STRUCTURE AND FUNCTION OF THE EPSTEIN-BARR VIRUS BZLF1 PROTEIN

Thesis submitted by

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Abstract

The BZLF1 protein of Epstein-Barr virus is a gene regulatory molecule and is thought to play a crucial role in the switch from latent to productive viral infection. BZLF1 is a sequence specific DNA binding protein capable of transactivating other EBV early genes.

BZLF1 has homology to the c-fos protein and was initially shown to bind AP-1 sites. Analysis of the binding specificity of BZLF1 demonstrates that, unlike other members of the c-fos family, it can bind sequences similar to both AP-1 and C/EBP binding sites. A consensus for BZLF1 binding has been deduced by comparison of naturally occuring binding sites and the results of point mutagenesis.

The DNA binding domain of BZLF1 has been defined by deletion analysis. This domain has a bipartite structure; it has a basic motif required for DNA binding, but not for dimerisation, and an adjacent structure required for dimerisation as well as DNA binding. Transient transfection experiments have identified a transactivation domain in the N-terminal part of the protein.

Unlike other members of the c-fos family the BZLF1 dimerisation domain does not possess a heptad repeat of leucines. However, site directed mutagenesis suggests that BZLF1 has a coiled-coil dimerisation domain lacking a leucine repeat. These results show that BZLF1 may be considered to be a member of the bZIP family of proteins. BZLF1 does not appear to heterodimerise with any other viral or cell protein.

Antibodies directed against BZLF1 are an important diagnostic indicator in nasopharyngeal carcinoma. Sera from NPC patients, a monoclonal antibody and several rabbit sera have been analysed for their reactivity against regions of BZLF1.

The levels of various lytic transcripts have been investigated after induction of the EBV positive cell line Akata in the presence or absence of an inhibitor of protein synthesis. This approach identifies some immediate early promoters of EBV.

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Declaration

All the work presented in this thesis is the result of my own work and in no other way forms part of any other thesis. This work was carried out while I was a student at the Ludwig Institute for Cancer Research, St.Mary's Hospital Medical School (a constituent college of Imperial College of Science, Technology and Medicine) under the supervision of Professor D.J. Jeffries (University of London) and Dr P.J. Farrell (Ludwig Institute for Cancer Research).



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Publications

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Kouzarides, T., Packham, G. and Farrell, P.J. (1991) The BZLF1 protein of EBV has a coiled coil dimerisation domain without a heptad leucine repeat but with homology to the C/EBP leucine zipper. Oncogene 6: 195-204

Young, L.S., Lau, R., Rowe, M., Niedobitek, G., Packham, G., Shanahan, F., Rowe, D.T., Greenspan, D., Greenspan, J.S., Rickinson, A.B. and Farrell, P.J. (1991) Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. J.Virol. 65: 2868-2874

Abbreviations

BL	Burkitt's Lymphoma
CAT	Chloramphenicol Acetyl Transferase
C/EBP	CCAAT/Enhancer Binding Protein
CREB	cAMP Response Element Binding Protein
CTL	Cytotoxic T-Lymphocytes
CMV	Cytomegalovirus
DTT	Dithiothreitol
DS_L	Duplicated Sequence Left
DS_R	Duplicated Sequence Right
EA	Early Antigens
EB	Epstein-Barr
EBER	Epstein-Barr Virus Encoded RNA
EBNA	Epstein-Barr Virus Nuclear Antigen
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetra-acetic acid
HEPES	2-Hydroxyethylpiperazine-2-ethanesulphonic acid
HD	Hodgkin's Disease
HSV	Herpes Simplex Virus
IM	Infectious Mononucleosis
LCL	Lymphoblastoid Cell Line
LMP	Latent Membrane Protein
LP	Leader Protein
MA	Membrane Antigen
MSH	2-mercaptoethanol
NPC	Nasopharyngeal Carcinoma
OHL	Oral Hairy Leukoplakia
PAGE	Polyacrylamide Gel Electrophoresis
PMSF	Phenylmethylsulphonyl Fluoride
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic Acid
TLC	Thin Layer Chromotography
TP	Terminal Protein
TPA	12-O-tetradecanoyl Phorbol-13 Acetate
TR	Terminal Repeat
VCA	Viral Capsid Antigen
VZV	Varicella-Zoster Virus

Chapter 1
Introduction

1.1 The Herpesviridae

More than 80 distinct members of the family Herpesviridae have been isolated and partially characterised (reviewed in Roizman, 1982). The virus particles of this family are characterised by the presence of a large linear double-stranded DNA genome (between 100 and 250kb in size) packaged within the core of an icosahedral capsid which is in turn surrounded by a host-derived membrane. Although these viruses have a characteristic morphology by electron-microscopic examination they vary greatly in biological properties, such as host range, genome size and speed of replication. However, all members have a capacity to maintain a latent infection within their host.

At least seven herpesviruses are known to infect humans; herpes simplex viruses type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpes viruses type 6 (HHV-6) and type 7 (HHV-7).

The herpesviruses are traditionally classified into three subfamilies (the alpha, beta and gamma Herpesviridae) on the basis of host range and cell tropism, as well as in vitro growth characteristics. The Epstein-Barr virus is the prototype of the gamma-herpesviruses and all members of this subfamily replicate in vitro in either B- or T-lymphocytes; some also cause lytic infections in epithelial and fibroblast cells. Other well characterised herpesviruses traditionally classified as members of the gamma subfamily include herpesvirus saimiri (HVS) and the Marek's disease Virus (MDV) which are both T-lymphotropic. Difficulty in classification of herpesviruses has arisen following molecular analyses of the viral genomes. For example, the genome arrangement and predicted protein sequences of MDV, traditionally classified as a gamma herpesvirus, is more closely related to that of the alpha-herpesvirus HSV than EBV (Buckmaster et al., 1988).

1.2 Epstein-Barr Virus

Epstein-Barr virus was discovered in 1963 in the lymphoblastoid cell line EB1 derived from a Burkitt's lymphoma biopsy (Epstein et al., 1964; Epstein and Barr, 1964). Electron microscopy revealed particles with characteristic herpesvirus morphology and subsequent analysis

demonstrated that the virus was a previously uncharacterised human herpesvirus.

1.2.1 The Virus Particle

As with other members of the Herpesviridae the immature EB virus particle consists of a dense central core of DNA and protein which is surrounded by an icosahedral nucleocapsid composed of 162 individual capsomeres (reviewed in Kieff et al., 1982; Epstein and Achong, 1979a). The mature virus particle is 120nm in diameter and consists of the capsid surrounded by a trilaminar membrane envelope. The space between the capsid shell and the envelope is filled with an amorphous tegument.

The mature virus contains more than 30 component polypeptides (Dolyniuk et al., 1976a,b), a number of which are likely to be generated by post-translational modifications of common precursors. The major protein in the nucleocapsid is a 160kD polypeptide which is a component of the viral capsid antigen (VCA; Vroman et al., 1985).

The capsid is assembled in the nucleus and viral DNA is packaged at this point before the host derived membrane is added by budding through cell membranes. The mature virus envelope includes three abundant glycoproteins (gp) of apparent molecular weights 350, 220 and 85 kD as well as an additional non-glycosylated protein of 140kD which are expressed in the late productive cycle (Thorley-Lawson and Edson, 1979; Qualtiere and Pearson, 1979). Together these polypeptides are known as the membrane antigen (MA). The exact mechanism of virus egress and glycoprotein addition is not clear and may involve a complex process of envelopment at the inner nuclear membrane followed by de-envelopment and subsequent re-envelopment at the plasma membrane or Golgi complex associated vesicles (Gong and Kieff, 1990; Rabanus et al., 1991).

1.2.2 Virus Genome

The EB virus genome is a double stranded DNA molecule about 170kb in length that is linear within the virus particle (reviewed in Farrell, 1989). The molecule circularises soon after infection of a cell by joining of the terminal repeat (TR) sequences and the DNA is associated with nucleosomes in latently infected cells (Dyson and Farrell, 1985; Shaw et al., 1979). The genome is usually maintained as an episome but has integrated in the Namalwa (Matsuo et al., 1984; Henderson et al., 1983) and IB4

(Henderson et al., 1983; Hurley et al., 1991b) cell lines and some degree of integration may occur in many EBV infected cells (Hurley et al., 1991a).

The genome is conveniently subdivided into four regions; the short and long unique regions (U_S and U_L respectively), separated by the major internal repeat (IR) and the terminal repeats (figure 1.1). These regions are not simply equivalent to the same four regions in HSV to which they are superficially similar. There is only one relative arrangement of the regions in EBV as compared with the four isomeric forms of HSV. In addition the repeats are not distinct genetic units since transcription units are now known to span all of the repeat boundaries. The number of copies of the TR and of the IR sequences in the circular viral genome is variable (Allan and Rowe, 1989; Raab-Traub and Flynn, 1986).

The sequence of the entire genome has been obtained for B95-8 virus (Baer et al., 1984; Laux et al., 1988b; Allan and Rowe, 1989). B95-8 has a sizeable (13.6kb) deletion relative to most other strains (Raab-Traub et al., 1980) and the sequence of the Raji strain has been determined in this region (Parker et al., 1990). The sequence of the EBV genome revealed an excess of G and C content (59.9%) in the genome (Baer et al., 1984) and selective methylation of CpG residues has been reported. This has been suggested to reflect direct control of EBV gene expression by methylation (Szyf et al., 1985; Li-Fu et al., 1991).

Restriction maps of individual EBV strains show some differences in restriction sites though when the sequence of equivalent sections of B95-8 and Raji DNA were compared the variation at the nucleotide level was low (typical variation 0.4%; Hatfull et al., 1988). However, significant variation in several genes has been reported. In particular, two EBNA-2 DNA hybridisation types (A and B or 1 and 2) exist that stem from sequence divergence within the EBNA-2 open reading frame (ORF; Dambaugh et al., 1984; Adldinger et al., 1985). These proteins are antigenically distinct (Rickinson et al., 1987) and EBV isolates can be classified as type A or B depending on their EBNA-2 gene. Distinction between the type A and B virus has now been extended to the EBNA-3 family (Rowe et al., 1989; Sculley et al., 1989; Sample et al., 1990), the EBER sequences (Arrand et al., 1989) and the 3' end of the EBNA-LP gene (Huen et al., 1988) and there are quantitative differences between virus strains in their biological activities, for example, type A is more efficient at immortalising B-cells (Rickinson et al., 1987) and this is due, in part, to variations in the EBNA-2 gene (Cohen et al., 1989b). The virus types have

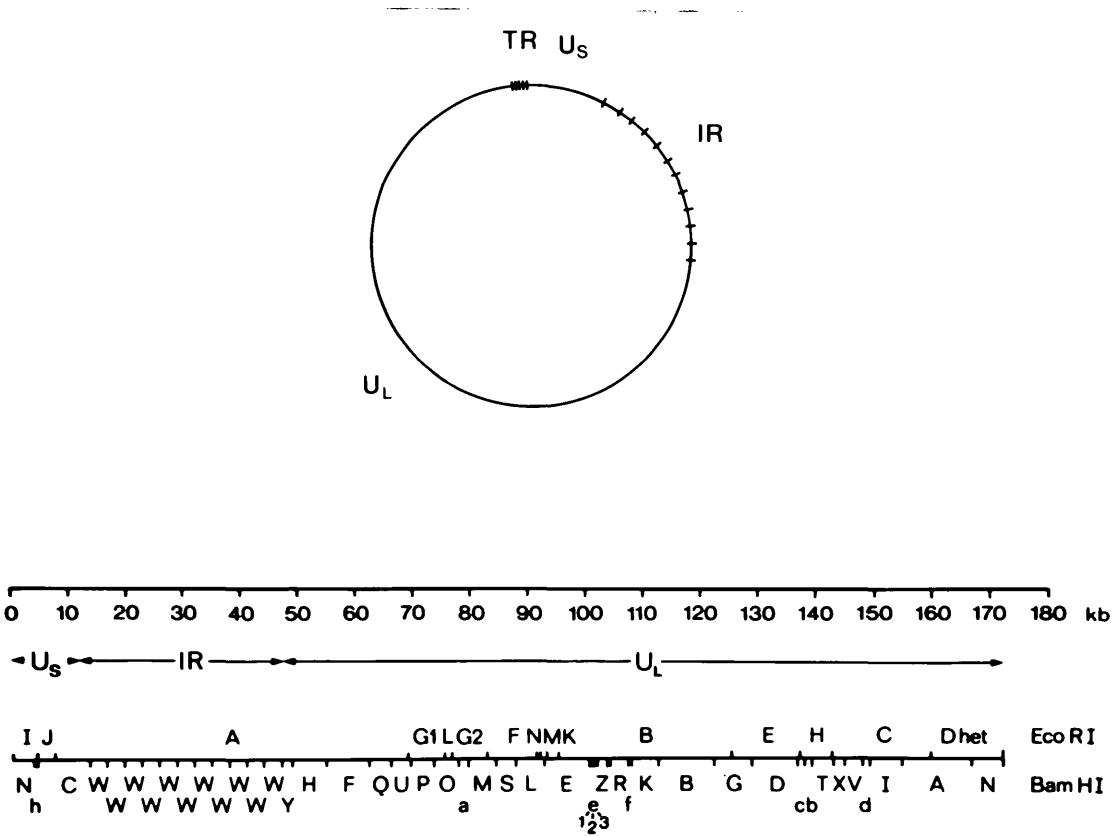


Figure 1.1 Epstein-Barr virus genome. The standard circular and linear representations of the B95-8 EBV genome are shown. The short unique (U_S), long unique (U_L), major internal repeat (IR) and terminal repeat (TR) are indicated. A restriction map for the enzymes EcoRI and BamHI is shown below. Principal open reading frames of EBV are named according to the BamHI fragment in which they begin, followed by L or R to indicate whether they are leftward or rightward on the standard map and then a number. For example, BZLF1 is the first leftward reading frame originating in the BamHI Z fragment of the genome.

different geographical distributions; type A virus is common worldwide while type B virus is more common in African populations (Zimber et al., 1986; Young et al., 1987). A further EBV variant that contains a restriction fragment length polymorphism (the f variant) is present in Chinese populations (Lung et al., 1990).

Some common EBV laboratory strains have deletions relative to each other and it is likely that at least some of these deletions have arisen in vitro. The prevalence of deletions in natural isolates has not been well documented. Some laboratory strains of EBV contain defective viral particles; the clearest example of this is the P3HR-1 strain of EBV. Some of these defectives do not act to interfere but promote the virus productive cycle (Rabson et al., 1983; Miller et al., 1984). This is because the defectives have rearranged EBV genomes which result in the constitutive expression of genes, including BZLF1 and BRLF1, which are critical for induction (see section 1.5.5). It has been reported that defectives can be found in viral isolates from certain patients although the significance of defectives in natural infection is unclear (Alfieri and Joncas, 1987; Patton et al., 1990).

1.2.3 Cell Infection and Cell Tropism

EBV has a narrow host range and infects humans and closely related primates. In humans the virus is known to infect B-lymphocytes and certain epithelial tissues of the oropharynx and cervix (Jondal and Klein, 1973; Wolf et al., 1984; Sixbey et al., 1986), although only the former tissues are readily infected in vitro. The lack of a simple in vitro system for studying infection in epithelial cells means that much of the work comes from the study of infected lymphocyte lines.

The EBV receptor on B-cells has been shown to be the cluster designation (CD) 21 antigen, a 145kD cell surface protein which acts as the receptor for the C3dg complement component (Fingeroth et al., 1984; Nemerow et al., 1985). CD21 (also termed CR2; complement receptor 2) is known to bind gp350/220 (Tanner et al., 1987) and gp350/220 and C3dg interact with the same part of CD21 (Lowell et al., 1989). Indeed, it has been shown that a part of gp350/220 that has sequence homology with C3dg plays a dominant role in ligand binding to CD21 (Nemerow et al., 1989). Following complexing of gp350/220 and CD21 the virus enters the cell by receptor-mediated endocytosis (Tanner et al., 1987). Antibodies specific for gp350/220 and CD21 have been shown to be able to act as neutralising antibodies and inhibit virus release (Thorley-Lawson and Geilinger, 1980;

Sairenji et al., 1988; Hoffman et al., 1980; Fingeroth et al., 1984). More recently, it has been demonstrated that both soluble CD21 and gp350/220 could also block EBV infection in vitro (Tanner et al., 1988; Nemerow et al., 1990). Any agent that is capable of interfering with virus infection has important implications for the development of an EBV vaccine (Morgan et al., 1988; Morgan et al., 1989). Once the virus has entered the cell, the genome is released from the virion and circularises via the TR. Infection of B-cells results in immortalisation of the cell and the virus establishes a latent infection, not replicating unless induced to do so.

Some monoclonal antibodies specific for CD21 also recognise a polypeptide on cervical epithelium (Young et al., 1986). However, the antigen on epithelial cells is a 200kD protein, different in size to the 145kD CD21 on B-cells (Young et al., 1989). Polymorphic variations in the CD21 coding sequence have been identified and these may result in distinct forms of CD21 being expressed on different cell types (Toothaker et al., 1989). Further research has shown unequivocally that EBV can infect epithelial cells in vitro (Sixbey et al., 1987) but the role of the 200kD protein is unclear. Recently it has been suggested that EBV specific IgA antibody-mediated transcytosis of virus may play a role in infection of epithelial cells (pers. comm. J. Sixbey).

1.3 Epstein-Barr Virus Gene Expression

EBV encodes about 80 genes of which all but two are translated into proteins (reviewed in Farrell, 1989). Transcription units span all of the unique regions and repeats and genes are classified as latent (those expressed in the latent cycle) or productive (those expressed after induction of the productive cycle). The virus relies heavily on the cell transcription, RNA processing and translation machinery although the virus does supply some factors which modulate gene expression.

1.3.1 Epstein-Barr Virus Latent Gene Expression

Eleven latent genes have been shown to be expressed in B-cells immortalised in vitro and they are therefore all candidates for being essential for or contributing to both initiation and maintenance of immortalisation (reviewed in Sugden, 1989). The latent gene products are the EBNAs (Epstein-Barr virus nuclear anigens) which are all localised in the nucleus of infected cells, the latent membrane proteins and the EBERs (Epstein-Barr virus encoded RNAs).

Expression of the EBNAs

The genes encoding the EBNAs are spread out over 100kbp of DNA and their transcripts form a family of mRNAs with some common 5' sequences which are joined to unique 3' sequences (reviewed in Speck and Strominger, 1989). The common 5' sequences span short exons derived from the IR and are sometimes capable of encoding EBNA-LP (depending on the exact splice pattern of these exons) while the other EBNAs are encoded by the unique 3' sequences. Therefore, EBNAs can be encoded by potentially bicistronic mRNAs (Wang et al., 1987). These transcripts are initiated at one of two promoters in the left-hand end of the genome, the BCR2 promoter in the U_S region (Bodescot et al., 1987) or the BWR1 promoter in the IR (Sample et al., 1986). Sequences immediately 3' to the promoters, the common 5' exons and the unique 3' exons are spliced together in a complex manner and splicing control may play an important role in control of expression of the EBNAs.

Use of the two promoters is mutually exclusive in clonal cell lines derived from Burkitt's lymphoma (BL) tumours or lymphoblastoid cell lines (LCLs) and the BCR2 promoter is the normal promoter in established latently infected cells (Woisetschlaeger et al., 1989). The BWR1 promoter is active soon after infection of B-lymphocytes in vitro and the virus subsequently switches to BCR2 (Woisetschlaeger et al., 1990). It is now known that BCR2 is transcriptionally activated by EBNA-1 (Sugden and Warren, 1989) and that there is an upstream enhancer responsive to EBNA-2 (pers. comm., C.M.Rooney; Sung et al., 1991). There is also a functional glucocorticoid responsive element within the BCR2 promoter and glucocorticoids may regulate latent gene expression (Kupfer and Summers, 1990; Schuster et al., 1991; Sinclair et al., 1992).

EBNA-1 can also be transcribed from a third promoter found in the U_L region of the genome (Sample et al., 1991). This promoter is used for expression of EBNA-1 in cells representative of BL cells in vivo (Sample et al., 1991; Schaefer et al., 1991) and in NPC cells (Brooks et al., 1991); it presumably allows the virus to express only EBNA1. There is a relatively low affinity EBNA1 binding site in the BamHI Q region close to this promoter (Jones et al., 1989) although it has not yet been demonstrated to regulate promoter activity through EBNA-1 binding.

EBNA1 and Plasmid Maintenance

EBNA1 is a DNA binding protein that is required for plasmid maintenance by binding to the latent origin of DNA replication, oriP (Yates et al., 1984; Reisman et al., 1985; Yates et al., 1985). The origin is located in the BamHI C region of the genome and consists of two essential cis-acting elements which are related in sequence. One consists of a region of about twenty 30bp tandem repeats and the other is a region of dyad symmetry which is close to the site of initiation of DNA replication (Gahn and Schildkraut, 1989). The tandem repeats contain the termination site and a replication fork barrier so bidirectional replication from oriP proceeds 99% unidirectionally and produces covalently closed, circular DNA molecules identical to the substrate. EBNA1 binds both elements (Jones et al., 1989) as a dimer (Ambinder et al., 1991) and higher order multimerisation between EBNA1 binding at the two elements may induce distortion in the DNA in this region leading to initiation of DNA replication. DNA replication has been thought to occur in the early S-phase of the cell cycle (Hampar et al., 1974) but a recent report demonstrates replication in the late-S phase (Carroll et al., 1991). The process requires host cell replication machinery (Yates et al., 1985) and maintains a stable number of plasmid molecules per cell (Yates and Guan, 1991).

