHRF1 locus in the EBV-mediated inhibition of apoptosis. This would be consistent with increasing the 'Bcl-2-like' pro-survival activity independent of the activating pathway.

6.6 Conclusions

Work presented in this thesis has shown that there are at least two disctinct pathways by which BL cells undergo apoptosis and which can be suppressed by latent EBV. The apoptotic pathway activated by genotoxic agents is dependent on high levels of Bim and hence, co-expression of EBNA3A and EBNA3C promotes survival. Non-genotoxic agents such as ionomycin and staurosporine (and probably anti-IgM) trigger apoptosis via the Noxa-associated pathway. Although the BHRF1 locus provides partial protection against ionomycin- and staurosporine-induced apoptosis, this is not achieved through the inhibition of Noxa induction. The mechanism by which EBV inhibits Noxa induction and the latent gene(s) involved has yet to be identified. We have also shown that in addition to EBNA3A and EBNA3C, the BHRF1 locus also contributes (perhaps less significantly) to the EBV-mediated rescue from apoptosis induced by the genotoxic agent etoposide. This suggests that there are probably additional pro-apoptotic cellular factors (besides

Bim and Noxa) involved in mediating apoptosis triggered by both sets of drugs. It is likely that EBV confers resistance by suppressing a range (and not just one) of pro-apoptotic factors activated through the actions of different latent gene products.

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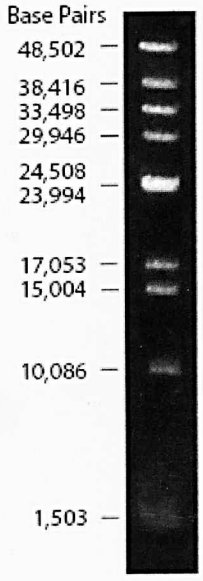
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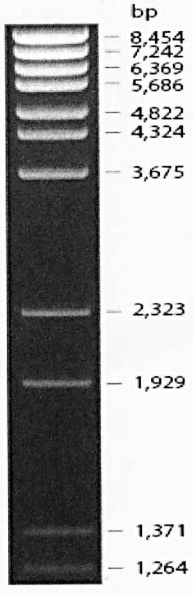
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Appendix A DNA Markers