The 30bp family of repeats also acts as a transcriptional enhancer of the BCR2 promoter when bound by EBNA-1 so EBNA-1 may regulate its own expression and that of the other EBNAs (Sugden and Warren, 1989). EBNA-1 is found in all EBV infected cells and is the only protein encoded by the virus to be expressed in cells representative of BL cells in vivo (Rowe et al., 1986a; Rowe et al., 1987; Rowe et al., 1988) reflecting its essential role in EBV plasmid maintenance.

EBNA-2

The importance of EBNA-2 in EBV mediated immortalisation of B-cells was first identified from the inability of P3HR-1 virus (which lacks the EBNA-2 gene) to immortalise cells (Delius and Bornkamm, 1978; Rabson et al., 1982; Jeang and Hayward, 1983). Recombination events which introduce an intact EBNA-2 gene are able to restore the immortalising potential of the P3HR-1 virus (Hammerschmidt and Sugden, 1989; Cohen et al., 1989b).

EBNA-2 has been shown to affect cell growth properties in several assays supporting the idea that EBNA-2 plays a role in immortalisation. Transfection of EBNA-2 into Rat-1 cells results in altered serum requirements (Dambaugh et al., 1986) and EBNA-2 alone can induce the expression of the B-cell activation markers CD21 and CD23 (Cordier et al., 1990). EBNA-2 may also stimulate the expression of the c-fgr proto-oncogene (Knutson, 1990). In addition EBNA-2 transactivates the promoters for the LMP-1, LMP-2A and LMP-2B genes and the BCR2 promoter (Abbot et al., 1990; Wang et al., 1990b; Zimber-Strobl et al., 1991; Sung et al., 1991) and these activities may also be involved in the immortalisation function of EBNA-2.

EBNA-3A, 3B and 3C

The EBNA-3A, 3B and 3C proteins are also known as EBNA-3, EBNA-4 and EBNA-6 respectively. The EBNA-3 family of proteins show considerable homology in their N-terminal sequences and their genes are likely to have arisen from triplication of a viral gene. Little is known about the functions of the EBNA-3 proteins though the EBNA-3C protein has been shown to induce the expression of the B-cell activation marker CD21 (Wang et al., 1990a). The EBNA-3B ORF has recently been shown to be non-essential for immortalisation (B.E.Tomkinson and E.Kieff, International Herpesvirus Workship, 1991) since an EBNA-3B deleted recombinant virus is still capable of immortalising cells.

EBNA-Leader Protein (EBNA-LP)

EBNA-LP is also known as EBNA5 and EBNA4. Transcription of the EBNA-LP mRNA spans exons derived from the IR and then splices into unique exons. The protein therefore has a repetitive N-terminus and a unique C-terminus. The mRNA can sometimes be potentially bicistronic for the other EBNAs and therefore the protein is called LP since it is encoded as a leader in mRNAs for the other EBNAs (Wang et al., 1987).

The function of EBNA-LP is not known. The 3' end of the gene (which encodes the unique C-terminus of the protein) is deleted along with the EBNA-2 gene in the non-immortalising virus strain P3HR-1 (Delius and Bornkamm, 1978; Rabson et al., 1982; Jeang and Hayward, 1983). Recombination of only the EBNA-2 sequences results in an immortalisation competent virus, however, such a virus immortalises

with a lower efficiency (Sugden, 1989). Therefore, although not absolutely required, EBNA-LP plays a subsidiary role in immortalisation by EBV.

Latent Membrane Protein 1

The latent membrane protein 1 (LMP1) is the most abundant latent cycle RNA polymerase II transcript in infected cells. Another RNA expressed in the productive cycle overlaps the LMP1 RNA and codes for a protein comprising the C-terminal 80% of LMP1 (Hudson et al., 1985). Structural predictions indicate that LMP1 has six membrane spanning domains and an acidic C-terminal globular domain (Fennewald et al., 1984); both the C- and N-termini are thought to be cytoplasmic (Liebowitz et al., 1986). LMP1 has been shown to be localised mainly in the plasma membrane (Liebowitz et al., 1986) and it is hypothesised LMP1 may act as an ion channel or as a cell surface receptor (Sugden, 1989).

The protein is phosphorylated (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987) and patches and caps in lymphocytes (Liebowitz et al., 1986; Liebowitz and Kieff, 1989) where it is associated with vimentin (Liebowitz et al., 1987; Liebowitz and Kieff, 1989). It has also been shown to induce expression of vimentin when expressed in EBV negative B-cells (Birkenbach et al., 1989). LMP1 is transforming in Rat-1 and Balb/3T3 rodent cells (Wang et al., 1985; Baichwal and Sugden, 1988). In addition, LMP1 is a broad activator of B-cell activation markers including CD23 and the cell surface adhesion molecules Lfa-3 and ICAM-1 (Wang et al., 1988; Wang et al., 1990a) and LMP1 is therefore considered to be important in immortalisation of B-cells. LMP1 also prevents the differentiation of human epithelial cells in culture (Dawson et al., 1990) and can transform a human keratinocyte line (Fahraeus et al., 1990), characteristics which may be important in the development of NPC. Recently, expression of LMP1 has been shown to protect B-cells from apoptosis by upregulating the bcl-2 gene (Gregory et al., 1991; Henderson et al., 1991). Hence, LMP1 may also play a role in enhancing B-cell survival.

Latent Membrane Proteins 2A and 2B

LMP-2A and 2B are also called TP1 and TP2 respectively. The LMP-2A and 2B genes are transcribed from exons derived from the terminal repeats and are therefore only made when the genome is circularised (Laux et al., 1988b; Sample et al., 1989). The two transcripts are generated from distinct promoters and have unique 5' exons, the remaining exons

are common to both mRNAs. Both proteins are predicted to be membrane spanning proteins (Laux et al., 1988b; Sample et al., 1989); the use of unique exons results in LMP-2B having a truncated N-terminus. LMP-2A has been shown to colocalise with LMP1 on the plasma membrane (Longnecker and Kieff, 1990). The function of these proteins is not clear although LMP2A has been shown to associate closely with a cell protein kinase and to be phosphorylated by this activity (Longnecker et al., 1991). Thus, LMP1 and LMP2A and LMP2B may form a complex at the cell membrane which functions to influence host cell phosphorylation activity.

EBERs

The two EBER transcripts (EBER-1 and -2) are small, untranslated, non-polyadenylated RNAs which associate with the La antigen and are the most abundant EBV transcripts in LCLs and EBV-positive BL cell types (Rymo, 1979; Arrand and Rymo, 1982; Lerner et al., 1981). The genes are transcribed constitutively in lymphocytes (Weigel et al., 1985) and are the only genes of EBV transcribed by RNA polymerase III although there are some RNA polymerase II regulatory elements near them which affect gene expression (Howe and Shu, 1989). The EBER RNAs are about 160 (EBER-1) and 180 (EBER-2) nucleotides in length, have sequence homology and have considerable stable stem-loop secondary structure (Arrand and Rymo, 1982; Glickman et al., 1988).

Functional analysis has been dominated by the observation that the EBERs can partly complement the functions of the viral associated (VA) RNAs in an adenovirus mutant defective for VA (Bhat and Thimmappaya, 1983; Bhat and Thimmappaya, 1985). The adenovirus VA RNAs act to inhibit an interferon induced dsRNA activated pathway by preventing the activition of a protein kinase known as the dsRNA-activated inhibitor of protein synthesis (DAI; O'Malley et al., 1986). Although there are differences between the RNAs (VA RNAs seem to be localised in the cytoplasm while the EBERs are localised only in the nucleus of infected cells; Howe and Steitz, 1986) it is possible that the EBERs have a similar role. Indeed, EBER-1 has been shown to inhibit the dsRNA activated pathway in vitro and to bind DAI (Clarke et al., 1990; Clarke et al., 1991). However, the EBERs have been shown to be dispensible for immortalisation of B-cells in vitro (Swaminathan et al., 1991) and experiments presented by S.Swaminathan and E.Kieff at the

International Herpesvirus Workshop, 1991 suggest that an EBER deleted virus can reactivate efficiently in the presence of interferons. Both EBER-1 and -2 have recently been shown to bind a highly conserved cell protein designated EAP (EBER associated protein) though the relevance of this interaction is not known (Toczyski and Steitz, 1991).

1.3.2 Epstein-Barr Virus Productive Cycle Gene Expression

The virus can be induced to enter the productive cycle in several ways (see section 1.5). Expression of the early and late productive cycle genes can be separated by the use of inhibitors of viral DNA synthesis; the early genes are expressed before viral DNA synthesis whilst the late genes are expressed after DNA synthesis. Some of the productive cycle genes have been identified by homology to other herpesvirus gene products. This presumably identifies evolutionarily conserved genes whose functions are likely to be necessary for many herpesviruses, for example, viral DNA replication and structural components of the virion.

Early Productive Cycle Gene Expression

Expression of the early genes has been shown to precede EBV productive cycle DNA replication. Many of the early gene products (including the DNA polymerase, major DNA binding protein, large and small sub-units of ribonucleotide reductase, thymidine kinase, DNase and dUTPase) are thought to be involved in productive cycle replication of EBV DNA and have been identified either functionally or by homology with other, better characterised herpesvirus gene products.

Other early gene products function as modulators of productive cycle gene expression and their activity and control of their expression is central to the switch from latent to productive infection. These genes include BZLF1, BRLF1, and BSLF2+BMLF1 and are discussed more fully in section 1.5. An additional productive cycle gene regulatory molecule, BI'LF4 has recently been described (Marschall et al., 1991), however, the protein is non-essential in the productive cycle since the ORF is deleted in the B95-8 virus strain.

An early gene, BHRF1 has some homology to bcl-2 (Pearson et al., 1987) and the BARF1 gene product has been shown to be transforming in rodent cell culture systems (Wei and Ooka, 1989). The significance of these observations has not been examined.

Productive Cycle DNA Replication

Two productive cycle origins of DNA replication (oriLyt) have been identified, one of which is located between bases 52642 and 53697 of the B95-8 genome (Hammerschmidt and Sugden, 1988). The other origin is deleted in the B95-8 but is found in other virus strains such as Raji (located at positions 3554-4609 of the B95-8 deletion; Hammerschmidt and Sugden, 1988; Parker et al., 1990). Clearly, only one origin is required since B95-8 virus can complete productive cycle DNA replication. Unlike HSV, the oriLyt of EBV is complex and requires several individual elements for activity. The origin overlaps a very efficient promoter/enhancer function active in the productive cycle that has been shown to be transactivated by both BZLF1 and BRLF1 (Chavrier et al., 1989; Chevallier-Greco et al., 1989; Cox et al., 1990). Therefore, as in the case of EBNA-1 and oriP and as for many other viruses, transcriptional activation and initiation of DNA replication may be linked. Despite structural differences in the oriLyt between HSV and EBV it is likely that DNA is replicated in the same way via a rolling circle mechanism using a virally encoded DNA polymerase which results in a head to tail concatameric molecule with fused TR (Hammerschmidt and Sugden, 1988; Sato et al., 1990; Roizman and Sears, 1990). The genome is cleaved at the TR to produce a linear DNA molecule presumably before packaging in virions.

Late Productive Cycle Gene Expression

The late productive cycle gene products include components of the VCA and MA including gp350 and gp220 which are known to be generated by alternate splicing (Hummel et al., 1984; Biggin et al., 1984; Beisel et al., 1985). The mechanism of control of expression of the late genes is not known but expression is dependent upon DNA replication since it is sensitive to phosphonoacetic acid and acyclovir, which act to inhibit the viral DNA polymerase (Nyormoi et al., 1976; Summers and Klein, 1976; Colby et al., 1980). Extensive homology has been reported between a late gene BCRF1 and a cDNA clone for human interleukin 10 (IL10; Vieira et al., 1991). The significance of this is not clear but may represent a way in which the virus can evade immune responses during the productive cycle.

1.4 Epstein-Barr Virus Immortalisation and Associated Disease

EBV is the causative agent of most infectious mononucleosis and is associated with nasopharyngeal carcinoma and Burkitt's lymphoma. More recently EBV has been linked to lymphomas in immunocompromised people, oral hairy leukoplakia, Hodgkin's disease and possibly some T-lymphomas. The virus also immortalises B-lymphocytes efficiently in vitro. These aspects of EBV biology are discussed fully below.

The majority of the population world-wide becomes infected with EBV within the first 5 years of life and then goes on to develop a life-long asymptomatic carrier state. Most EBV carriers periodically shed virus particles into the buccal cavity so that horizontal transmission of virus is most likely to occur through salivary transfer (Yao et al., 1985a).

Several of the latent gene products have proved to be non-essential for immortalisation of B-cells in vitro. However, it seems possible that all these products contribute to the overall in vitro or in vivo immortalisating potential of EBV.

1.4.1 Epstein-Barr Virus Immortalisation of B-cells

EBV efficiently immortalises B-cells in vitro (reviewed in Sugden, 1989; Klein, 1989). In fact, virus binding to the CD21 EBV receptor and internalisation alone are sufficient for activation of a B-cell from G_0 to G_1 of the cell cycle even by a non-immortalising strain of virus (Gordon et al., 1986). This is followed by initiation of host cell DNA synthesis, amplification of the circularised viral genomes and expression of the latent gene products (Hurley and Thorley-Lawson, 1988; Alfieri et al., 1991). Small foci of transformed cells appear after 3-4 days and these expand to form a lymphoblastoid cell line which typically expresses all eleven latent gene products. Such cells have the ability to divide indefinitely in culture though they are not tumourigenic in nude mice.

Activation of cell genes is likely to be an important way in which EBV acts to immortalise a B-cell and appearance of CD23 on the cell surface is one of the earliest B-cell activation markers detected after infection with an immortalising strain of virus (Thorley-Lawson and Mann, 1985). Appearance of CD23 is a prerequisite for immortalisation by EBV although this can be overcome by addition of B-cell growth factor (Thorley-Lawson and Mann, 1985; Azim et al., 1990). The membrane bound form of CD23 can function as a low affinity IgE Fc receptor but it is not clear if this is significant for its function in the cell. CD23 is cleaved to

give a soluble protein, sCD23, which acts as an autocrine factor stimulating infected cells to proliferate (Thorley-Lawson et al., 1986; Gordon et al., 1984; Swendeman and Thorley-Lawson, 1987). CD23 is now known to be cooperatively upregulated by EBNA-2 and LMP1 (Wang et al., 1990a). In addition to activation of CD23, EBV infection induces expression of a number of other B-cell activation markers (e.g. CD21, CD30, CD39 and the Ki-24 antigen; Rowe et al., 1986b; Calendar et al., 1987). Although these antigens are expressed transiently on normal activated B-cells EBV induced B-cell activation is associated with constitutive expression of these antigens. EBV infection is also thought to induce the cell surface adhesion molecules Lfa-1, Lfa-3 and ICAM-1 (Gregory et al., 1988) and LMP1 has been shown to activate expression of Lfa-3 and ICAM-1 (Wang et al., 1988).

The cell proto-oncogenes, c-fgr (Cheah et al., 1986) and c-myc (Lacy et al., 1987; Wennborg et al., 1987) are also reported to be upregulated (though not rearranged as in BL). Upregulation of c-fgr is thought to require expression of the EBNA-2 gene but the precise mechanism of activation is not known (Knutson, 1990). C-fgr is a tyrosine kinase of the c-src family (Katamine et al., 1988) Although the function of c-fgr is not known, other members of the c-src family have been shown to be involved in signal transduction and c-fgr may act in this way.

1.4.2 Immunesurveillance of Epstein-Barr Virus Infected Cells

In the normal EBV positive host the number of circulating B-cells carrying EBV is estimated to be less than 1 in 10^6 to 10^7 cells (Rocchi et al., 1977). Infection in the majority of people is asymptomatic and this reflects an efficient mechanism of host immune surveillance against EBV.

Following primary infection, antibodies specific for the early antigens (EA, derived from early productive cycle gene products), MA and VCA, and later antibodies specific for the EBNAs are generated (reviewed in Rickinson et al., 1985). These antibodies, with the exception of those directed against EA and the EBNAs are maintained for life. Antibodies against gp350/220 act as neutralising antibodies, blocking infectivity and release of virus (Thorley-Lawson and Geilinger, 1980; Sairenji et al., 1988; Hoffman et al., 1980).

Rigorous cell mediated responses are also required to prevent the outgrowth of EBV immortalised cells in vivo and class 1 major

histocompatibility complex (MHC) restricted cytotoxic T-lymphocytes (CTLs) develop which recognise antigens on infected B-cells (Rickinson et al., 1981). These antigens have been termed LYDMA (Svedmyr and Jondal, 1975) and are probably derived from a number of latent gene products. CTL clones have been shown to recognise epitopes on the LMP, EBNA-2 and EBNA-3A proteins as well as the productive cycle product gp350/220 (Reiss et al., 1987; Thorley-Lawson and Israelsohn, 1987; Murray et al., 1990; Burrows et al., 1990; Wallace et al., 1991). The immune response against EBV antigens is influenced by virus strain (Moss et al., 1988) and CTL clones have been isolated which recognise the EBNA-2 and -3A genes from type A virus only (Murray et al., 1990; Burrows et al., 1990).

In vivo the virus evades host immune surveillance by downregulating latent gene expression and in the case of B-cells, B-cell activation markers and cell surface adhesion molecules. However, all EBV infected cells express EBNA-1; no CTL response specific for EBNA-1 has been described and this may be why cells expressing EBNA-1 avoid immune surveillance. In addition, the EBERs, which are not translated into proteins, are expressed in many EBV infected cell types.

1.4.3 Lymphoma in the Immunocompromised Host

The immune system plays a central role in preventing outgrowth of EBV immortalised cells in the normal host. However, it is apparent that CTL responses against EBV infected B-cells are impaired in people who are immunosuppressed (Masucci et al., 1981; Yao et al., 1985b; Ragona et al., 1986). This may occur in AIDS patients, transplant patients who are receiving immunosuppressive drugs or individuals with primary immunodeficiency.

Transplant recipients receiving immunosuppressive drugs are at a higher risk than the normal population of developing cancers, particularly polyclonal lymphomas. Such lymphomas often contain EBV DNA and express the EBNAs though they do not have a c-myc translocation (Crawford et al., 1980; Hanto et al., 1981; Saemundsen et al., 1982; Thomas and Crawford, 1989). Similar polyclonal EBV positive lymphomas occur with a relatively higher incidence in AIDS patients (Thomas and Crawford, 1989; Hamilton-Dutoit et al., 1991). Lymphomas of this kind generally resemble LCLs in their latent gene expression and B-cell phenotype and presumably occur in severely immunosuppressed patients.

EBV positive Burkitt's-like lymphomas usually occur in patients who have less severe immune disfunction (Ziegler et al., 1982; Kalter et al., 1985).

Males with X-linked lymphoproliferative disease (XLP), a congenital immunodeficiency disease, commonly develop chronic infectious mononucleosis, malignant lymphomas, aplastic anaemia and hypogammaglobulinemia, often during childhood (Purtillo et al., 1982). B-cells from these individuals often contain EBV DNA and express EBV latent antigens (Saemundsen et al., 1981; Falk et al., 1990) and have very low titres of antibodies against EBV antigens suggesting that they have an inherited immune defect in immune responses against EBV.

1.4.4 Infectious Mononucleosis

EBV plays a causal role in most cases of infectious mononucleosis (IM; reviewed in Epstein and Achong, 1979b; Roizman, 1982). Primary infection by EBV usually occurs in early childhood and is asymptomatic but where primary infection is delayed until adolescence or later IM may develop. IM is predominantly found in Western populations and particularly in upper socio-economic groups where primary infection may be delayed. In Africa, where the vast majority of children are infected in childhood, IM is practically absent.

In an IM patient the number of EBV infected circulating B-cells is higher than in a normal carrier (Rocchi et al., 1977; Lam et al., 1991) and this elicits a vigorous CTL response which, in the acute phase of the disease results in a doubling of the number of leukocytes in the blood. This results in the symptoms of sore throat, fever and lymphadenopathy and is why the disease is commonly known as glandular fever in the UK.

Some individuals develop a chronic IM infection (Schooley et al., 1986). Such patients have extremely high titres of EBV specific antibodies yet fail to develop the typical anti-EBNA-1 response. Patients' B-cells commonly contain EBV DNA and express latent cycle antigens (Saemundsen et al., 1981; Falk et al., 1990). Acyclovir has some effects in controlling this syndrome again suggesting a causal role for EBV.

1.4.5 Burkitt's Lymphoma

Burkitt's lymphoma (BL) is an undifferentiated B-cell tumour which occurs sporadically world-wide but has a 20-100 fold higher incidence in Equatorial Africa and New Guinea where it is described as endemic

(reviewed in Epstein and Achong, 1979b; Roizman, 1982). BL is the commonest cancer in African children occuring in 5-15 children/100,000/year. Sporadic BL occurs worldwide at low incidence and 85% of these tumors are EBV negative. The few EBV negative tumours (5%) in areas of endemic BL probably represents the underlying incidence of sporadic EBV negative tumours world-wide.

EBV is implicated in development of endemic BL since the majority of patients (95%) have EBV DNA and express EBNAs in the tumour cells (zur Hausen et al., 1970; Lindahl et al., 1974). Gene expression in BL is restricted and generally only EBNA-1 and the EBERs are expressed (Rowe et al., 1986a; Rowe et al., 1987). LMP2A may be expressed in a limited number of cell lines representative of BL cells in vivo (Rowe et al., 1990). BL is thought to be a clonal tumour with respect to both the B-cell and the virus (Bechet et al., 1974; Raab-Traub and Flynn, 1986) which implies that the virus infection was an early event in the development of BL. Moreover, since less than 1% of circulating B-cells contain virus, yet over 95% of endemic tumours are EBV positive then the chance of transformation must be higher in EBV infected cells.

BL is characterised by a translocation at or near the c-myc proto-oncogene locus on chromosome 8 (reviewed in Lenoir and Bornkamm, 1987). Commonly this reciprocal translocation occurs between chromosomes 8 and 14 and less frequently 8 and 2 or 8 and 22. Such translocations always involve one of the three immunoglobulin loci on chromosomes 2, 14 or 22. In the 8:14 translocation the myc gene is fused to the Ig heavy-chain constant gene on 14. In the 8:2 and the 8:22 translocations one of the two light chain loci are moved to the myc gene. In all cases the translocation break points are close to those used for normal physiological immunoglobulin rearrangements (Cory, 1986).

The precise function of c-myc is unclear though the protein is thought play a central role in controlling progression through the cell cycle. Mice rendered transgenic for the translocated Eμ-c-myc gene commonly develop lymphomas at an enhanced rate although additional genetic alterations are required to complement c-myc in this activity (Adams et al., 1985; Haupt et al., 1991; van Lohuizen et al., 1991).

The translocations described above may result in deregulated myc expression in a number of ways (reviewed in Lenoir and Bornkamm, 1987) and EBV can also cause deregulation of myc in the absence of a translocation (Lacy et al., 1987; Wennborg et al., 1987).

The geographical distribution of endemic BL overlaps that of infection by Plasmodium falciparum, the parasite causing malaria. Repeated malarial infections lead to immune disfunction and during the acute phase of malaria the CTL response directed against EBV infected cells is reduced (Whittle et al., 1984; Lam et al., 1991). This immune disfunction and repeated immune challenge which would expand the B-cell pool are likely to be critical events in the development of EBV associated BL. However, the precise sequence of events in genesis of BL are not known (for discussion see Lenoir and Bornkamm, 1987; Klein, 1987). For example, it is not known whether EBV infects the developing BL cell prior to or after the myc translocation.

1.4.6 Nasopharyngeal Carcinoma

Undifferentiated nasopharyngeal carcinoma (NPC) is an epithelial tumour associated with EBV infection (reviewed in Epstein and Achong, 1979b; Roizman, 1982). Like BL, NPC has a distinct geographical distribution being predominant in South-East Asia where it is the most common cancer in young to middle aged men (up to 95 cases/100,000/year in 45-54 year old males).

EBV is implicated in the development of NPC since undifferentiated NPC tumour cells almost always contain EBV DNA and express EBNAs (zur Hausen et al., 1970; Wolf et al., 1973; Klein et al., 1974; Fahraeus et al., 1988) and patients exhibit an unusual antibody response against EBV antigens.

Analysis of the TR of the virus in a BL cell indicates that the tumour is clonal with respect to the virus (Raab-Traub and Flynn, 1986). EBNA-1 is typically expressed in all cases, while the other EBNAs are absent (Fahraeus et al., 1988; Young et al., 1988; Hitt et al., 1989). LMP1 may be expressed in about 50% of biopsies. Transfection of LMP1 can prevent the differentiation of epithelial cells in vitro (Dawson et al., 1990) and alters the surface phenotype of human keratinocyte lines (Fahraeus et al., 1990) suggesting a role for this protein in development of NPC.

Research has been hampered by an inability to passage NPC tissues in culture but recently analysis of the xenograft line C15 has revealed a novel virus-cell interaction which suggests that the mode of immortalisation may be different for B-cell and epithelial cell associated tumours. Transfection of certain EBV genomic sequences into epithelial cells has been reported to result in immortalisation (Griffin and Karran, 1984). This

general region of EBV is also expressed in the C15 line and this might have some relevance to the growth of NPC cells.

Environmental and genetic factors may also play a role in genesis of NPC. Traditional foods and medicines contain factors capable of activating EBV which might affect the fine balance between virus and the immune system (reviewed in Ito, 1986). Certain HLA phenotypes have been associated with NPC and linkage data suggests the presence of a susceptibilty gene(s) closely linked to the HLA locus (Lu et al., 1990).

The serological responses directed against EBV are an important marker of disease in a number of EBV associated malignancies, including NPC and seroepidemiological studies have shown that such patients exhibit a characteristic and unusual immune response to EBV antigens (Zeng, 1985). Patients with undifferentiated NPC have high antibody titres to the EBV EA, MA and VCA complexes (Henle et al., 1973) and to the virus encoded enzymes DNase, DNA polymerase and thymidine kinase (Cheng et al., 1980; Tan et al., 1986; Turenne-Teissier et al., 1989). In NPC the antibodies are generally specific for the diffuse component of the EA complex (EA-D) which contrasts with antibodies from BL patients which are generally specific for the restricted component, EA-R (Henle et al., 1973). These antibodies characteristically include both the IgA and the IgG classes (Henle and Henle, 1976). Moreover, antibody titres in NPC patients increase with progression of the disease from stage I to stage IV and are detectable before the disease is clinically apparent (Henle et al., 1973). In addition, post-treatment, long term survivors have reduced titres against most of these antigens.

These serological characteristics have formed the basis for prognostic and diagnostic screening in South-East Asia (Zeng, 1985). NPC is particularly suited to large scale screening because of its high incidence, because undifferentiated NPC is relatively aggresive and because effective treatment is possible if the disease is diagnosed in its early stages.

Early assays to determine the titre of EBV specific antibodies in patients' sera used EBV infected cell lines as a target to detect antibody (IgA) to EBV VCA and EA by indirect immunofluorescence tests. Other tests involve the neutralisation of viral enzymic activity (e.g. DNase) by patients' sera to detect raised antibody titres (Chen et al., 1989). More recently viral antigens produced in recombinant systems have been employed in enzyme-linked immunoabsorbant assays (ELISAs) to measure specific antibody titres (Littler et al., 1991).

1.4.7 Hodgkin's Disease, T-lymphomas and Oral Hairy Leukoplakia

Hodgkin's Disease

Hodgkin's disease (HD) is a lymphoma characterised by the presence of the Reed-Sternberg cell, a cell of unknown lineage. Serological evidence has implicated EBV in the genesis of Hodgkin's disease (Mueller et al., 1989).

EBV genomes have been found in the Reed-Sternberg tumour cells by in situ hybridisation and up to 68% of cases may contain EBV DNA (Herbst et al., 1991). The Reed-Sternberg cells often express LMP1 and the EBERs but not EBNA-2 or gp350/220 (Wu et al., 1990; Herbst et al., 1991; Pallesen et al., 1991). The cell is clonal with respect to the virus (Anagnostopoulos et al., 1989) and it appears that EBV may play a causal role in HD.

T-cell Lymphomas

Some peripheral T-lymphocytes express a 145kD CD21 molecule (Fischer et al., 1991) and the presence of EBV genome positive T-lymphomas indicates that EBV may be involved in the genesis of some T-lymphomas (Jones et al., 1988; Kikuta et al., 1988; Harabuchi et al., 1990). EBV positive T-lymphomas have been shown to express EBNA-2 and LMP1 (Harabuchi et al., 1990) and the cell is clonal with respect to both T-cell receptor rearrangements and the viral genome (Jones et al., 1988).

Oral Hairy Leukoplakia

Oral hairy leukoplakia (OHL) is an oral, epithelial lesion which commonly presents on the lateral margin of the tongue (Greenspan et al., 1984); despite characterisation as a leukoplakia there is no evidence that the lesion is premalignant. The lesion was first identified in AIDS patients but it is likely that all immunosuppressed individuals may be susceptible to OHL. The lesion has been shown to contain virus particles and EBV DNA, and to express viral antigens associated with productive infection (Greenspan et al., 1985). The lesion undergoes remission following acyclovir treatment suggesting that a herpesvirus, presumably EBV, plays a causal role in OHL (Friedman-Kien, 1986). Productive cycle gene expression occurs in localised areas of the upper, more differentiated tissue layers and there is no evidence for EBV infection in the basal tissues (Young et al., 1991); the

detailed interaction between host cells and virus in this disease is not understood.

1.5 Epstein-Barr Virus Latency

1.5.1 Herpesvirus Latency and Replication

All members of the Herpesviridae are characterised by an ability to establish a life long infection within the host. This ability to develop a latent infection and then to reactivate at intervals has endowed the herpesviruses with a great survival advantage. The exact strategy by which herpesviruses have achieved these ends varies and the large number of host organisms and cell types which are infected in vivo is testament to this variability. However, herpesviruses have apparently evolved a system whereby in general they can develop a latent infection in one cell type (or tissue site as in the case of HSV and VZV) and replicate efficiently in another cell type or tissue site. EBV is thought to establish latency in B-cells and to replicate most efficiently in epithelial cells (see below).

Herpesviruses share some common genome structures and these genetic units tend to encode conserved functions. Genes whose products are involved in productive cycle DNA replication or are structural components of the virion are highly conserved between herpesviruses (Davison and Taylor, 1987; reviewed in Chee and Barrell, 1990). Conserved genes are generally restricted to the long unique regions of the genome where they tend to occupy blocks of homology that have been rearranged during the evolution of the herpesviruses from a common ancestor. However, each herpesvirus is uniquely adapted to its host system and encodes functions enabling the virus to establish latency and replicate in its specific host. These functions are not shared with other herpesviruses. For example, unlike EBV, no EBNA-1-like oriP maintenance function has been described for HSV, presumably because HSV does not need to replicate in the neuron since that cell type does not divide. EBV also has unique genes which appear to be related to host cell immune system regulators, for example BCRF1 is related to the host interleukin 10 (Vieira et al., 1991) and this may enable the virus to avoid immune surveillance during certain stages of its life cycle. The latent genes have no homologues in other herpesviruses and presumably encode unique functions not shared with other herpesviruses that enable the virus to establish latency in B-cells.

1.5.2 Epstein-Barr Virus Latency in the Host

The identity of the cell in which EBV establishes latency in the host has been the subject of much discussion. However, recent analysis of the EBV status of patients who have received allogeneic bone marrow transplants strongly implicates the lymphoid tissues, presumably B-cells, as a reservoir for EBV (Gratama et al., 1988). In one case, a seropositive recipient seroconverted to the virus strain of the donor following bone marrow transplantation. The eradication of the resident virus by the combined drug and irradiation treatment which accompanies the operation suggests that the virus persists in lymphoid tissues. Reactivation of the virus from lymphocytes would presumably lead to infection of the epithelial tissues. However, the mode and site of transport of the virus between epithelial and lymphoid tissues remains unclear.

The phenotype of the EBV infected cell in the normal infected host is not known. EBV latent gene expression differs between the three main EBV-carrying cell prototypes; in vitro immortalised LCLs, EBV positive BL cells and NPC cells. LCLs typically express all eleven latent gene products whereas BL and NPC cells have a more restricted latent gene expression pattern (see section 1.4). In addition EBV positive BL cells characteristic of BL cells in vivo have downregulated B-cell activation markers and cell surface adhesion molecules. It is thought that EBV latent cycle gene expression is dependent on the host cell activation state such that an activated B-cell would express an LCL phenotype while a resting B-cell would express a BL cell type phenotype (Klein, 1989). B-cells with the LCL phenotype are found in vivo in some lymphomas in immunocompromised people and presumably in infectious mononucleosis.

Analysis of the EBV status of density separated B-cells from normal individuals suggests that immortalised cells can grow out directly from high density B-cells (Lewin et al., 1987). It is hypothesised that the B-cell that carries EBV in the normal host is a small, high density, resting B-cell that expresses a phenotype similar to that of BL cells in vivo. Such a cell would be resistant to CTL responses, compared to the in vitro immortalised LCL, as are the BL cells. BL cells do not generally express the immunogenic LYDMA complex, some BL cells downregulate certain class 1 MHC complexes (Masucci et al., 1987) and cell surface adhesion molecules involved in T-cell interactions (Gregory et al., 1988). Similar mechanisms may permit the persistence of EBV in the normal host. It

would be advantageous for the virus to reside in a long lived resting B-cell (such as a memory B-cell) and a transient ability of the virus to protect the cell against apoptosis may be important in the virus gaining access to this pool of cells (paradoxically, this requires LMP1 expression which is a target for CTL responses).

Reactivation from B-cells presumably has to occur since the virus is thought to persist in the lymphoid compartment. The stimulus that results in reactivation of EBV in B-cells in vivo is not known though it is thought to be associated with B-cell maturation (see section 1.5.3). Little is known about infection and virus production in epithelial cells in normal people but EBV may be detected in the buccal cavity of infected people and the productive cycle can readily be seen in OHL (Yao et al., 1985a; Young et al., 1991) and this may be the site where the majority of EBV production occurs.

1.5.3 Epstein-Barr Virus Latency in Vitro

The term latency is not only applied to the ability of the virus to maintain a latent infection in the host but also to characterise those virus-cell interactions which occur in vitro in which the virus is maintained in infected cells and virus production rarely occurs. However, it is important to realise that these two forms of latency may be different. The latently infected B-cell in vivo is thought to express a phenotype similar to that of BL cells in vivo while EBV infected B-cells grown in vitro (e.g. an LCL) often express a number of the latent cycle genes. Such cells are unlikely to represent the latently infected cell in the host as they express targets for CTL responses. Despite these apparent differences EBV is an attractive system in which to study latency and reactivation because latently infected B-cells can be cultured in vitro and the switch to the replicative cycle can be effected, albeit relatively inefficiently by several agents.

Latently infected B-cells can be induced to enter the productive cycle at a rather low efficiency by treatment with chemical or biological inducers. Infected B-cells with either a phenotype representative of the BL in vivo or with an LCL phenotype can be induced to the productive cycle and it is known that BL cells do not need to express the full complement of the latent genes prior to induction (pers. comm., A. Rickinson).

Within an LCL there is a continuing maturation of cells such that a minority of more differentiated cells are present. Such cells no longer

express the EBNAs and the loss of associated immortalising functions may enable the virus to complete the productive cycle and to release infectious virus particles prior to cell death (Crawford and Ando, 1986; Wendel-Hanson et al., 1987). This is thought to result in the small number of spontaneously productive cells (i.e. cells which have entered the productive cycle without treatment with inducing agents) observed in such cultures. In addition, effective inducers of the productive cycle include TPA, sodium butyrate, iododeoxyuridine, TGFβ (transforming growth factor β) and anti-immunoglobulin (zur Hausen et al., 1978; Luka et al., 1979; Gerber, 1972; Bauer et al., 1982; Tovey et al., 1978) and such inducers may act to induce cell differentiation. Many other chemicals including inhibitors of cell DNA, RNA and protein synthesis, hormones and cyclic nucleotides have some inducing activity (Joncas et al., 1973; Hampar et al., 1976); some of these agents are growth inhibitory and the virus may be induced to replicate when host cell viability is threatened. A number of inducing agents act synergistically and this indicates that there are probably multiple points at which virus replication can be induced (Bauer, 1983).

Permissive infection by HSV has been well studied and trans-acting factors and their response elements have been identified. During this infection, which is not equivalent to reactivation from a latent state, viral immediate early productive gene expression is activated by a complex involving a cell protein and a viral protein derived from the virion particle (O'Hare et al., 1988). The equivalent type of infection has not yet been studied in EBV.

Induction of the Productive Cycle in the Akata Cell Line

In this thesis the Akata cell line has been used as a system to investigate EBV reactivation from B-cells. Akata cells are derived from an EBV positive BL from an Japanese patient (Takada et al., 1991). The cells express surface IgG (sIgG) and can be induced to the productive cycle by treatment with anti-immunoglobulin (Takada, 1984; Takada and Ono, 1989). Cross-linking of the sIg presumably activates the B-cell; this is accompanied by an inhibition of cell DNA synthesi (Takada, 1984). Akata cells have a phenotype characteristic of BL cells in vivo (pers. comm., A.B.Rickinson) and may represent a transformed version of the B-cell latently infected in vivo. So reactivation in response to B-cell activation

signals may be physiologically relevant, making such cells an attractive system to study reactivation in vitro.

Following induction of the productive cycle (for example, by treatment of Akata cells with anti-immunoglobulin or B95-8 cells with TPA) the cells express the productive cycle genes and produce virus particles (Hummel and Kieff, 1982; Takada and Ono, 1989). In Akata cells mRNAs capable of expressing BZLF1 are among the first RNAs induced. Hence, control of the expression of these transcripts is likely to be central to the mechanism of the switch from latent to productive infection.

Induction of the productive cycle by TPA in Raji cells is sensitive to inhibitors of protein synthesis and therefore requires de novo protein synthesis (Laux et al., 1988a). The requirement for protein synthesis in the induction of the expression of productive cycle mRNAs in Akata cells following anti-immunoglobulin treatment is studied in Chapter 7.

It is not known whether the mechanism of induction of the productive cycle involves induction of a positively acting factor(s) or removal of a viral or cell repressor(s). There is no evidence for gene products consistently associated with latent infection, i.e. EBNA-1 or the EBERs, acting as repressors of productive cycle gene expression. The affect of various second messenger pathway modulators on induction by anti-Ig in Akata cells indicates that induction is mediated by the Ca^{2+}/calmodulin and diacylglycerol (DAG) pathways suggesting that the virus is in fact sensitive to the B-cell activation pathways (Daibata et al., 1990). It also appears that the cAMP pathway suppresses induction though the identity of factors required for the switch from latent to productive infection are not known.

1.5.4 Role of BZLF1, BRLF1 and BSLF2+BMLF1 Gene Regulatory Molecules in Induction of the Productive Cycle

As discussed in section 1.3.2 the early productive cycle gene regulatory molecules, BZLF1, BRLF1 and BSLF2+BMLF1 are thought to play a crucial role in the switch from viral latency to productive cycle. The BZLF1 protein is considered to be central to the mechanism of induction and is the major subject of investigation in this thesis. The other gene regulatory molecules are thought to act in concert with BZLF1 to enable efficient induction of full productive cycle gene expression and their function is discussed below. The BRLF1 and BZLF1 genes have no homologues in

other herpesviruses and presumably encode unique functions required for reactivation from B-cells and possibly for virus growth in epithelial cells.

BRLF1

The BRLF1 protein is also known as R and Rta. BRLF1 was first implicated as being important in the early stages of the productive cycle when both the BRLF1 ORF and the BZLF1 ORF was found to be rearranged and constitutively expressed in P3HR-1 virus superinfected Raji cells (Biggin et al., 1987). BRLF1 was subsequently shown to be a strong transcription transactivator (Hardwick et al., 1988) and is thought to cooperate with BZLF1 in inducing the productive cycle.

The BRLF1 ORF is contained within a 2.8kb mRNA which is bicistronic for BRLF1 and BZLF1 (see figure 1.2). It contains three splices and includes a short 5' leader (Biggin et al., 1987; Manet et al., 1989). The promoter for this transcript has been identified and has been shown to be activated by both BZLF1 and BRLF1 itself but not by TPA (Sinclair et al., 1991).

BRLF1 is an approximately 90 kD protein by SDS-PAGE (Marschall et al., 1989) that functions as a sequence specific DNA binding protein (Gruffat et al., 1990a). BRLF1 seems to act several hundred basepairs away from the promoter and is thus like an enhancer factor (Chevallier-Greco et al., 1989; Kenney et al., 1989a). BRLF1 is capable of transactivating a number of EBV promoters although together BZLF1 and BRLF1 sometimes appear to act synergistically (Sinclair et al., 1991; Buisson et al., 1989; Kenney et al., 1989a; Holley-Guthrie et al., 1990; Chavrier et al., 1989; Chevallier-Greco et al., 1989; Cox et al., 1990).

BMLF1 and BSLF2

The BSLF2+BMLF1 protein is also known as EB2 and MS-EA. Several productive cycle mRNAs span the region of the genome containing the BMLF1 and BSLF2 ORFs and the structure of the active mRNA is unclear. Two 5' coterminal mRNAs have been described (see figure 1.2); one RNA splices together BSLF2 and BMLF1 and could express both BMLF1 and BSLF2+BMLF1, another unspliced RNA could express only BMLF1 (Buisson et al., 1989). The spliced transcript is the more abundant in productively infected cells.