λ-DNA mono cut mix

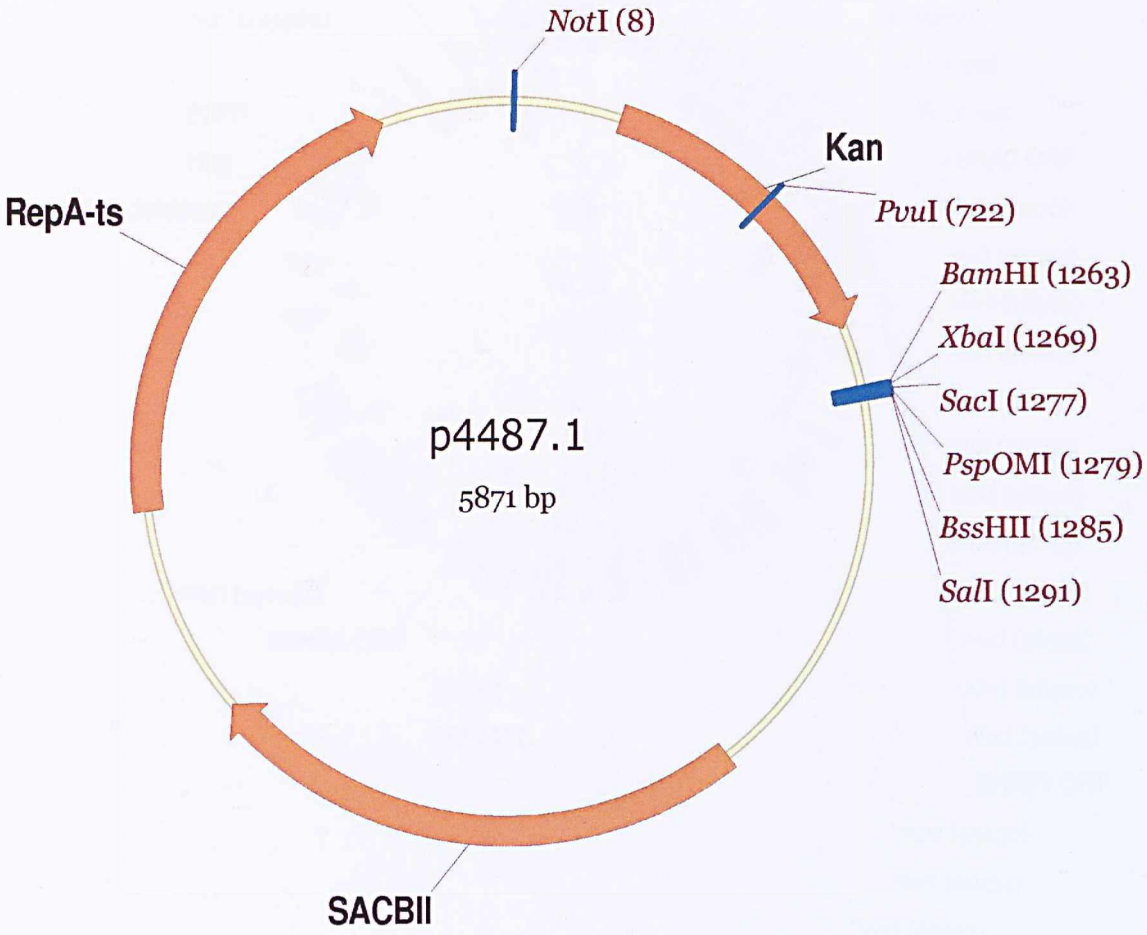


λ-DNA *Bst*EII



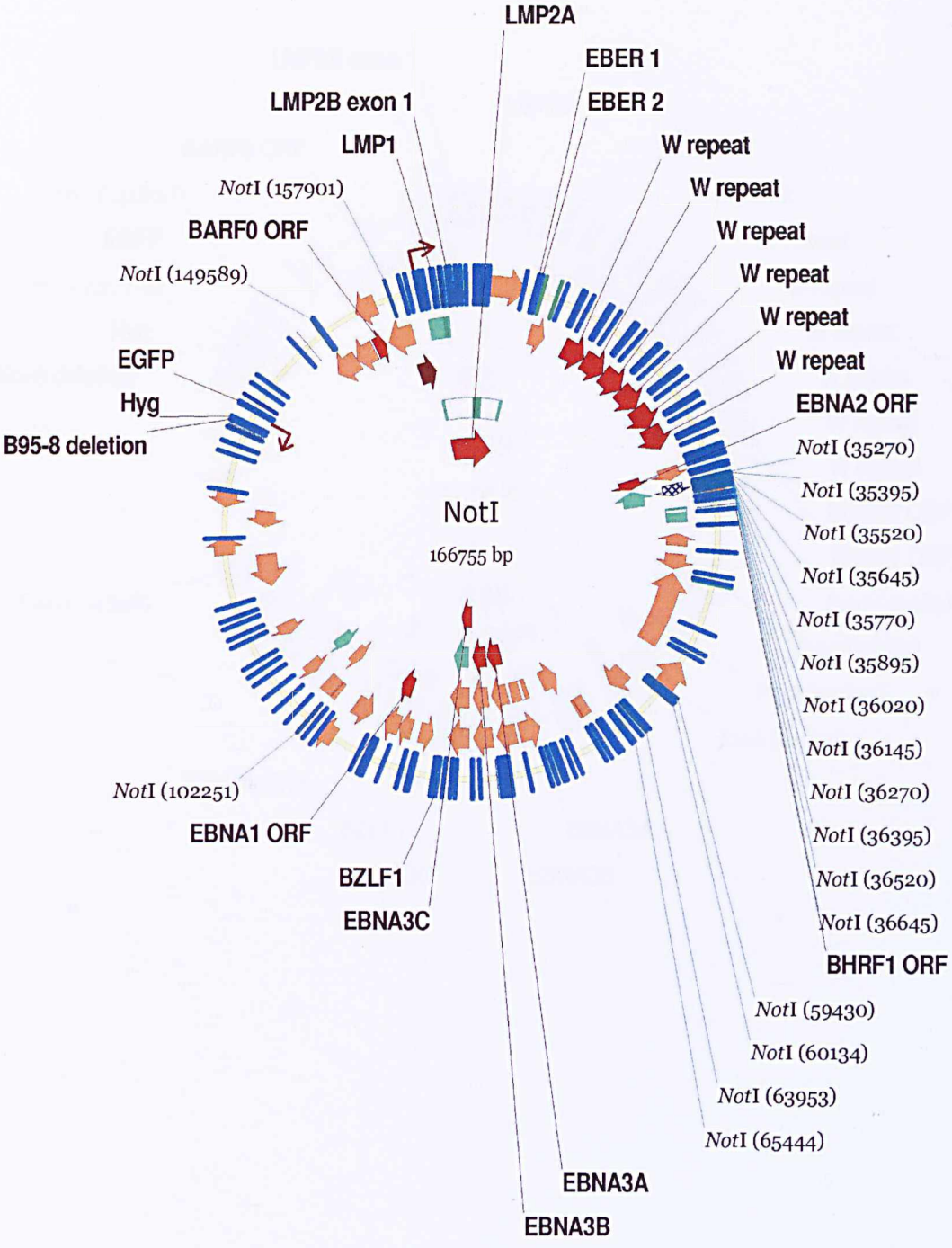
Appendix B Plasmid maps