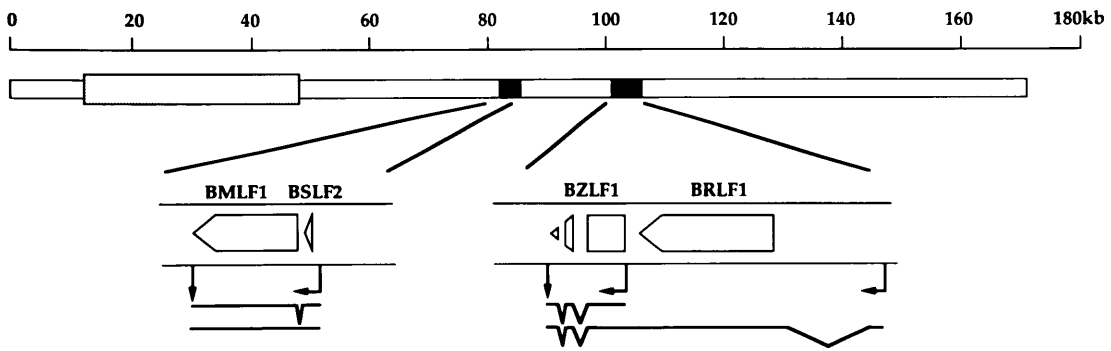


Figure 1.2 Positions of the early productive cycle gene regulatory molecules in the Epstein-Barr virus genome.

The B95-8 virus genome is represented in its linear form (the major internal repeat is indicated by shading). The approximate position and structure of the BZLF1, BRLF1, BMLF1 and BSLF2 open reading frames are shown below along with the structures of the abundant mRNAs encoding the BRLF1, BZLF1, BMLF1 and BSLF2+BMLF1 proteins. The genes are transcribed in a leftward direction from the U_L region of the genome; polyA signals are indicated by vertical arrows and promoter sequences by bent arrows (see text for references).

The promoter for these transcripts (the BSLF2+BMLF1 promoter) has been identified and is known to be responsive to BZLF1, BRLF1 and TPA (Buisson et al., 1989; Kenney et al., 1989a; Urier et al., 1989).

The BMLF1 protein was thought to act as a promiscuous transcription activator since it could transactivate many promoters when linked to the CAT reporter gene (Lieberman et al., 1986). BMLF1 has a low degree of homology to the ICP27 (IE63) immediate early gene of HSV (McGeoch et al., 1990) and other herpesvirus genes and that protein has now been shown to act post-transcriptionally. BMLF1 appears to have little effect on transcription (Buisson et al., 1989; Kenney et al., 1989c). Although no function of BMLF1 or BMLF1+BSLF2 has been demonstrated directly by mutagenesis of the virus, by analogy with HSV the protein is thought to be likely to play an important role in the activation of the EBV productive cycle cooperating with BZLF1 and BRLF1 to switch on other early genes.

1.5.5 Structure and Function of the BZLF1 Protein

The significance of the BZLF1 gene was first realised in superinfection studies using virus strains in the P3HR-1 cell line which were capable of inducing cells latently infected with EBV into the productive cycle (Rabson et al., 1983). It was shown that P3HR-1 virus contains defective forms of EBV known as het (heterogeneous DNA) virus (Miller et al., 1984), which contain gross deletions and rearrangements (Cho et al., 1984; Jenson et al., 1987) and that these het particles were responsible for this effect (Rabson et al., 1983; Miller et al., 1984). The defective virus was subcloned and a fragment of the genome containing the BamHI Z fragment of the standard genome was identified as being uniquely capable of inducing the productive cycle (Countryman and Miller, 1985). The BamHI Z fragment of B95-8 virus was shown to function in the same way when placed under the control of a constitutive, heterologous promoter (Takada et al., 1986) and it was established that the BZLF1 gene product was responsible for this induction (Countryman et al., 1987; Chevallier-Greco et al., 1986; Rooney et al., 1989). The rearrangements result in the constitutive expression of BZLF1 (Rooney et al., 1988; Taylor et al., 1989); some defective EBV in P3HR-1 also expresses the BRLF1 gene (Biggin et al., 1987). It is clear that expression of BZLF1 is sufficient to induce the replicative cycle though the contribution that other genes, e.g. BRLF1, make to the inducing activity of superinfecting P3HR-1 has not been addressed directly. To date, BZLF1 is

the only EBV gene shown to be capable of inducing the productive cycle alone (Chevallier-Greco et al., 1986; Grogan et al., 1987; Cox et al., 1990).

BZLF1 Gene Expression

The BZLF1 protein is also known as Z, Zta, EB1 and ZEBRA. The spliced BZLF1 gene product is active in inducing the productive cycle and comprises 3 exons all of which are coding (Biggin et al., 1987; Rooney, 1989). The BZLF1 protein is potentially expressed from two mRNA species (see figure 1.2); a 1.0kb transcript and a 2.8kb transcript that is bicistronic for BZLF1 and BRLF1 (Biggin et al., 1987; Manet et al., 1989). A third mRNA has been described which splices the 5' part of the BRLF1 frame to the 3' part of BZLF1 (Manet et al., 1989) though this is a minor mRNA species and its significance has not been investigated.

The promoters for both the 1.0kb and the 2.8kb mRNAs have been identified and the promoter for the 2.8kb bicistronic BRLF1+BZLF1 mRNA is described above. The promoter for the 1.0kb mRNA has been extensively analysed and deletion mapped. The promoter is autoactivated by BZLF1 and is responsive to TPA (Urier et al., 1989; Flemington and Speck, 1990b; Flemington and Speck, 1990c). The promoter includes some negative elements which may act to suppress the promoter (Montalvo et al., 1991).

Both the 1.0kb and the 2.8kb mRNAs are detected soon after anti-immunoglobulin treatment of Akata cells and are some of the first transcripts expressed (Takada and Ono, 1989; Sinclair et al., 1991). It is likely that at least one of the promoters for these transcripts is directly responsive to cross-linking of surface Ig in Akata cells but this has not been investigated satisfactorily.

BZLF1 Protein Structure

The BZLF1 protein has a predicted molecular weight of 26.9kD but the protein migrates on SDS polyacrylamide gels with an apparent molecular weight of about 39kD (Rooney et al., 1989). The reason for this anomalous migration is not known. BZLF1 has been reported to be post-translationally modified (Marschall et al., 1989) but other workers have reported no difference in size between BZLF1 made by in vitro translation and made in cells (Rooney et al., 1989). There is also a slight difference in the size of the protein between strains (Countryman et al., 1987; Taylor et al., 1989).

Protein sequence analysis of BZLF1 has identified two regions of homology with the c-fos protein (figure 1.3.A; Farrell et al., 1989). Residues 153-196 of BZLF1 are similar to the basic DNA binding structure and the adjacent casein kinase II site of c-fos. This region is encoded within the 35 amino-acid second exon of BZLF1 and a 36 amino-acid exon of c-fos, suggesting that the exons may be of common evolutionary origin. BZLF1 residues 36-110 are also homologous to N-terminal protein sequences of c-fos. The c-fos protein is a member of a family of related DNA binding proteins which share sequence similarity in their DNA binding domains; therefore, BZLF1 is related to all members of this family of proteins (figure 1.3.B). However, the additional N-terminal homology with c-fos argues that BZLF1 is most like c-fos.

Structure of the bZIP Proteins

The c-fos protein is a member of the bZIP family of proteins (Vinson et al., 1989). Members of this family of proteins are sequence specific DNA binding proteins that often play a central role in transcription regulation. For example, the c-fos and c-jun proteins are part of a cell protein complex, AP-1 that regulates the activity of many promoters. The bZIP proteins are characterised by a bipartite DNA binding domain (Landschulz et al., 1989; Kouzarides and Ziff, 1989; Agre et al., 1989) consisting of a basic region and an adjacent dimerisation domain. The basic motif shows a high degree of homology between members of this family and the dimerisation domain is characterised by a heptad repeat of leucine residues (figure 1.3 B) in a region of the protein that adopts an α-helical secondary structure. This dimerisation structure is termed a leucine zipper and is present in a number of proteins (Landschulz et al., 1988b; reviewed in Busch and Sassone-Corsi, 1990).

Each turn of an α-helix spans approximately 3.5 amino-acids residues and a leucine zipper domain would be predicted to form a helical backbone with leucine residues located every second turn along one face of the helix. The positions of amino-acid residues in one of these heptad repeats are termed (a)-(g) (see figure 6.1); in this way the leucines are found at the (d) positions. The positions at the (a) positions are also occupied by hydrophobic residues. This creates a structure which is α-helical and presents a hydrophobic face consisting of alternating leucine and other hydrophobic residues. A leucine zipper typically comprises four or more such repeats. The leucine zipper mediates both

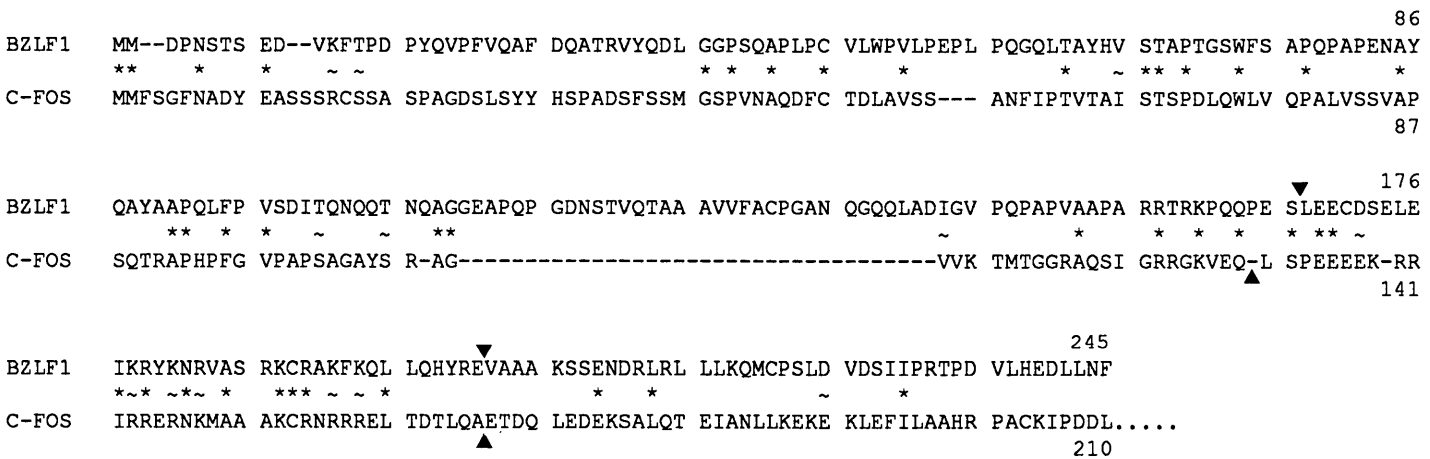


Figure 1.3 (A) Alignment of the protein sequences of BZLF1 and part of c-fos. Amino acid identities are shown as stars and conservative substitutions (S-T, R-K, I-V, E-D) as (~). The numbers indicate the residues of each protein and amino-acids are shown using the one letter code. Triangles mark the exon boundaries of the second exon of BZLF1 and the third exon of c-fos (adapted from Farrell et al., 1989).

```
                                        R    R    RC   RLER
                                        KR-RNK-AA-KSR-RKRQL

JUN-B  248  SRDATPPVSPINMEDQER--IKVERKRLRNRLAATKCRKRKLERIARLEDKVKTLKAENAGLSSAAGLLREQVAQL  321  JUN-B
                 :   ::          :: ::  :: ::: :::::   :      :      :      :      :
C-JUN  224  MPGETPPLSPIDMESQER--IKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQL  317  C-JUN
                 :   ::          :: ::  :: ::: :::::   :      :      :      :      :
GCN4   210  RKQRSIPLSPIVPESSDP----AALKRARNTEAARRSRARKLQRMKQLEDKVEELLSKNYHLENEVARLKKLVGER  281  GCN4
                 :  :::          :: ::  :  :::::  ::
BZLF1  153  AAPARRTRKPQQPESLEECDSELEIKRYKNRVASRKCRAKFKQLLQHYREVAAAKSSENDRLRLLLKQMCPSLDVD  228  BZLF1
            :    :  :  :  : ::      ::: ::: :  ::: : :::
C-FOS  120  AQSIGRRGKVEQ-LSPEEEE-KRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQTEIANLLKEKEKL  193  C-FOS
                 :    :  : ::       :: ::: :: ::: :::::   :      :      :      :      :
FRA-1   90  PPPGVRRRPCEQ-ISPEEEE-RRRVRRERNKLAAAKCRNRRKELTDFLQAETDKLEDEKSGLQREIEELQKQKERL  163  FRA-1
```

Figure 1.3 (B) Alignment of parts of the protein sequences of Jun-B, c-jun, GCN4, BZLF1, c-fos and fra-1. Amino-acid identities to BZLF1 or close matches to the consensus sequence for the basic region (shown above), the leucine zipper and the consensus casein kinase II site (SLEECDSELE in BZLF1) are indicated by double dots. The numbers indicate the residues of each protein illustrated and amino-acids are shown using the one letter code (adapted from Farrell et al., 1989).

homodimerisation and heterodimerisation through the coiled-coil arrangement of helices (Landschulz et al., 1988b; O'Shea et al., 1989a,b). In this structure the leucines of one helix interdigitate between the leucines of the second helix and interact with hydrophobic parts of residues at position (a). The helices are thought to be arranged in a parallel fashion (Gentz et al., 1989; O'Shea et al., 1989b) and an exact spatial requirement places the adjacent DNA binding structures in the correct position for sequence specific DNA binding (Vinson et al., 1989; Agre et al., 1989; O'Neil et al., 1990).

Recently BZLF1 has been identified as having sequence similarity with part of the leucine zipper dimerisation domain of C/EBP (figure 6.1; Kouzarides et al., 1991). BZLF1 has no apparent leucine zipper; the significance of this homology is discussed in chapter 6. However, this does argue that BZLF1 is closely related to both C/EBP and c-fos. The C-terminal half of BZLF1 (which includes the sequences homologous to the c-fos DNA basic motif) has been shown to be sufficient for DNA binding but not for transcriptional activity (Farrell et al., 1989; Rooney et al., 1989). Part of this thesis presents a more extensive deletion analysis of BZLF1.

Function of the BZLF1 Protein

BZLF1 is localised in the nucleus of cells (Marschall et al., 1989) and is a sequence specific DNA binding protein which transcriptionally regulates other early EBV genes by binding to BZLF1 responsive elements. The protein was first shown to bind an AP-1 site in the promoter for the BZLF1 responsive gene BSLF2+BMLF1 (Farrell et al., 1989). A number of BZLF1 binding sites have now been identified and the binding specificity of BZLF1 appears complex (see Table 3.1; Farrell et al., 1989; Packham et al., 1990; Lieberman et al., 1990; Lieberman and Berk, 1990; Flemington and Speck, 1990d; Gruffat et al., 1990b). The protein is considered to be a promoter factor since moving the BZLF1 responsive element several hundred basepairs destroys the function of the protein (Urier et al., 1989).

In addition to autoregulation of the BZLF1 promoter the protein has been shown to transactivate the promoter for the BRLF1, BSLF2+BMLF1 and BMRF1 genes and the DS_L and DS_R promoter regions (Sinclair et al., 1991; Buisson et al., 1989; Kenney et al., 1989a;

Holley-Guthrie et al., 1990; Chevallier-Greco et al., 1989; Chavrier et al., 1989; Cox et al., 1990; Lieberman et al., 1989). The protein is capable of acting synergistically with BRLF1 and this is likely to be important in reactivation for efficient transactivation. Under certain conditions, when BZLF1 is not bound to DNA, BZLF1 inhibits BRLF1 transactivation (Giot et al., 1991). BZLF1 has also been shown to transactivate the promoter for the c-fos gene (Flemington and Speck, 1990d) and the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR; Mallon et al., 1990). Following induction of the productive cycle, latent gene expression has been shown to be downregulated (Rooney et al., 1989; Sinclair et al., 1992) and BZLF1 has been shown to inhibit the activity of the BWR1 and BCR2 promoters and the promoter for the LMP1 gene (Kenney et al., 1989b). Differential effects on EBV latent and productive cycle gene expression by BZLF1 may be important in the switch to the productive cycle.

In this thesis experiments are presented which investigate the structure and function of the BZLF1 protein. The DNA binding specificity of the protein is analysed and point mutagenesis of a BZLF1 binding site and inspection of naturally occuring sites are used to define a consensus for BZLF1 sequence specific DNA binding. The structure of the protein is investigated using deletion, point and domain swap mutants and several functional domains are identified. BZLF1 is shown to homodimerise and the structure and function of this dimerisation domain are investigated. The mutant BZLF1 proteins are used to analyse the antibody response against BZLF1 in patients with NPC. Finally, transcription of mRNAs encoding BZLF1 are analysed in the cell line Akata following induction by anti-immunoglobulin treatment.

Chapter 2
Materials and Methods

All solvents and chemicals were supplied by BDH Chemicals, UK unless otherwise indicated. Radiochemicals were from Amersham International, UK and nucleotide triphosphates were from Pharmacia, Sweden.

2.1 Solutions and Buffers

Buffer O

100mM	Tris-Cl pH 7.5
100mM	$MgCl_2$
1mg/ml	gelatin

Bacteriology Reagents

2x TY

16g	tryptone (Difco, USA)
10g	yeast extract (Difco, USA)
5g	sodium chloride
	water to 1 litre, pH 7.4

TYE plates

10g	tryptone
5g	yeast extract
8g	sodium chloride
15g	agar (Difco, USA)
	water to 1 litre

ELB

250mM	sodium chloride
50mM	HEPES pH 7.0
5mM	EDTA
0.1% (v/v)	Nonidet-P40
1mM	PMSF
0.5mM	DTT

TE

10mM	Tris-Cl pH 7.5
1mM	EDTA

TBE

10.8g	Tris base
5.5g	boric acid
4.0ml	0.5M EDTA pH 8.0
	water to 1 litre

5x Transcription Buffer

200mM	Tris-Cl pH 7.5
30mM	$MgCl_2$
10mM	spermidine-HCl

Tris-Saline-Tween (TST)

10mM	Tris-Cl pH 8.0
150mM	sodium chloride
0.05% (v/v)	Tween-20

Phosphate Buffered Saline (PBS)

8.00g	sodium chloride
0.20g	potassium chloride
1.44g	Na_2HPO_4
0.24g	KH_2PO_4
	water to 1 litre; pH 7.2

2.2 Cell Lines

2.2.1 Bacterial Cell Strains

The E.coli strains TG1 (Gibson, 1984) and HB101f (Boyer and Roulland-Dussoix, 1969) were used for plasmid DNA preparations.

2.2.2 B-Lymphocyte Cell Lines

The EBV negative cell line Ramos used in the transient transfection studies was derived from an American Burkitt's lymphoma (Klein et al., 1975). The cell line Akata used in the induction studies was derived from a Japanese EBV positive Burkitt's lymphoma (Takada et al., 1991). The cell line B95-8 used in the induction studies is a lymphoblastoid cell line made by transforming marmoset B-lymphocytes with the B95-8 strain of EBV (Miller et al., 1972). The cell lines were maintained in RPMI 1640 (Gibco, UK) supplemented with 10% (v/v) fetal

calf serum (Flow Laboratories, UK), 2mM glutamine, 200µg/ml streptomycin and 60µg/ml penicillin (all from Gibco, UK) at 37°C, 10% CO_2. The fetal calf serum was heat inactivated at 56°C for 1 hour before use. Cell numbers were determined using a haemocytometer (Weber, England).

2.3 Gel Electrophoresis

2.3.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was in 0.8-1.5% (w/v) electrophoresis grade agarose gels (Bethesda Research Laboratories, USA) in TBE containing 10µg/ml ethidium bromide. Samples were loaded in a solution containing a final concentration of 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol FF and 3% (w/v) Ficoll type 400. Electrophoresis was at 7 to 10 volt/cm.

2.3.2 Polyacrylamide Gel Electrophoresis

Denaturing Gels (6%)

24g	urea
2.5ml	10x TBE
7.5ml	38.8% (w/v) acrylamide, 1.2% (w/v) bisacrylamide
	H_2O to 50ml
50µl	TEMED (tetramethylethylene diamine)
50µl	25% (w/v) ammonium persulphate

Samples were loaded in formamide loading buffer containing a final concentration of 90% (v/v) formamide (FSA Laboratory Supplies, UK), 0.02% (w/v) bromophenol blue and 0.02% (w/v) xylene cyanol FF.

Non-denaturing Gels (4%)

2.5ml	10x TBE
5.0ml	38.8% (w/v) acrylamide, 1.2% (w/v) bisacrylamide
	H_2O to 50ml
50µl	TEMED
50µl	25% (w/v) ammonium persulphate

2.3.3 SDS-PAGE and Western Immunoblotting

SDS-PAGE was employed to analyse in vitro translation products and proteins from cell extracts. Cells (1-2x10^6 cells) were collected by centrifugation at 1,300 rpm (500g) for 5 min at 4°C in an MSE Centaur centrifuge and were washed once in ice-cold PBS. The cells were resuspended in SDS sample buffer (30-50µl) and lysed by sonication for 30 sec at 4°C.

The proteins were separated electrophoretically on polyacrylamide gels by the method of Laemmli, 1970. Proteins were boiled for 5 min immediately before loading onto the gel system and electrophoresis was performed overnight at 3 volts/cm. Protein molecular weight markers were 'Rainbow' [^{14}C]methylated proteins from Amersham International, UK.

The gel was subjected to fluorography to detect ^{35}S-labelled proteins. The gel was fixed in 45% (w/v) ethanol, 10% (w/v) glacial acetic acid (3 x 30 min) before being impregnated for 25 min with 'Enlightening' (Biotechnology Systems, USA). The gel was dried using a heated vacuum gel drier (120 min at 80°C) and exposed to X-ray film.

Western immunoblotting was performed using previously described protocols (Towbin et al., 1979; Burnette, 1981). Proteins were transferred electrophoretically on to nitrocellulose (Schleicher and Schuell, Germany) and the filter was incubated with blocking agent (5% (w/v) skimmed milk in PBS) to block non-specific binding of antibody to the filter. The filters were incubated overnight at 4°C with the antibody at the recommended dilution in blocking agent. The filters were washed in TST (15 min, two washes) and were then incubated with [^{125}I]protein A (30mCi/mg, final activity 50nCi/ml in blocking agent) for 4 hours at room temperature. The filters were washed in TST (at room temperature for 15 min, at least 4 washes) blotted dry and exposed to X-ray film.

2.4 DNA Manipulations

Standard subcloning techniques were employed (Maniatis et al., 1989). Restriction endonucleases and other enzymes were obtained from New England BioLabs, USA unless indicated. Plasmid vectors were linearised and dephosphorylated prior to ligation and DNA fragments were obtained by digestion of suitable plasmids and were gel purified. Following ligation, DNA was transformed into competent bacteria and clones analysed by restriction mapping of small scale DNA preparations. Plasmids were

purified by centrifugation on caesium chloride gradients containing ethidium bromide (Birnboim, 1983). The cloning strategy for each construction is described fully in section 2.5.

Restriction Endonuclease Digestion

A typical reaction to linearise vector DNA would be;

16µl	DNA (1-2µg) in H_2O
10 units	restriction enzyme
4µl	appropriate 5x restriction buffer
20µl	

Reactions were incubated at the recommended temperature for 1-2 hours with an approximately 10 fold excess of restriction enzyme. Partial digests were obtained by serial dilution of the restriction enzymes and analysis of the products by agarose gel electrophoresis.

Vector Dephosphorylation

Following linearisation, 5' phosphate groups were removed from the vector DNA. Calf intestinal alkaline phosphatase (25 units; Boehringer Mannheim, Germany) was added to the restriction reaction and incubated at 37°C for 30 min. The enzymes were heat inactivated at 65°C for one hour and the DNA was purified by extraction with phenol/chloroform and ethanol precipitation, as below. An equal volume of water saturated phenol (Rathburn Chemicals Ltd., UK)/chloroform (1/1, v/v) was added to the reaction mix. The mixture was vortexed and the phases were separated by centrifugation at 13,000 rpm for 3 min in a microfuge. The top aqueous phase was removed and extracted as above with an equal volume of chloroform. The top aqueous phase was again removed and the DNA was precipitated by addition of 1/10 volume 3M sodium acetate pH 5.2 and 2.5 volumes of ethanol. The sample was vortexed and then incubated at -20°C for 10-60 min and DNA recovered by centrifugation at 13,000 rpm for 5-20 min in a microfuge. The pellet was washed using ice-cold 70% (v/v) ethanol and dried using a Speed Vac Concentrator (Savant, USA). All subsequent ethanol precipitations were performed in this way.

Gel Purification of DNA Fragments

DNA fragments, obtained by restriction digestion of appropriate plasmids, were separated by agarose gel electrophoresis and the DNA was

purified using a Biotrap (Schleicher and Schuell, Germany). The Biotrap was placed in a horizontal electrophoresis tank filled with TBE and the agarose gel block placed in the central elution chamber. An electrical field was applied (120 volts for 2 hours). The DNA molecules migrate from the elution chamber into a small chamber (approximately 300µl) formed by two different types of membrane, both of which allow the flow of current. The first membrane acts as a prefilter which excludes large particles, whilst the second retains the DNA molecules. Fragments from 14bp to 150kbp can be purified in this way. The contents of the chamber were removed and ethanol precipitated.

PCR Amplification of DNA fragments

DNA fragments were also prepared by PCR amplification of EBV sequences which had been subcloned into M13mp8 as part of the EBV sequencing project. A typical polymerase chain reaction (PCR) amplification reaction would be,

1µl	single stranded M13 DNA containing EBV sequences (approximately 50ng)
2µl	oligonucleotide primers (25 pmoles each)
10µl	500mM KCl, 100mM Tris-Cl pH 8.5, 15mM $MgCl_2$
16µl	dATP, dCTP, dGTP, dTTP mix (1.25mM each)
1µl	Taq DNA polymerase (2.5 units, Cetus, USA)
70µl	H_2O
100µl	

Thirty cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 2 min) were performed using a Cetus Thermal DNA Cycler. After amplification the DNA was purified by phenol/chloroform extraction and ethanol precipitation before digestion to release the amplified sequences. The fragment was gel purified as above.

Removal of Protruding Termini from DNA

It was often necessary to blunt the protruding 3' and 5' termini produced by restriction with certain endonucleases. DNA with protruding 5' termini was repaired using the large fragment of DNA polymerase I

(Klenow fragment) from E.coli, (supplied by Boehringer Mannheim, Germany). A typical reaction would be,

21µl	DNA (1-5µg) in H_2O
3µl	buffer O
1µl	60mM MSH
4µl	dATP, dCTP, dGTP, dTTP mix (2mM each)
1µl	Klenow enzyme (5 units)
30µl	

The reaction was incubated at room temperature for 30 min and the enzyme heat inactivated at 65°C for 10 min.

DNA with recessed 5' termini was made blunt using T4 DNA polymerase which removes single stranded DNA. A typical reaction would be,

23µl	DNA (1-5µg) in H_2O
3µl	10x T4 polymerase buffer
3µl	dATP, dTTP, dGTP, dCTP mix (0.5mM each)
1µl	T4 polymerase (1 unit)
30µl	

The reaction was incubated at 37°C for 30 min and the enzyme heat inactivated at 70°C for 10 min. After protruding temini had been made blunt the DNA was purified by phenol/chloroform extraction and ethanol precipitation.

DNA Ligations

The purified vector and DNA fragments were analysed on an agarose gel before ligation. A ligation reaction using typical vector DNA (3kbp) and DNA fragment of approximately 1kbp would be,

50ng of dephosphorylated vector DNA	
200ng of purified DNA fragment	
2µl	0.5M Tris-Cl pH 7.5, 100mM $MgCl_2$ 100mM DTT
2µl	10mM ATP
1µl	T4 DNA ligase (400 units)
	H_2O to 20µl

The reaction was incubated overnight at 16°C. In some cloning procedures it was necessary to include synthetic double-stranded oligonucleotides as adaptors (Oswel, UK). One of the strands was phosphorylated using T4 polynucleotide kinase to allow ligation to the dephosphorylated vector. The other strand was used unphosphorylated since the purified fragment would carry a 5' phosphate group and excessive adaptor self-ligation could be prevented. A typical kinase reaction would be,

6µl	oligonucleotide (1µg) in H_2O
1µl	buffer O
1µl	60mM MSH
1µl	10mM ATP
1µl	T4 polynucleotide kinase (1 unit)
10µl	

The reaction was incubated at 37°C for 30 min before the enzyme was heat inactivated at 65°C for 10 min.

After phosphorylation the oligonucleotide was hybridised with its complementary strand. Equimolar amounts of the two oligonucleotides were mixed and heated to 65°C for 5 min before gradual cooling to room temperature over 15 min. Adaptors were included in ligation reactions at a 25 fold molar excess over vector DNA.

Plasmid Transformation into Competent Bacteria

Competent bacteria were prepared after the method of Cohen et al., 1972. A culture of TG1 or HB101 cells was prepared by innoculating a bacterial colony into 2x TY (1ml). The culture was incubated with shaking overnight at 37°C. The following day some of the cells were diluted 100 fold into 30ml of fresh 2x TY and were incubated for approximately two hours until the cells had reached an absorbance at 600nm of 0.3-0.5. The cells were collected by centrifugation at 3,000 rpm (2,000g) for 10 min at 4°C in an MSE Centaur centrifuge and resuspended in 10ml of ice-cold 100mM calcium chloride. The cells were left on ice for 15 min and then collected as above before being resuspended in ice-cold 100mM calcium chloride (1ml). This procedure produces competent bacteria which yield about $1x10^6$ transformants per microgram of supercoiled plasmid DNA.

A typical transformation reaction would be,

5µl	ligation mix (50ng DNA)
5µl	100mM Tris-Cl pH 7.5, 100mM $MgCl_2$, 100mM $CaCl_2$
40µl	H_2O
100µl	competent bacteria
150µl	

The tubes were placed on ice for 30 min before being heat-shocked at 37°C for 2 min. The tubes were placed back on ice for a further 10 min before addition of 300µl of 2x TY. The cells were incubated at 37°C for 30 min with shaking before a portion (typically 50µl) was plated on to TYE plates with ampicillin (100µg/ml). The plates were incubated overnight at 37°C.

Small Scale DNA Preparations

Individual colonies were innoculated into 2x TY (2ml) with ampicillin (100µg/ml) and were grown overnight at 37°C in a shaking incubator. The following day the cells were collected by centrifugation at 13,000 rpm (11,600g) for 1 min in a microfuge and were resuspended in 100µl of 20% (w/v) sucrose, 100mM Tris-Cl pH 8.0, 20mM EDTA with lysozyme (2mg/ml; Sigma, USA). The tubes were incubated for 5 min at room temperature before addition of 200µl of 1% (w/v) SDS, 0.2M NaOH. After thorough mixing, sodium acetate (3M, 150µl) was added and the tubes placed at -20°C for 10 min. The samples were then centrifuged at 13,000 rpm for 15 min in a microfuge and the supernatant transferred to a new tube. The plasmid DNA was ethanol precipitated and redissolved in 50µl of TE. The DNA was heated at 65°C for 10 min and then incubated with RNase A (20µg; Boehringer Mannheim, Germany) at 37°C for 15 min.

Positive clones were identified by restriction enzyme digestion and taken for large scale DNA preparation. Where necessary the authenticity of constructions was confirmed by double-stranded DNA sequencing.

2.4.1 Double-Stranded DNA Sequencing

Sequencing was performed with the Sequenase Version 2 Kit (United States Biochemical Corporation, USA). Plasmid DNA (5µg) was denatured with 0.2M NaOH, 2mM EDTA at 37°C for 30 min. The DNA was ethanol

precipitated as before and resuspended in the annealing buffer with the appropriate sequencing primer (final concentration, 0.5pmol/µl, Oswel, UK). The primer was hybridised by heating at 65°C for 5 min followed by gradual cooling to room temperature over 15 min. The template-primer mix was incubated in the labelling mix at room temperature for 4 min before being added to each of the four extension termination mixes. The reactions were incubated at 37°C for 4 min before the reactions were stopped by addition of sample loading buffer. The samples were heated to 95°C for 2 min and were loaded onto a 6% denaturing polyacrylamide gel. Electrophoresis was for 120 min at 40 watts. After electrophoresis the gel was fixed for 15 min in 10% (v/v) glacial acetic acid, 10% (v/v) methanol. The gel was dried using a heated vacuum gel drier (30 min at 80°C) and exposed to X-ray film.

2.5 Plasmid Constructs

The construction of the plasmids used is given below,

Construction of the BZLF1 deletion mutants

The BZLF1 deletion mutants were derived from the plasmid SP64-BZLF1 (gift of P.J.Farrell) which is an 870 bp BamHI-EcoRI cDNA encoding the whole of the BZLF1 coding sequence cloned between the BamHI and EcoRI sites of SP64 (Rooney et al., 1989).

SmaI-C; SP64-BZLF1 was digested with SmaI and the large C-terminal/vector fragment was isolated. This fragment was ligated with a double stranded (ds) oligonucleotide of sequence CATCGATG to introduce the initiating methionine and the construct was cloned.

N-HincII; SP64-BZLF1 was digested with HincII and a ds oligonucleotide of sequence TAGAATTCTA was ligated to the ends. After digestion with EcoRI the plasmid was circularised and cloned.

N-PstI; SP64-BZLF1 was digested with PstI and a ds adaptor of sequence,

```
GCGAATTCTTACTGCA
CGCTTAAGAATG
```

was ligated to the ends. After digestion with BamHI and EcoRI, the resulting BamHI-EcoRI fragment was cloned between the BamHI and EcoRI sites of SP64.

N-StyI; SP64-BZLF1 was digested with StyI and the ends were made blunt with mung bean nuclease. A ds oligonucleotide of sequence TAGAATTCTA was ligated to the ends. After digestion with EcoRI the plasmid was circularised and cloned.

HindIII delta; SP64-BZLF1 was digested with HindIII giving three pieces. The N-terminal fragment was cloned in the appropriate orientation into the large vector/C-terminal piece, omitting the small central HindIII fragment.

BsmI-C; SP64-SmaI-C was digested with EcoRI and BsmI. The ds adaptor,

```
GATCCAAGATGTGCG
    GTTCTACAC
```

was used to join the BsmI end to the BamHI site in the large BamHI to EcoRI fragment of SP64 and to introduce the initiating methionine. The authenticity of the construction was confirmed by DNA sequencing.

BsmI-HincII; SP64-BsmI-C was digested with PstI. The smaller fragment was cloned in the appropriate orientation into the larger PstI fragment of SP64-N-HincII.

BsmI-PstI delta; SP64-BZLF1 was digested with PstI and both the large C-terminal/vector and the small N-terminal pieces were isolated. The N-terminal fragment was partially digested with BsmI and the PstI to BsmI fragment extending from the 5' end of the BZLF1 gene down to the second BsmI site was isolated. This was ligated to the large C-terminal/vector fragment using the ds adaptor,

```
  ATTCTCTGCA
GCTAAGAG
```

to join the BsmI and PstI sites within the gene. The authenticity of the construction was confirmed using DNA sequencing.

The plasmids SmaI-C, N-HincII, N-PstI and N-StyI were a gift of P.J.Farrell.

The St.CAT and p48Z.CAT plasmids were used in transient transfection assys and contained the BSLF2+BMLF1 and BZLF1 promoters respectively fused to the CAT reporter gene.

St.CAT was constructed by inserting a BamHI to BglII fragment of B95-8 DNA (EBV genome coordinate numbers 84234 to 87053) into the promoterless CAT vector pSVOCAT (Gorman et al., 1982) with the BamHI end (EBV number 84234) next to the CAT gene. The HindIII site of pSVOCAT had previously been converted to a BglII site with a synthetic linker. The plasmid was a gift of P.J.Farrell.

p48Z.CAT was constructed by cloning the insert of the EBV M13 clone, 48.BAZ into the HindIII site of pSVOCAT. 48.BAZ was an M13mp8 clone containing B95-8 sequences (EBV genome coordinates 103188 to about 103650) at the SmaI site. The insert was released by digestion with HindIII and EcoRI, and the EcoRI was converted to HindIII with synthetic linkers. The insert in pSVOCAT was orientated with the 103188 end next to the CAT gene. The plasmid was a gift of P.J.Farrell.

The vector p3.1W was used to drive expression of the BZLF1 deletion mutants in B-cells from the EBV BWR1 promoter. The plasmid is derived from pUC19 and consists of the BWR1 promoter and the SV40 early promoter polyA signals. The polyA signals were cut out of pSV2CAT (Gorman et al., 1982) as a 135bp BamHI-HpaI fragment. The BamHI site was repaired with Klenow DNA polymerase and the fragment was cloned into the AatII site of pUC19 which had been made blunt with T4 DNA polymerase to give plasmid pUC19.25. Plasmid A94.58 (gift of C.M.Rooney), which contains B95-8 sequences spanning the BamHI-W repeat, was digested with HindIII and XbaI and the 3.1 kb BWR1 promoter fragment was cloned into pUC19.25 which had been digested with HindIII and XbaI to give plasmid p3.1W. BZLF1 and the deletion mutants, BsmI-PstI delta, HindIII delta, N-PstI and N-HincII were subcloned from SP64 into p3.1W as XbaI-EcoRI fragments.

The following plasmids were used to generate riboprobes to detect EBV mRNAs. The plasmids contained sequences derived from M13 clones from the EBV sequencing project.

pGEM-3Z-374 is derived from the M13 clone 374.RF, which contains EBV sequences from 84487 to about 83800 inserted at the SmaI site of M13mp8, orientated with the 84487 end close to the BamHI site of the polylinker. The clone was digested with EcoRI and BamHI site at EBV genome number 84233) and the resulting fragment was cloned between the EcoRI and BamHI sites of pGEM-3Z. The plasmid was digested with BamHI and transcribed using T7 RNA polymerase.

SP64-Z44 is derived from the M13 clone BAZ.44.RF, which contains EBV sequences from 102984 to about 103650 inserted at the SmaI site of M13mp8, orientated with the 102984 end close to the BamHI site of the polylinker. The clone was digested with EcoRI and PstI and the resulting fragment was cloned between the EcoRI and PstI sites of SP64. The plasmid was digested with SphI (site at EBV genome number 103419) and transcribed using SP6 RNA polymerase.

pGEM-4Z-288.HET is derived from the M13 clone 288.HET, which contains EBV sequences from 164608 to about 164838 inserted at the SmaI site of M13mp8, orientated with the 164608 end close to the BamHI site of the polylinker. The fragment was amplified using PCR, digested with EcoRI and BamHI and the resulting fragment was cloned between the EcoRI and BamHI sites of pGEM-4Z. The plasmid was digested with EcoRI and transcribed using T7 RNA polymerase.

2.6 In Vitro Transcription and Translation

The BZLF1 deletion mutants were cloned into the SP64 plasmid (Promega, USA) in which transcription is directed from the SP6 promoter. This system allows the synthesis of large amounts of RNA for in vitro translation. Other proteins were made by in vitro translation and transcription from similar plasmids.

Prior to transcription the plasmid DNA (10μg) was linearised at a restriction site downstream of the cloned sequences. The DNA was purified by phenol/chloroform extraction and ethanol precipitation and was resuspended in TE at 0.5μg/μl. A typical transcription reaction would be,

5µl	linearised plasmid (2.5µg)
10µl	5x transcription buffer
5µl	ATP, UTP, CTP, GTP mix (each 5mM)
5µl	0.1M DTT (Promega, USA)
2.5µl	$m^7G(5')ppp(5')G$, 2mM (Boehringer Mannheim, Germany)
1.25µl	RNasin Ribonuclease inhibitor (50 units, Promega, USA)
2.5µl	SP6 RNA polymerase (25 units)
18.75µl	H_2O
50µl	

The reaction was incubated at 40°C for 30-60 min. The RNA was incubated with DNase I (10 units, Pharmacia, Sweden) at 37°C for 15 min to remove the DNA template and RNA was then purified by phenol/chloroform extraction and ethanol precipitation.

A portion of the RNA was sometimes made radioactive and analysed by gel electrophoresis to demonstrate that the transcripts produced were of the correct size. A portion (5µl) of the complete transcription reaction mix before incubation was transferred to a new tube with [α^{32}P]UTP (5µCi, 800Ci/mmol). The reaction was incubated and RNA purified as above.

The radiolabelled RNA was analysed by agarose gel electrophoresis. A portion of the RNA (4µl) was mixed with 7.2µl of glyoxal mix (105µl DMSO, 4.2µl 0.5M sodium phosphate and 30µl glyoxal) and incubated at 50°C for 10 min before being resolved on a 1.4% agarose gel in 10mM sodium phosphate. Electrophoresis was at 40mA for 2 hours. The buffer was recycled and the gel cooled using an LKB 2117 Multiphor II electrophoresis unit. After electrophoresis the gel was dried using a heated vacuum gel drier (2 hours at 60°C) and exposed to X-ray film.

Small RNAs were analysed by denaturing polyacylamide gel electrophoresis. The RNA was diluted in formamide loading buffer and heated at 90°C for 10 min before being resolved on an 8% polyacrylamide gel (see section 2.3.2). After electrophoresis the gel was fixed for 15 min in 10% (v/v) glacial acetic acid, 10% (v/v) methanol, dried using a heated vacuum gel drier (30 min at 80°C) and exposed to X-ray film.

The RNA was translated using wheat germ extracts (Amersham International, UK) or rabbit reticulocyte lysate systems. Where it was

necessary to generate radiolabelled translation products, either [^{35}S]cysteine or [^{35}S]methionine was added to the wheat germ extract mixes. Unradiolabelled proteins were generated using the complete rabbit reticulocyte lysate system (N90, Amersham International, UK) whilst amino-acid depleted lysates (Promega, USA) were used to generate radiolabelled translation products. Where amino-acid depleted systems were used, a mix of all amino-acids (i.e. alanine, asparagine, aspartic acid, arginine, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) except the radiolabelled one was added to the reaction. Typical translation reactions using [^{35}S]cysteine as a radiolabel are shown below,

Wheat Germ Extract Translations

10µl	1M potassium acetate
10µl	1mM amino-acid mix minus cysteine
75µl	wheat germ extract
10µl	[^{35}S]cysteine (100µCi, 1000Ci/mmol) or H_20
15µl	H_20
120µl	

1µl of RNA (1µg) was added to 12µl of the translation mix.

Complete Rabbit Reticulocyte Lysate Translations

80µl	rabbit reticulocyte lysate (N90)
10µl	H_2O
90µl	

1µl of RNA was added to 9µl of the mix.

Depleted Reticulocyte Lysate Translations

85µl	amino-acid depleted lysate
2.5µl	1mM amino-acid mix minus cysteine
2µl	[^{35}S]cysteine (100µCi, 1000Ci/mmol)
30.5µl	H_2O
120µl	

1µl of RNA was added to 9µl of the mix.