Shuttle plasmid p4487.1



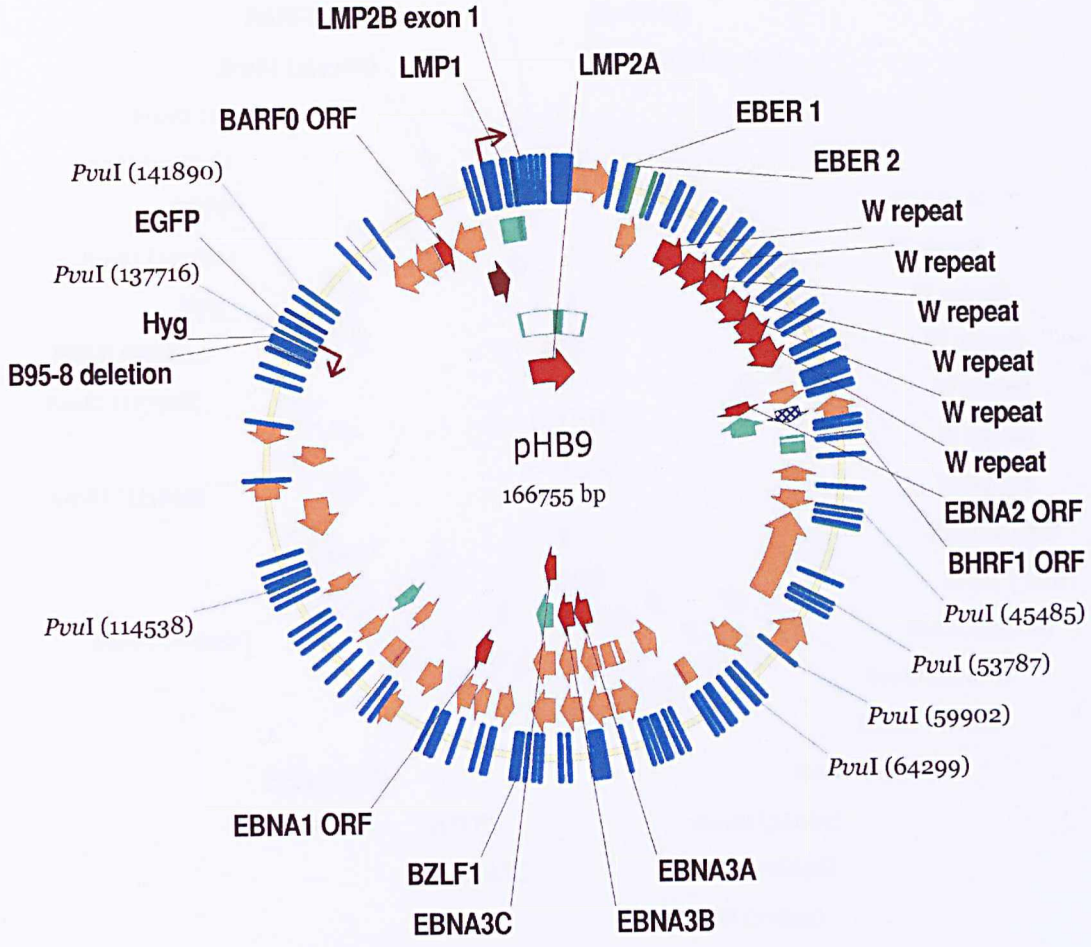
pHB9 EBV-BAC

*Not*I restriction sites



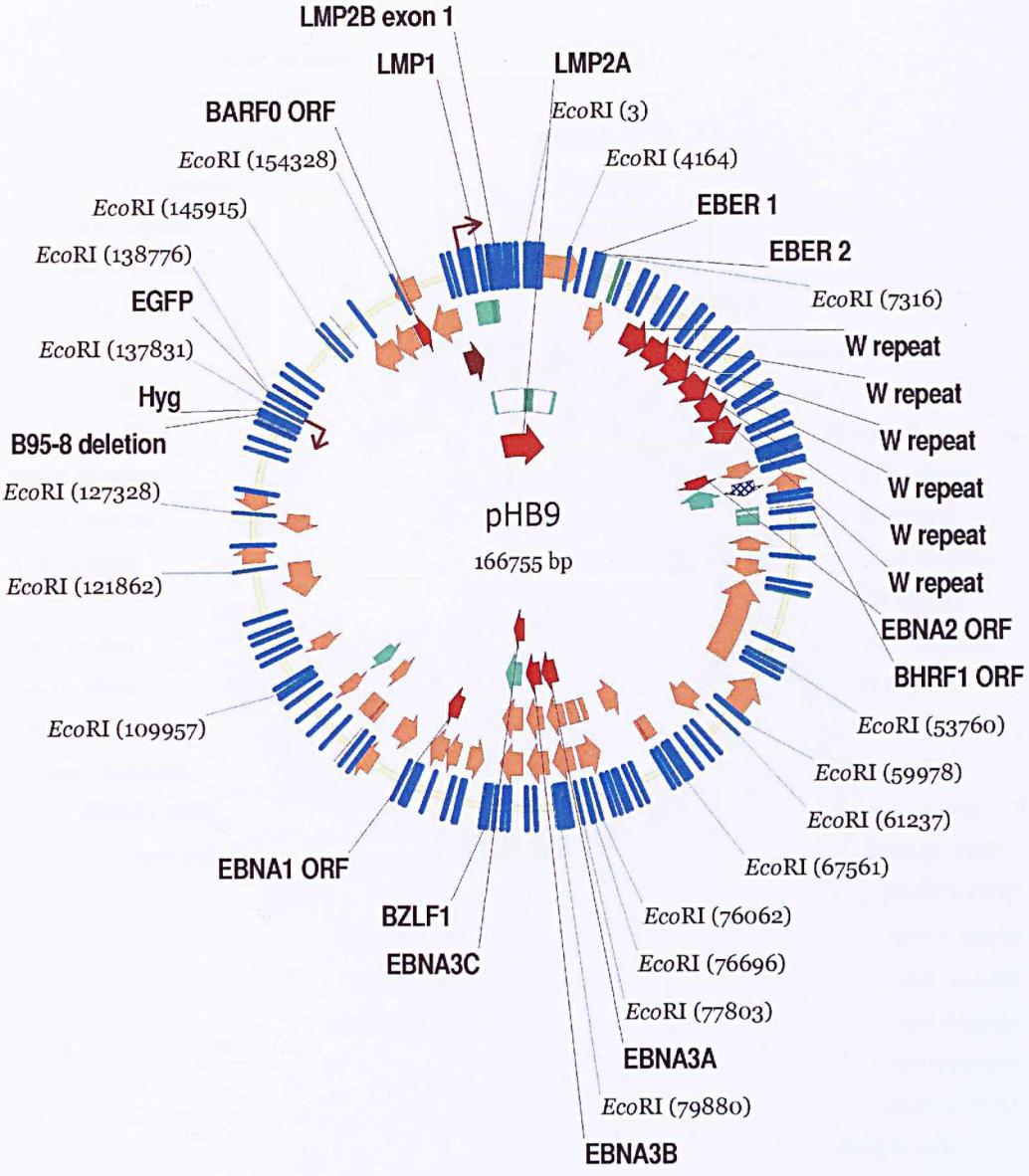
pHB9 EBV-BAC

*Pvu*I restriction sites



pHB9 EBV-BAC

*Eco*RI restriction sites



pHB9 EBV-BAC

*Age*I restriction sites