Control translations contained water in place of RNA. Cotranslations were made by mixing complete lysates before incubation.

Translations in the rabbit reticulocyte lysate systems were incubated at 30°C for 30-45 min. Translations in the wheat germ extract system were incubated at 25°C for 30-60 min. The translation reactions were terminated by addition of 5 volumes of SDS sample buffer (0.0625M Tris-Cl pH 6.8, 2% (w/v) SDS, 100mM DTT, 10% (v/v) glycerol, 0.001% bromophenol blue) or were stored at -70°C.

2.7 Gel Retardation Assays

The DNA substrate in the gel retardation assays was either a 261bp DNA fragment spanning a known BZLF1 binding site or double-stranded (ds) oligonucleotides (Oswel, UK). Double-stranded oligonucleotides were made by hybridisation of complementary strands at 65°C for 10 min followed by gradual cooling to room temperature over 15 min. Substrate DNA was radiolabelled using T4 polynucleotide kinase. A typical reaction would be,

28µl	ds oligonucleotide (300ng) in H_2O
4µl	buffer O
4µl	60mM MSH
2µl	[γ^{32}P]ATP (20µCi, 5000Ci/mmol)
2µl	T4 polynucleotide kinase (2units)
40µl	

The reaction was incubated at 37°C for 30 min and the enzyme removed by phenol/chloroform extraction. Unincorporated nucleotides were removed by gel filtration through Sephadex G50 (Pharmacia, Sweden) equilibrated with TE.

The sequences of the ds oligonucleotides used in gel retardation assays are given below;

292/293 (M site)

CTTCATGAGTCAGTGCTTC
GAAGTACTCAGTCACGAAG

475/476 (Z2 site)

GTACATTAGCAATGC
CATGTAATCGTTACG

AP-1 (fat specific element; Distel et al., 1987)

GATCCATGACTCAGAGGAAAACATACG
CTAGGTACTGAGTCTCCTTTTGTATGC

C/EBP (Landschulz et al., 1988a)

AATTCAATTGGGCAATCAGG
GTTAACCCGTTAGTCCTTAA

ds458 (control)

GCGAAGCACTCTCGAGTGAAGGTGAC
CGCTTCGTGAGAGCTCACTTCCACTG

Point mutagenesis of M site; all the ds oligonucleotides were derived by point changes in both strands of the wild type M sequence below,

TTCATGAGTCAGTGC
AAGTACTCAGTCACG

427/428 (octanucleotide AP-1 site in promoter for BZLF1)

ACCATGACATCACAGAGG
TGGTACTGTAGTGTCTCC

The concentrations of the oligonucleotides were determined by measuring their absorbance at 260nm using a Philips PU8620 spectrophotometer. It was assumed that an OD_{260} of 1 corresponded to a concentration of oligonucleotide of 20µg/ml.

Proteins made by in vitro translation (1-3µl) or fusion proteins (0.1µg) were diluted into 18µl of BC100 (100mM KCl, 20mM HEPES pH 7.9, 10% glycerol, 0.2mM EDTA, 4mM DTT) and to this 4µl of premixed DNA (i.e. poly(dIC) (Boehringer Mannheim, Germany), radiolabelled substrate DNA and any competitor DNA) was added. The final concentration of poly(dIC) was 120µg/ml. Similar assays were performed using radiolabelled proteins made by in vitro translation and unlabelled substrate DNA. After incubation at room temperature for 25 min, 3µl of dyes (0.05% (w/v) xylene cyanol FF and 0.05% (w/v) bromophenol blue in BC100) was added and the sample was loaded on to either a 1% agarose gel or a 4% non-denaturing polyacrylamide gel (see section 2.3). Electrophoresis was at 120 volts for 2 hours in 0.5x TBE or in buffer G (380mM glycine, 50mM Tris-Cl pH 8.5, 5mM EDTA). Complexes with

radiolabelled DNA were detected by autoradiography of the dried agarose gels or the wet polyacrylamide gels. Polyacrylamide gels were subjected to fluorography to detect ^{35}S-labelled proteins. The gel was fixed in 45% (w/v) ethanol, 10% (w/v) glacial acetic acid (3 x 30 min) before being impregnated for 25 min with 'Enlightening' (Biotechnology Systems, USA). The gel was dried using a heated vacuum gel drier (120 min at 80°C) and exposed to X-ray film.

2.8 Immunoprecipitations

Proteins were immunoprecipitated from in vitro translation systems and from cell lysates. Cell proteins were made radioactive by metabolic labelling of tissue culture cells. Two cell lines were used, B95-8 and Akata, both of which could be induced to the productive cycle to increase the expression of BZLF1.

On the day before the immunoprecipitation, B95-8 cells were diluted into fresh growth medium ($1x10^7$ cells; $0.25x10^6$ cells/ml) with TPA (40ng/ml; Sigma, USA) and were incubated overnight at 37°C, 10% CO_2. The following day the cells were collected by centrifugation at 1,300 rpm (500g) for 5 min at 20°C in a Sorvall H-6000A rotor and were washed twice in warm cysteine depleted growth medium. Finally the cells were resuspended in cysteine depleted growth medium (5ml) at $2x10^6$ cells/ml in small tissue culture flasks. Akata cells ($1x10^7$ cells; 0.6 x 10^6 cells/ml) were induced by treatment for 6 hours with 1% (v/v) rabbit anti-human IgG antibody (A423, Dakopatts, Denmark). The cells were collected and resuspended in cysteine depleted growth medium as above.

B95-8 and Akata cells were radiolabelled by incubation for 3 hours at 37°C, 10% CO_2 with [^{35}S]cysteine (1000Ci/mmol stock; final activity, 0.2mCi/ml of cysteine depleted growth medium).

The cells were collected by centrifugation at 4°C and were washed twice with ice-cold PBS. The cells were resuspended in ELB (1ml) and lysed by sonication. The cell lysate was placed on ice for 15 min before cell debris was removed by centrifugation at 13,000 rpm (11,600g) for 10 min at 4°C in a microfuge. The cell lysate was precleared before immunoprecipitation by incubation with protein A Sepharose beads (200µl of a 1:1 slurry in ELB; Pharmacia, Sweden) at 4°C on a rotating mixer. The beads were removed by brief centrifugation and the precleared lysate transferred to a new tube. Unradiolabelled precleared cell lysates for the Western immunoblot analyses were prepared in a parallel fashion without addition of a

radiochemical. Proteins made by in vitro translation (typically 5-10µl) were diluted into ELB (250µl).

Antibodies and anti-sera were added to the ELB/protein mixtures at the required dilution and were mixed gently. This mix was incubated at 4°C for 20 min on a rotating mixer. Protein A Sepharose beads (65µl of a 1:1 slurry in ELB) were added to immunoprecipitations containing rabbit or human antibodies. Where the monoclonal antibody BZ1 (Young et al., 1991) was used, the beads were precoated with a rabbit anti-mouse Ig antibody (Z259; Dakopatts, Denmark). The beads were precoated by incubating 200µl of a 1:1 slurry of beads in ELB with 12µl Z259 at 4°C for 30 min. The beads were washed three times in ELB and 12.5 µl of a 1:1 slurry of these pre-coated beads was used in each immunoprecipitation. The beads were collected by centrifugation and washed 3 to 5 times in ELB. Where immunoprecipitation was from cell lysates the beads were transferred to a new tube after two washes as this was found to reduce the background. After washing, the proteins bound to the beads were eluted in SDS sample buffer and analysed by SDS-PAGE with fluorography or by Western immunoblotting.

2.9 Transfections

B-cells were transfected using conditions previously described (Buschle et al., 1991). On the day prior to transfection the cells were collected by centrifugation at 1,300 rpm (500g) for 5 min at 20°C in a Sorvall H-6000A rotor. The cells were resuspended in an equal volume of fresh growth medium and incubated overnight. The following day the cells were collected by centrifugation at 4°C and resuspended in cold growth medium at $32x10^6$ cells/ml. The supernatant was saved as conditioned medium. The cell suspension (250µl, $8x10^6$ cells) was transferred into 0.4cm electrode gap electroporation cuvettes (Bio-Rad, USA) and the plasmid DNA was added (10-20µg). The cells and DNA were mixed by gentle agitation and the cuvettes placed on ice for 15 min. The cells were electroporated with a Bio-Rad gene pulser using a capacitance extender at 960µF, 250V and then incubated at 37°C for 20 min. The cells were transferred to tissue culture flasks and the cuvettes rinsed using the conditioned medium. The cells were diluted with conditioned medium to a final volume of 10ml and incubated for 24hr before assaying for gene expression. The cell survival 24hr post-transfection was approximately 70%.

2.10 CAT Assays

The CAT activity in transfected cells was determined 24 hours post-transfection after the method of Gorman et al., 1982. The cells were collected by centrifugation at 1,300 rpm (500g) for 5 min at 4°C in an MSE Centaur centrifuge and washed once in ice-cold PBS. The cells were resuspended in 0.25M Tris-Cl pH 7.5 (50µl) and lysed by repeated freeze-thawing (4 min at 37°C, then 4 min in a dry ice/ethanol bath; at least 5 cycles). The extracts were incubated at 65°C for 10 min and cell debris was removed by centrifugation at 13,000 rpm (11,600g) for 10 min at 4°C in a microfuge. The supernatant was transferred to a fresh tube and diluted, if necessary, to ensure that the assay was in the linear range. The CAT assay reaction mix is shown below,

25µl	cell extract
2µl	[^{14}C]chloramphenicol (50nCi, 50mCi/mmol)
5µl	4mM acetyl-CoA (Boehringer Mannheim, Germany)
18µl	H_2O
50µl	

The reaction was incubated at 37°C for one hour. Chloramphenicol and the acetylated derivatives were extracted with ethyl acetate (1ml) and transferred to a new tube. This was evaporated using a Speed Vac Concentrator (Savant, USA) and the chlorampenicol redissolved in a small volume of ethyl acetate (15µl). The ethyl acetate was spotted onto silica gel-60 TLC plates (Merck, Germany) and the chromatograph developed using chloroform/methanol (95/5, v/v). The plates were air-dried and exposed to X-ray film. The conversion was quantified by determining the radioactivity in the unconverted and acetylated forms of chloramphenicol. The areas of the TLC plate containing these two forms were removed and the radioactivity determined in a Beckman LS 3801 liquid scintillation counter. The percentage conversion was calculated using the equation;

$$\% \text{ conversion} = \frac{\text{acetylated chloramphenicol cpm} \times 100}{\text{total cpm}}$$

All conversions were less than 30% which is considered to be within the linear range of the assay.

2.11 Inhibition of Protein Synthesis in B-cells

Akata cells ($0.5x10^6$ cells/ml) were treated with anisomycin (100μM; Sigma, USA) or with cycloheximide (50μg/ml; Sigma, USA). Treated cells and control cells ($1x10^6$ cells) were incubated at 37°C, 10% CO_2 in 2ml culture wells. The incorporation of [^{35}S]methionine by control cells was measured at this point to determine the amount of protein synthesis at the time of treatment.

Cells ($1x10^6$ cells) were incubated with [^{35}S]methionine (1000Ci/mmol stock; final activity, 10μCi/ml of growth medium) for 10 min at 37°C, 10% CO_2 before being transferred to centrifuge tubes and diluted with ice-cold PBS (10ml). The cells were collected by centrifugation at 1,300 rpm (500g) for 5 min at 4°C in an MSE Centaur centrifuge and washed twice in ice-cold PBS. The cells were resuspended in water (100μl) and vortexed before addition of 50μl of 3mg/ml methionine, 1M NaOH. The lysate was incubated at 37°C for 15 min before addition of 25% (w/v) TCA (100μl) and cooling on ice. The precipitate was collected on glass microfibre filters (Whatman, UK) using a sample manifold (Millipore, USA) and the filters were washed in 10% (w/v) TCA (3x1ml) and 95% (v/v) ethanol (2x1ml) before being air-dried. The radioactivity was determined in a Beckman LS 3801 liquid scintillation counter.

The protein synthesis was measured in treated and control cells 2, 4 and 6 hours after the addition of anisomycin or cycloheximide.

2.12 RNA Isolation and RNase Protection Assays

RNA was isolated from Akata cells which had been treated with anti-immunoglobulin in the presence or absence of anisomycin. Akata cells ($100x10^6$ cells, $0.5x10^6$ cells/ml) were treated with anisomycin (100μM; Sigma, USA) at 37°C, 10% CO_2. Control untreated cells were incubated in a parallel fashion. After incubation for 6.5 hours the cells were induced by treatment with 1% (v/v) rabbit anti-human IgG antibody (A423, Dakopatts, Denmark).

Some treated and control cells ($25x10^6$ cells) were immediately placed on ice prior to RNA isolation. The remaining cells were returned to the incubator and samples (approximately $25x10^6$ cells) were taken at 1, 4 and 20 hours after treatment with anti-immunoglobulin.

RNA Isolation

RNA was isolated using the 'RNAgents Total RNA Isolation Kit', (Promega, USA). The cells were placed on ice immediately after removal from the incubator. The cells were collected by centrifugation at 1,300 rpm (500g) for 5 min at 4°C in an MSE Centaur centrifuge and were resuspended in ice-cold PBS (25ml). A sample of the cells ($1x10^6$ cells) was taken for Western immunoblot and indirect immunofluorescence analyses. The remaining cells were collected as above and resuspended in 6ml of cold denaturing solution (4M guanidium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M MSH) before addition of 2M sodium acetate pH 4.0 (600µl) and phenol/chloroform/isoamyl alcohol (25/24/1, v/v) (6ml). The mixture was vortexed and placed on ice for 15 min before being centrifuged at 8,000 rpm (7,600g) for 20 min at 4°C in a Sorvall SS34 rotor. The top aqueous layer was transferred to a new tube and precipitated by addition of isopropanol (6ml). The RNA was pelleted as above and resuspended in 2.5ml of denaturing solution before being ethanol precipitated two further times. The final RNA pellet was washed several times in cold 75% (v/v) ethanol and redissolved in water.

Contaminating DNA was removed by digestion with RQ1 DNase (10 units; Promega, USA) at 37°C for 15 min in 40mM Tris-Cl pH 7.5, 6mM $MgCl_2$. The RNA was then purified by phenol/chloroform extraction and ethanol precipitation.

RNAse Protection Assays

The amount of specific transcripts was determined using the 'Ribonuclease Protection Assay (RPA) Kit' (Ambion, USA).

Riboprobes were made by in vitro transcription of plasmids containing EBV sequences in the presence of [α^{32}P]UTP. Plasmids were linearised and purified as before (see section 2.6). A typical reaction would be,

0.5µl	linearised plasmid (0.5µg)
2µl	5x transcription buffer
1µl	0.1M DTT (Promega, USA)
0.5µl	RNasin Ribonuclease Inhibitor (20 units, Promega, USA)

(continued overleaf)

2µl	ATP, GTP, CTP mix (each 2.5mM)
2.5µl	[α^{32}P]UTP (50µCi, 800Ci/mmol)
0.5µl	SP6 RNA polymerase (10 units, Promega, USA)
1µl	H_2O
10µl	

The reaction was incubated at 40°C for 60 min. The RNA probe was incubated with RQ1 DNase (1 unit) at 37°C for 15 min to remove the DNA template. The probe was ethanol precipitated and redissolved in formamide loading buffer before being heated at 85°C for 4 min and loaded onto a 6% denaturing polyacrylamide gel (see section 2.3.2). After electrophoresis the part of the gel containing full length probe was removed and the probe eluted by incubation at 37°C for 2 hours in 0.5M ammonium acetate, 1mM EDTA, 0.1% (w/v) SDS. The radioactivity in the probe was determined by Cerenkov counting in a Beckman LS 3801 liquid scintillation counter.

Sample RNA and/or yeast RNA (20µg total) were mixed with the riboprobe (50,000 cpm) and ethanol precipitated. The RNA was redissolved in 20µl of hybridisation buffer (80% (v/v) formamide, 40mM PIPES pH 6.4, 400mM sodium acetate, 1mM EDTA) and heated at 85°C for 4 min before being incubated overnight at 42°C. The following day the RNA was incubated with RNase A (0.01 units) and RNase T1 (2 units) at 37°C for 30 min in RNase digestion buffer (10mM Tris-Cl pH 7.5, 5mM EDTA, 300mM sodium chloride). The enzymes were inactivated by incubation with proteinase K (50µg/ml) in 0.8% (v/v) SDS. The RNA was purified by phenol/chloroform extraction and ethanol precipitation and was redissolved in formamide loading buffer (4µl). The RNA was heated at 85°C for 4 min before being resolved on a 6% denaturing polyacrylamide gel (see section 2.3.2). After electrophoresis the wet gel was transferred to Whatman 3MM paper, covered with Saran wrap and exposed to X-ray film at -70°C.

2.13 Indirect Immunofluorescence

Akata cells ($0.5x10^6$ cells) were taken at 0, 1 and 4 hours after treatment with anti-immunoglobulin with or without anisomycin. The cells were collected by centrifugation at 6,500 rpm for 1 min at 4°C in a microfuge and were washed once in ice-cold PBS before being spotted on

to ethanol washed microscope slides. The slides were air-dried at 37°C and then fixed in acetone at -20°C for 5 min. The cells were rehydrated in PBS and incubated with the monoclonal antibody BZ1 (Young et al., 1991; 10µl of a 1/100 dilution of BZ1 ascites fluid in PBS) at room temperature for 30 min in a humid chamber. The cells were washed briefly in PBS before being incubated with a fluorescein isothiocyanate conjugated (FITC) goat anti-mouse Ig antibody (10µl of a 1/30 dilution in PBS; Southern Biotechnology Associates, USA) as above. The slides were washed at least three times for 10 min in PBS before being mounted in Citifluor (Citifluor Ltd, UK). Fluorescence was observed using a Zeiss immunofluorescence microscope.

Chapter 3
Sequence Specific DNA Binding by BZLF1

The BZLF1 protein has significant homology to the basic DNA binding structure of the c-fos protein and was originally demonstrated to bind specifically to a consensus AP-1 binding site, TGACTCA in the promoter for the BMLF1+BSLF2 gene (M site; Farrell et al., 1989). Since then a large number of BZLF1 binding sites have been reported (Table 3.1). The positions of the BZLF1 binding sites in the promoters for the BSLF2+BMLF1 and BZLF1 genes are shown in figure 3.1. Many of the BZLF1 binding sites show homology to the consensus AP-1 binding site; other sites show homology to C/EBP (CCAAT/enhancer binding protein) binding sites. One BZLF1 binding site in the promoter for the BZLF1 gene (Z2 ; TACATTAGCAATG) is very similar to the binding site for C/EBP (TTNNGCAAT). Also the ZRE5 binding site in the DS_L promoter AATGTGCAAA shows close homology to a site known to bind C/EBP in the SV40 core enhancer sequence, GGTGTGGAAA (Johnson et al., 1987). The following experiments were designed to investigate the sequence specificity of the DNA binding activity of BZLF1.

3.1 BZLF1 binds AP-1 and C/EBP sites

Since BZLF1 has been shown to bind sequences similar to both AP-1 and C/EBP binding sites within the viral genome it was determined whether BZLF1 could bind canonical AP-1 and C/EBP binding sites.

BZLF1 made by in vitro transcription and translation was used as a source of protein in these experiments. The plasmid SP64-BZLF1 (which contains a full length BZLF1 cDNA cloned into SP64) was linearised and transcribed in vitro using SP6 RNA polymerase. The RNA produced was purified and translated in vitro in the rabbit reticulocyte lysate system (see also section 4.2). To demonstrate translation of BZLF1 RNA [^{35}S]cysteine was added to a portion of the translation reaction and the radiolabelled translation products were analysed by SDS-PAGE (figure 3.2 A). Lysates containing unlabelled BZLF1 were used in the DNA binding studies. Control lysates where water had been added to the lysate in place of RNA prior to translation were used to control for possible endogenous DNA binding activities within the lysate.

BZLF1 protein was assayed for its ability to bind the AP-1-like M site and the C/EBP-like Z2 site from EBV (see figure 3.1) and canonical AP-1

	Site	Promoter	Ref.
M	TGAGTCA	BMLF1+BSLF2	(1,2)
Z1	TGAGCCA	BZLF1	(2)
Z2	TTAGCAA	BZLF1	(2)
R1	TGAGCGA	BRLF1	(2)
R2	TGAGCCA	DS_L	(2)
ZRE1	TGTGTAA	DS_L	(3,4)
ZRE2	TGAGCAA	DS_L	(3,4)
ZRE3	TGTGTCA	DS_L	(3,4)
ZRE4	TGTGTCA	DS_L	(3,4)
ZRE5	TGTGCAA	DS_L	(3,4)
ZRE6	TGTGTCA	DS_L	(3,4)
ZRE-B	TGTGTCA	DS_R	(5)
FZ1	TGTGTAA	c-fos	(6)
FZ2	TGCGTCA	c-fos	(6)
FZ3	TGAGTGT	c-fos	(6)
	TGAGTCA TCC	AP-1 consensus	(7)
	TTNNGCAAT G T G	C/EBP consensus	(8)

Table 3.1 Alignment of BZLF1 binding sites

The BZLF1 binding sites identified in the EBV genome and the c-fos promoter are shown aligned with respect to the heptanucleotide AP-1 consensus M site. The location of each site is indicated. The consensus binding sites for jun+fos (AP-1 site) and C/EBP are shown below.

References; 1, Farrell et al., 1989; 2, Packham et al., 1990; 3, Lieberman et al., 1990; 4, Lieberman and Berk, 1990; 5, Gruffat et al., 1990b; 6, Flemington and Speck, 1990c; 7, Risse et al., 1989; 8, Ryden and Beemon, 1989.

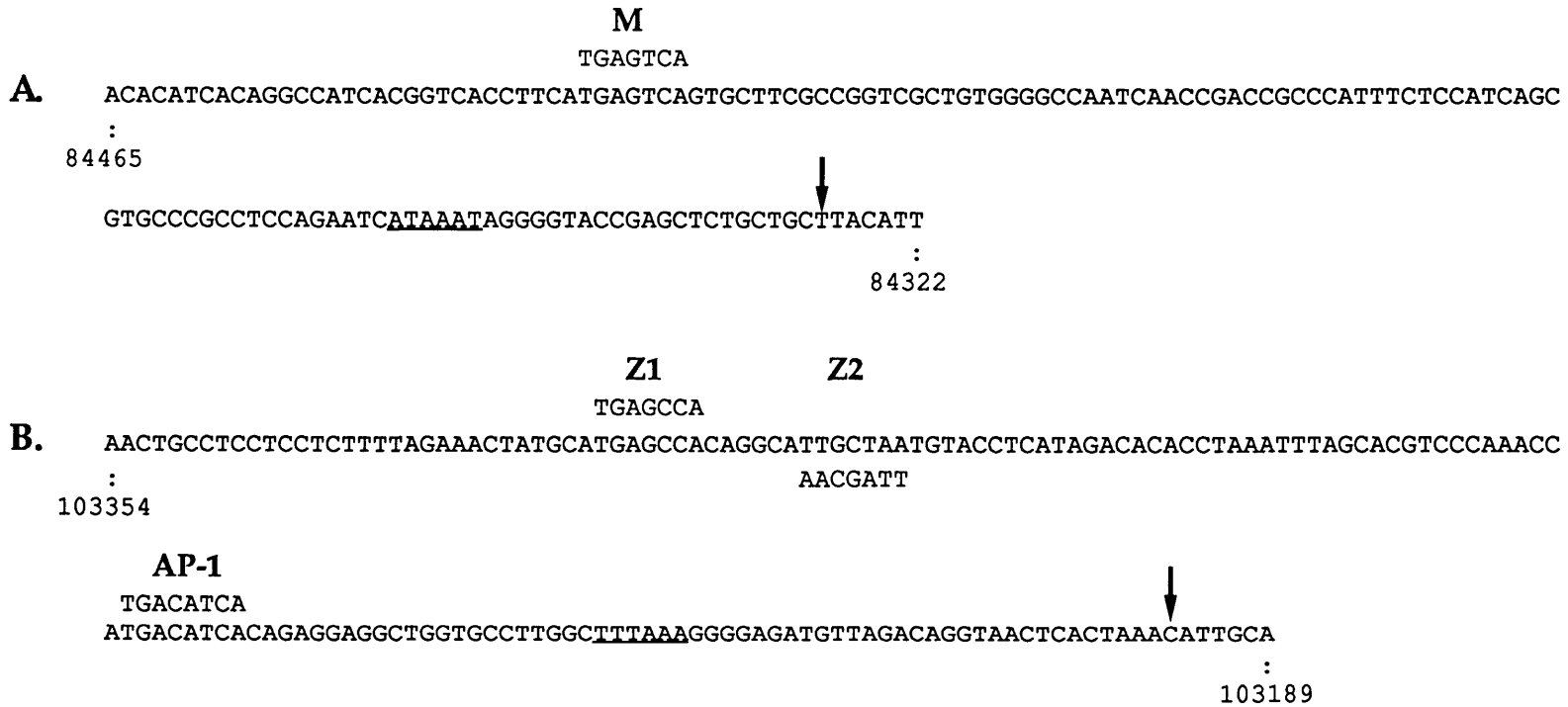


Figure 3.1 Positions of the M, Z1 and Z2 BZLF1 binding sites and the octanucleotide AP-1 binding site.

The nucleotide sequence of part of the promoters for the (A) BSLF2+BMLF1 and (B) BZLF1 genes are shown. The EBV DNA sequence numbers are indicated. The 7bp core of the BZLF1 binding sites are shown (see also Table 3.1) and the TATA boxes for each promoter are underlined. The major transcription start point is indicated by an arrow. The octanucleotide AP-1 site which has been demonstrated to be involved in TPA activation of the promoter for the BZLF1 gene is indicated (see text for references).

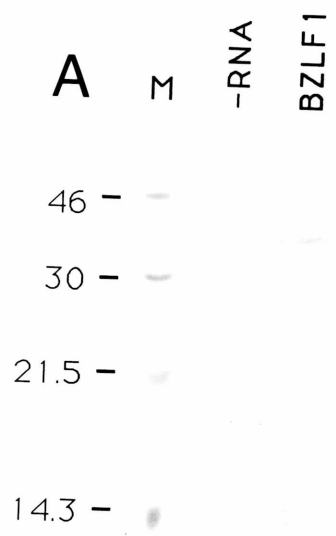


Figure 3.2 Gel retardation assay demonstrating relative affinity of BZLF1 for different binding sites

BZLF1 was made by in vitro translation in the rabbit reticulocyte lysate system with or without [^{35}S]cysteine.

Panel A; The radiolabelled translation products were resolved on a 12.5% SDS polyacrylamide gel and were detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD.

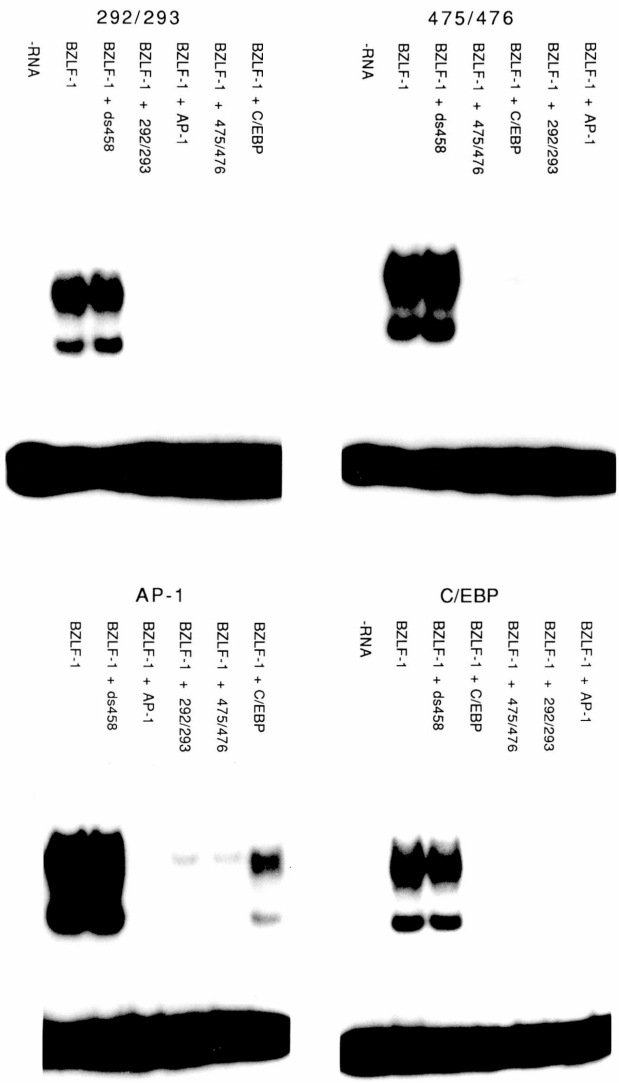


Figure 3.2 Panel B; Lysate containing BZLF1 or lysate with no added RNA (2µl each) was complexed with 2ng of each of the four radiolabelled oligonucleotides (a) 292/293, M site (b) 475/476, Z2 site (c) a canonical AP-1 site and (d) a canonical C/EBP site. Where indicated BZLF1 binding was competed by addition of 200ng of each of the four unradiolabelled oligonucleotides or a control oligonucleotide (ds458). Complexes were resolved on 4% polyacrylamide gels in 0.5x TBE and were detected by autoradiography.

and C/EBP sites in a gel retardation assay (figure 3.2 B). In each case BZLF1 binding was competed with a large excess of each of the unradiolabelled oligonucleotides as well as the oligonucleotide ds458 as a control. Oligonucleotide ds458 is essentially a mutated version of the M binding site (oligonucleotide 292/293); the mutations have been shown to abolish BZLF1 binding (Packham et al., 1990). Several exposures were made on preflashed film to ensure that the autoradiographs would be in the linear response range of the film. The autoradiographs were then scanned using a densitometer to quantitate complex formation and to allow the relative affinity of BZLF1 for each site to be determined.

BZLF1 is capable of binding to canonical AP-1 and C/EBP binding sites as well as the AP-1-like M binding site and C/EBP-like Z2 binding sites of EBV. The binding is considered to be specific since it is competed by an excess of the identical oligonucleotide but not by an excess of the control oligonucleotide. No binding was observed using lysates without added RNA demonstrating that the DNA binding activity observed is specific for BZLF1. Densitometric quantitation of the autoradiograph suggests that BZLF1 binds to the M and Z2 sites with about a five fold higher affinity than the canonical C/EBP site and about a four fold lower affinity than the canonical AP-1 site.

3.2 Consensus for BZLF1 DNA binding

The previous experiment demonstrates that BZLF1 can bind two different sequences. To understand better the sequence specificity of BZLF1 and to assess the importance of individual residues within the BZLF1 binding site, site directed mutagenesis of one BZLF1 binding site was performed. The binding site within the promoter for the BMLF1+BSLF2 gene (M site) was chosen since this is identical to a consensus AP-1 binding site.

Fourteen double stranded oligonucleotides were obtained (Oswel, UK) each with a single point mutation in the M site. The oligonucleotides were radiolabelled using T4 polynucleotide kinase and analysed on a non-denaturing polyacrylamide gel (data not shown) to ensure that the amount of each of the double stranded oligonucleotides was approximately equivalent. The affinity of BZLF1 for each of these mutated sites was measured by assaying their ability to compete BZLF1 binding to the wild type sequence. BZLF1 protein was made by in vitro translation and was complexed with radiolabelled 292/293 (M site)

oligonucleotide in a gel retardation assay. The wild type binding was competed with a 10 fold molar excess of each of the mutated oligonucleotides (figure 3.3). This gave an approximate measurement of their activities as competitors.

In order to more accurately measure the affinity of BZLF1 for each of the mutated sites, the oligonucleotides were then added as competitors in a dilution series (figure 3.4). The previous experiment indicated the concentration range in which each mutant oligonucleotide was effective as a competitor. A number of exposures were made on preflashed film and the autoradiographs were scanned using a densitometer. The concentration of competitor required to inhibit 50% of the binding of BZLF1 to the wild type sequence was calculated and the results are expressed in the histogram as $\log_{10}$ of the fold excess in concentration over the radiolabelled target for each competitor. Mutations which decrease the affinity of BZLF1 for the binding site require a higher concentration of competitor to inhibit wild type binding and are therefore represented as taller bars.

The results indicate that the binding site consists of a seven base pair core. All of the positions within this core are important to some extent but some mutations do not effect BZLF1 binding. A consensus for BZLF1 binding, derived from the results of the point mutagenesis and by comparison of naturally occuring sites is shown along with the consensus binding sites for fos+jun (AP-1) and C/EBP (figure 3.4).

3.3 C/EBP does not bind the Z2 site in the promoter for the BZLF1 gene

BZLF1 and C/EBP appear to be functionally related to some extent since both proteins can bind some C/EBP sites and it is possible that C/EBP might be capable of binding to the C/EBP-like sites within EBV. To address this question purified C/EBP (gift of S.McKnight) was assayed for its ability to bind an oligonucleotide spanning the Z2 site in a gel retardation assay as well as a canonical C/EBP site and the ds458 oligonucleotide as controls (figure 3.5). Complex formation was in the presence of bovine serum albumin (0.25mg/ml). BZLF1 binding to the C/EBP-like Z2 site (and the adjacent Z1 site) has been shown to be involved in auoregulation of this promoter (Flemington and Speck., 1990c).

In this assay C/EBP is capable of binding with high affinity to a canonical C/EBP site but is unable to bind the Z2 site of EBV. C/EBP

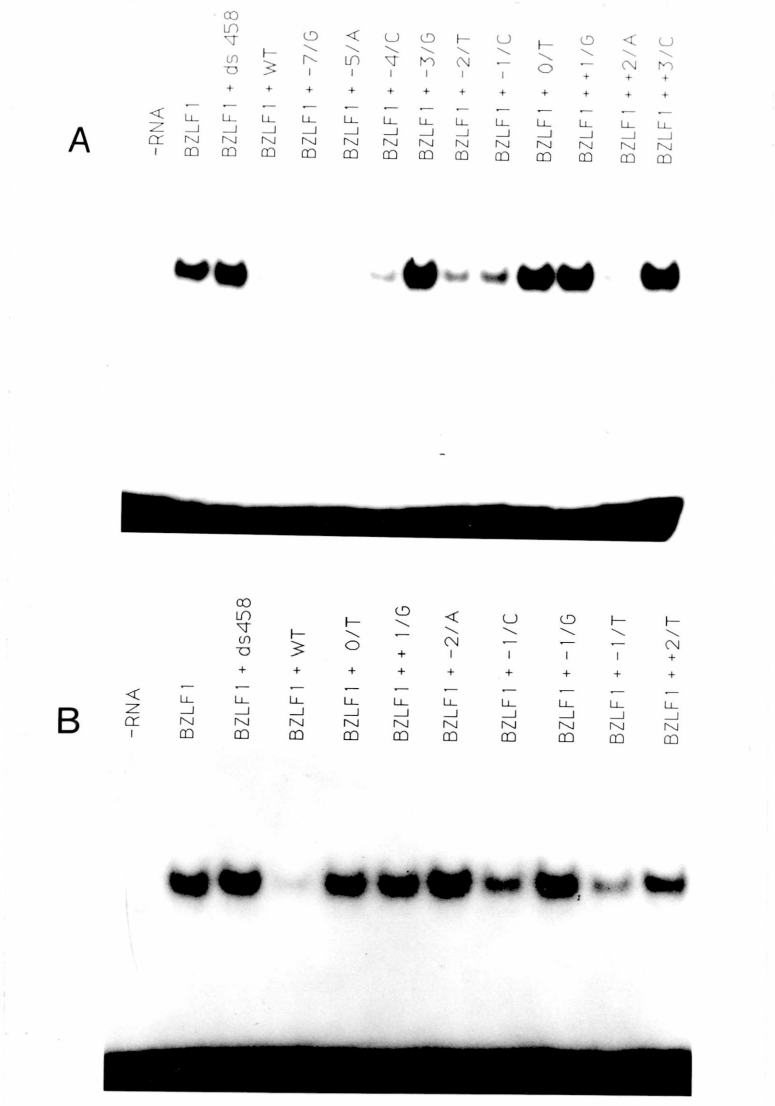


Figure 3.3 Effect of single point mutations on DNA binding by BZLF1

Proteins were made by in vitro translation in the rabbit reticulocyte lysate system. Lysate containing BZLF1 or lysate with no added RNA (3µl each) was complexed with 1.25ng of radiolabelled 292/293 (M site) oligonucleotide. Where indicated BZLF1 binding was competed by addition of a 10 fold molar excess of each of the unradiolabelled oligonucleotides. The competing oligonucleotides were derived by point mutagenesis of the wild type M site sequence; both strands were altered. The position and base change for each mutant is shown above (WT, wild type, see also figure 3.3). The non-specific competitor ds458 was a negative control oligonucleotide. The complexes were resolved on a 4% polyacrylamide gel in 0.5x TBE and detected by autoradiography.

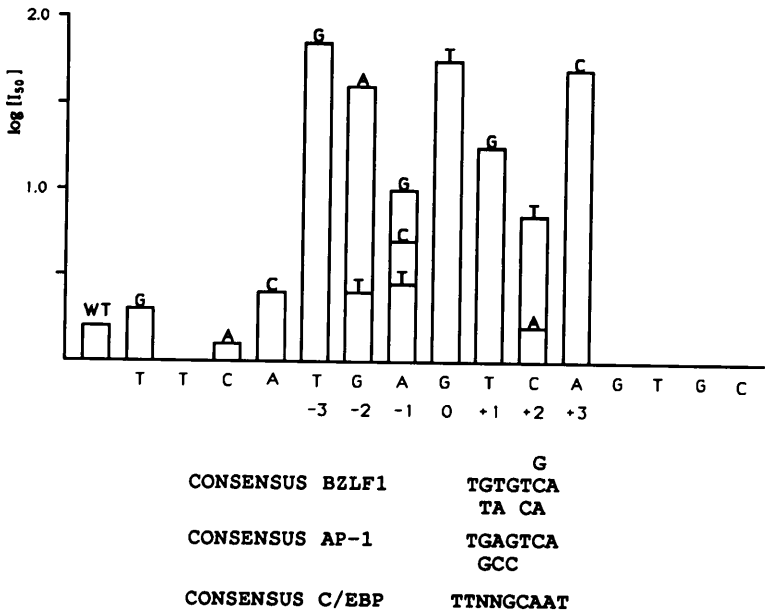


Figure 3.4 Effect of single point mutants on the affinity of DNA binding by BZLF1

BZLF1 was made by in vitro translation in the rabbit reticulocyte lysate system. Lysate containing BZLF1 (3µl) was complexed with 1.25ng of radiolabelled 292/293 (M site) oligonucleotide. Double-stranded oligonucleotides derived by point mutagenesis of the wild type site were tested for their ability to compete binding of BZLF1 to the wild type sequence in a dilution series. The amount of mutant or wild type oligonucleotide required to achieve a 50% inhibition in BZLF1 binding is plotted in the histogram as $\log_{10}$ of the fold excess in concentration over the radiolabelled target. The change made relative to the wild type M sequence is shown above each bar; the wild type (WT) sequence is given below. The consensus binding sites for BZLF1, fos+jun (AP-1 site) and C/EBP are shown (Risse et al., 1989; Ryden and Beemon, 1989).

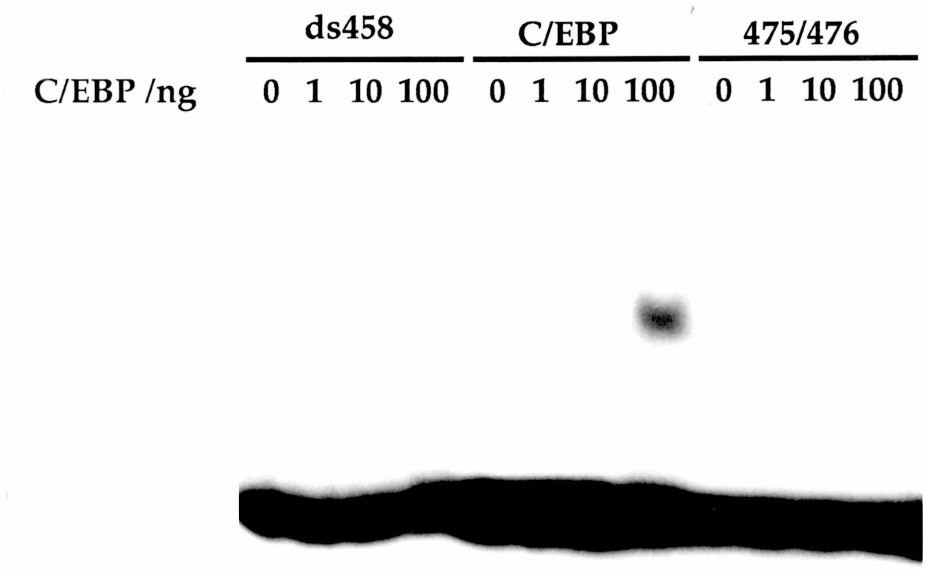


Figure 3.5 C/EBP does not bind the Z2 site in a gel retardation assay.

Purified C/EBP protein (0.1, 1.0 10 and 100ng) was complexed with 5ng of each of the radiolabelled oligonucleotides (a) ds458 (b) a canonical C/EBP binding site and (c) 475/476, Z2 site where indicated. Complex formation was in the presence of bovine serum albumin, fraction V (0.25mg/ml). The complexes were resolved on a 4% polyacrylamide gel in 0.5x TBE and detected by autoradiography.

binding is observed when 100ng of C/EBP is added to the DNA; it remains unclear why complex formation is not observed with 10ng of protein.

3.4 BZLF1 does not bind the octanucleotide AP-1 site in the promoter for the BZLF1 gene

The promoter for BZLF1 has been analysed by both deletion and site-directed mutagenesis and sequences required for TPA activation of the promoter have been identified (Urier et al., 1989; Flemington and Speck, 1990b). One site demonstrated to be important in TPA activation has the sequence CATGACATCACA (see figure 3.1) which is identical to a TPA responsive, high-affinity AP-1 binding site in the promoter for c-jun (TGACATCA; Angel et al., 1988). The site in the BZLF1 promoter has also been shown to bind jun+fos (Flemington and Speck, 1990b). Since BZLF1 is structurally and functionally related to c-fos, the protein was assayed for its ability to bind the octanucleotide TPA responsive element.

BZLF1 was made by in vitro translation in the rabbit reticulocyte lysate system and assayed for its ability to bind oligonucleotide 427/428 which spans the octanucleotide AP-1 binding site in the BZLF1 promoter in a gel retardation assay (figure 3.6). BZLF1 was also assayed for binding to the M site (oligonucleotide 292/293) as a positive control. BZLF1 does not bind the octanucleotide TPA response element from the BZLF1 promoter although as expected BZLF1 can bind the M site.

In addition, this site (TGACATCA) shows homology to a consensus CREB (cAMP response element binding protein) binding site, TGACGTCA (Roesler et al., 1988). CREB-A and CREB-B proteins (Berkowitz and Gilman, 1990) were made by in vitro translation and tested for binding to this site (the plasmids used to generate CREB proteins were a gift of L.Berkowitz), however, no DNA binding was observed (data not shown).

3.5 Discussion

The BZLF1 protein has been demonstrated to bind several sites within the EBV genome (Table 3.1). The identified sites appear to be quite diverse and include both AP-1-like and C/EBP-like binding sites. These experiments have investigated the sequence specific DNA binding activity of BZLF1.

BZLF1 is capable of binding with high affinity to not only an AP-1 site but also to a canonical C/EBP site. Unlike BZLF1, other proteins which bind an AP-1 site with high affinity, for example, jun+fos, cannot bind to

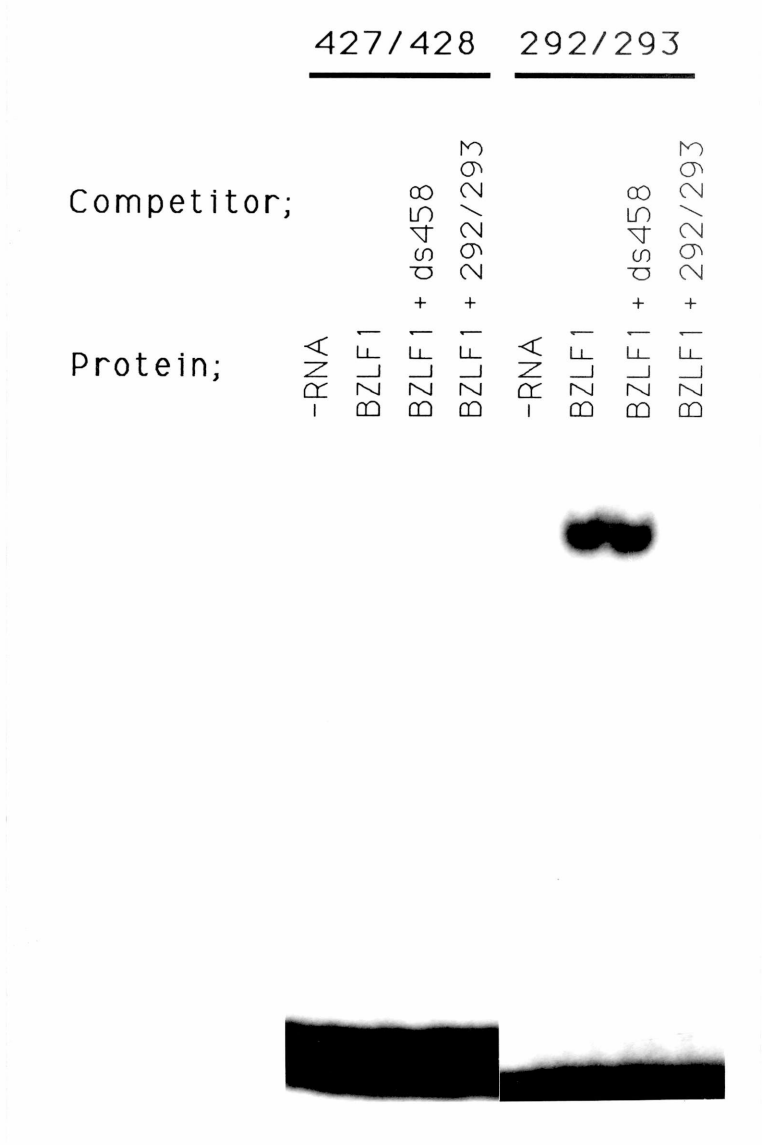


Figure 3.6 BZLF1 does not bind the octanucleotide AP-1 site within the promoter for BZLF1

BZLF1 was made by in vitro translation in the rabbit reticulocyte lysate system. Lysate containing BZLF1 or lysate with no added RNA (3µl each) was complexed with 2ng of the radiolabelled oligonucleotides 427/428 (site) and 292/293 (M site) as indicated. Binding was competed with a 20 fold molar excess of each of the competing oligonucleotides. The complexes were resolved on a 4% polyacrylamide gel in 0.5x TBE and detected by autoradiography.

a C/EBP site. In addition C/EBP cannot bind to an AP-1 site. BZLF1 therefore seems to be unusual in that it can bind to sequences which are normally bound by members of these two different families of bZip DNA binding proteins. In addition, BZLF1 does not bind the octanucleotide AP-1 binding site, TGACATCA in the promoter for BZLF1 indicating that the DNA binding specificity of BZLF1 overlaps, but does not include all jun+fos binding sites.

Comparison with the published consensus for jun+fos binding (Risse et al., 1989) suggests that only the M site will bind AP-1, since changes in the natural sites no longer conform to the consensus sequence. It has been confirmed that jun+fos is able to bind the M site, but not the R1, R2, Z1, Z2 sites and the sites in the DS_L promoter by gel retardation assays (Kouzarides et al., 1991; Chang et al., 1990; Sinclair et al., 1991). The ability of jun+fos to bind at the M site may have significance for the regulation of this promoter. Mutation of the AP-1 site increases the basal activity of the promoter; it is possible that AP-1 (or a related factor) acts as a repressor at this site which is relieved by BZLF1 (Packham et al., 1990).

The AP-1 site used in these experiments is derived from the human fat specific element (Distel et al., 1987). The results imply that BZLF1 may bind at a number of AP-1 and C/EBP sites throughout the human genome and may be able to influence cell gene expression. Recently BZLF1 has been shown to bind to AP-1-like sites in the promoter for the c-fos gene and to upregulate expression from this promoter (Flemington and Speck, 1990d; Sinclair et al., 1992). Although it is not clear that this upregulation is important in the control of expression of EBV productive cycle genes it demonstrates that BZLF1 can potentially effect cell gene expression.

The point mutagenesis of the BZLF1 binding site gives a quantitative value to the effects of individual base changes on BZLF1 binding. The results indicate that the binding site consists of a central seven base pair core and that all positions within this core are important to some extent. Mutations outside of this core have little effect on BZLF1 binding. This has been confirmed by Chang et al., 1990.

An alternative method for analysing the DNA binding specificity of a protein has been described (Pollock and Treisman, 1990). The method involves the generation of a set of oligonucleotides consisting of conserved flanking sequences with a random sequence core. The subset of oligonucleotides that bind the protein are selected by repeated binding of the protein, immunoprecipitation and PCR amplification of the

immunoprecipitated oligonucleotides. In this way a population of oligonucleotides is selected on their basis of their ability to bind the protein and can be analysed by sequencing. This technique has the advantage that many sites can be rapidly identified and that the effects of multiple substitutions can be analysed. This method could be applied to further analyse the DNA binding specificity of BZLF1.

Although the BZLF1 binding site sequence can be symmetrical the mutagenesis suggests that the two half sites are functionally asymmetric; equivalent changes in different half sites have a different effect on BZLF1 binding. For example, if the two half sites were symmetrical then the mutations -1/C and +1/G would be expected to have similar effects on the affinity of BZLF1 for the site. The results show that +1/G has a greater effect on BZLF1 binding than does -1/C. Likewise the mutation -2/A has a greater effect than the equivalent mutation +2/T. This property has also been recognised in jun+fos binding to the AP-1 site and in the GCN4 protein (Risse et al., 1989; Hill et al., 1986).

A consensus for jun+fos binding has also been reported obtained by point mutagenesis of an identical sequence (Risse et al., 1989). Comparison of the consensus sequences indicates that BZLF1 and jun+fos have slightly different specificities, especially within the 3' half site. At position +2 jun+fos has an absolute requirement for C; the substitution at this position of any other residue completely abolishes DNA binding. In contrast BZLF1 can tolerate an A at position +2 with no loss in affinity. At position +1 BZLF1 can tolerate a C in place of the T residue whereas a C at the +1 position of the jun+fos site completely abolishes binding. These differences in specificity at positions +1 and +2 make the consensus BZLF1 binding site compatible with the consensus AP-1 binding site and C/EBP which is consistent with the DNA binding data presented here.

A computer search of the EBV genome for sequences which match the derived consensus for BZLF1 binding identifies over 200 exact matches. Obviously the degeneracy in the consensus sequence limits its use in identifying meaningful new BZLF1 binding sites.

C/EBP was shown to be unable to bind the C/EBP-like Z2 site in the promoter for the BZLF1 gene in a gel retardation assay. It has been reported that C/EBP binds this site in a footprint assay, though the affinity of this interaction may be low (Kouzarides et al., 1991). Since it is difficult to estimate the effective concentration of C/EBP within the cell it is hard to assess the significance of this interaction.

Therefore, the DNA binding specificity of BZLF1 includes a canonical C/EBP site and some but not all jun+fos binding sites. Mutational analysis of a BZLF1 binding site and comparison of naturally occuring binding sites has been used to identify a consensus sequence for BZLF1 binding to DNA. This consensus sequence is compatible with both jun+fos and C/EBP binding sites which is consistent with the previous observations. BZLF1 was originally shown to bind a consensus AP-1 site with high affinity (Farrell et al., 1989); the demonstration that the protein can also bind a canonical C/EBP binding site indicates that BZLF1 is functionally related to two different families of bZip proteins, that of c-fos and C/EBP.

Chapter 4
Structural Analysis of BZLF1

Preliminary deletion analysis of BZLF1 has shown that approximately the C-terminal half of the protein is sufficient for sequence specific DNA binding but will not activate transcription (Farrell et al., 1989; Rooney et al., 1989). N-terminal sequences are therefore required for full activity of the protein. To define more accurately structural elements within the protein a more extensive deletion analysis was performed.

4.1 Generation of BZLF1 deletion mutants

The BZLF1 deletion mutants were derived from the plasmid SP64-BZLF1 (gift of P.J.Farrell) which is an 870 bp BamHI-EcoRI cDNA encoding the whole of the BZLF1 coding sequence cloned between the BamHI and EcoRI sites of SP64 (Rooney et al., 1989). Various deletions were prepared by restriction digestion of the parent plasmid and ligation of synthetic oligonucleotides where required to repair resected ends. In this way an initiating methionine was added to the N-terminal deletions and a translation terminator was added to the C-terminal deletions. The construction of the BZLF1 deletion mutants is described fully in section 2.5 and a schematic representation of the mutants is shown in figure 4.1.

All of the deletions were precise truncations of the BZLF1 sequence and no extra amino-acids were added. The nomenclature for the mutants refers to the restriction site(s) used in the cloning and to whether the proteins have an intact N- or C-terminus. Internal deletions are indicated by the word 'delta' following the site(s) used to generate the deletion. The mutants cannot be named according to amino-acid residue numbers since there are potentially two initiating methionines for BZLF1.

The resulting deletions of the BZLF1 cDNA could be transcribed and translated in vitro to generate truncated proteins. The plasmids were linearised with EcoRI and the BZLF1 deletions transcribed in vitro using SP6 RNA polymerase. To ensure transcripts were of the expected size, a small portion of the transcription reaction was radiolabelled and analysed by gel electrophoresis (figure 4.2). The RNA was translated in vitro in the presence of [^{35}S]cysteine or [^{35}S]methionine and the products analysed by SDS-PAGE (figures 4.3, 4.5 and 4.7) using the rabbit reticulocyte lysate or wheat germ extract systems. It was necessary to use the wheat germ extract system to demonstrate translation of the smaller proteins because

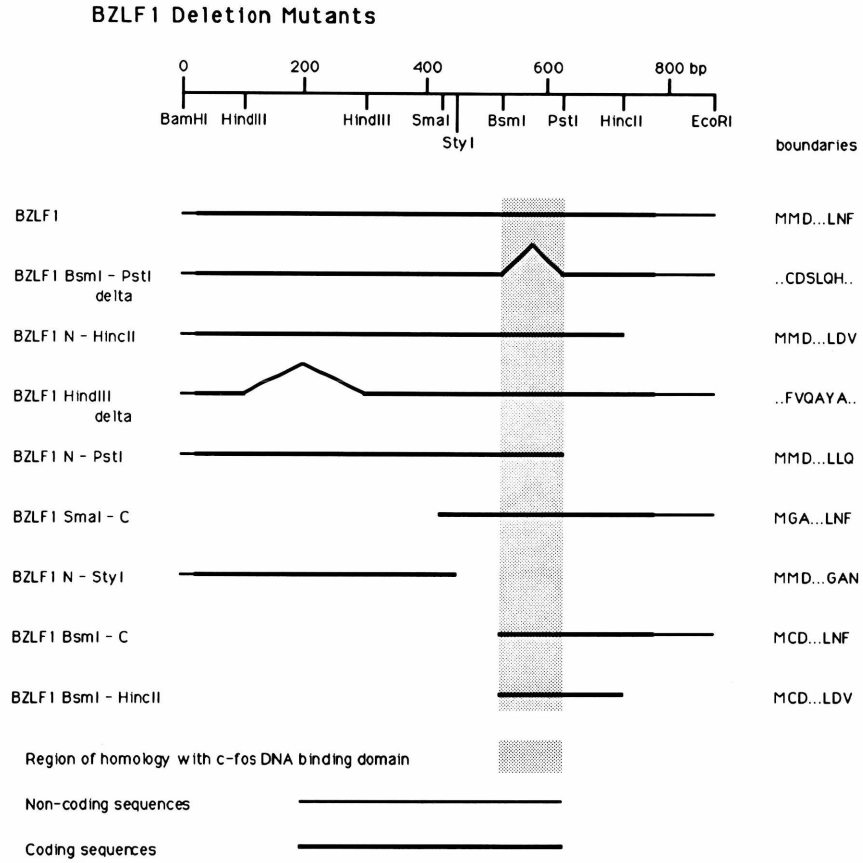


Figure 4.1 Structures of the BZLF1 deletion mutants

The structures of the BZLF1 deletion mutants are shown beneath a restriction map of the EBV sequences in the BZLF1 cDNA (SP64-BZLF1). The amino-acids at the boundaries of the truncations and deletions are shown using the single letter code. The sequences homologous to the DNA binding domain of c-fos are indicated.

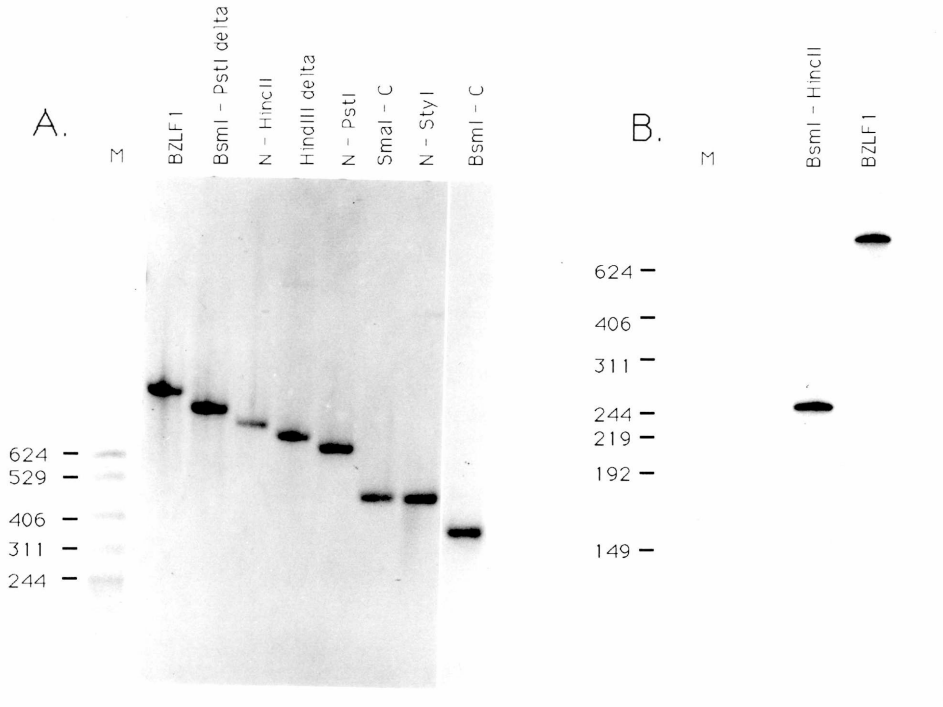


Figure 4.2 RNA made by in vitro transcription

The plasmid SP64-BZLF1 and the deletion mutants derived from it were linearised with EcoRI and in vitro transcribed using SP6 RNA polymerase. A small portion of each transcription reaction contained [α^{32}P]UTP to radiolabel the RNA obtained.

Panel A; Radiolabelled RNA made from the plasmids indicated was reacted with glyoxal and analysed on an 1.4% agarose gel.

Panel B; Radiolabelled RNA made from SP64-BZLF1 and SP64-BsmI-HincII was analysed on an 8% denaturing polyacrylamide gel. Size markers (M) are a MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in base pairs are indicated.

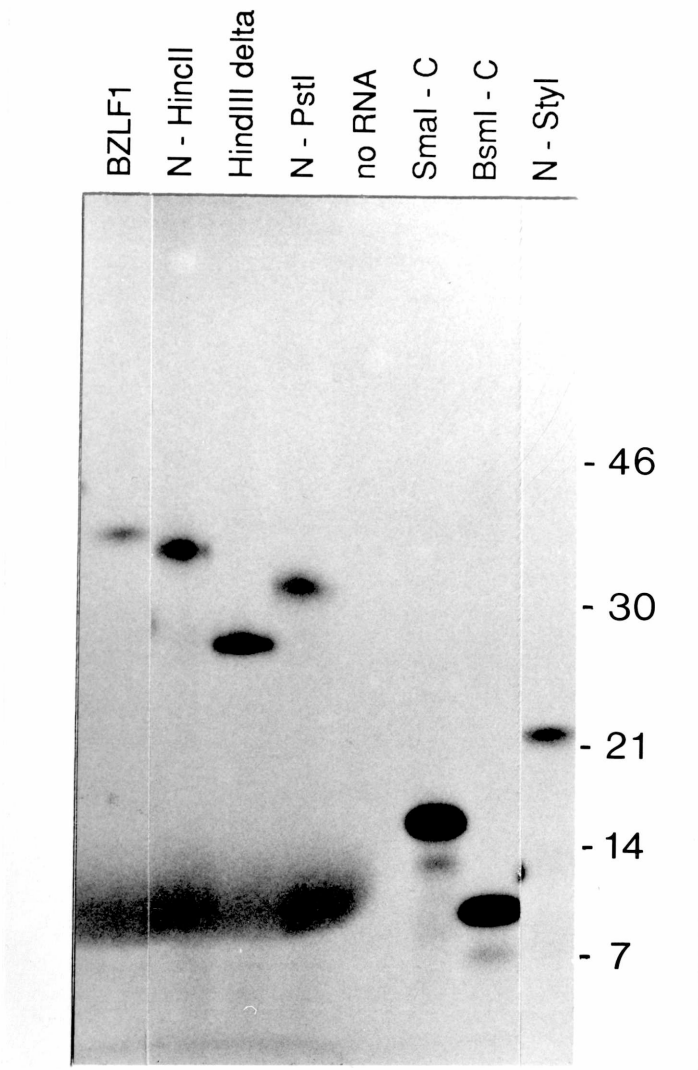


Figure 4.3 BZLF1 proteins made by in vitro translation

Radiolabelled proteins were made by in vitro translation with [^{35}S]cysteine. The proteins BZLF1, N-HincII, HindIII delta and N-PstI were made in rabbit reticulocyte lysates. The proteins SmaI-C, BsmI-C and N-StyI were made in wheat germ extracts. Translation products were resolved on a 17.5% SDS polyacrylamide gel and detected by fluorography. The sizes of the molecular weight markers are indicated in kD.

the gel is distorted in this size range by the high concentration of globin in the reticulocyte lysates. The full length BZLF1 protein has an apparent molecular weight of 38kD, in close agreement with previous reports (Rooney et al., 1989).

4.2 Identification of the part of BZLF1 required for DNA binding

The part of BZLF1 required for sequence specific DNA binding was identified using the deletion mutants. Wild type BZLF1 and the various deletion mutants were made by in vitro translation as described above but in the absence of a radiochemical. The proteins were then assayed for their ability to bind a fragment of EBV DNA in a gel retardation assay (figure 4.4). The target DNA was a 261bp fragment (EBV genome coordinates 84234 to 84487) derived from an M13 clone 374.RIF from the EBV sequencing project which spans the promoter for the BMLF1+BSLF2 gene and includes the previously identified BZLF1 binding site, (M site, see figure 3.1). In each case the binding was competed separately with an oligonucleotide spanning the binding site and an unrelated oligonucleotide to demonstrate specificity of binding.

Full length BZLF1 bound to the target DNA, as did the mutants HindIII delta, N-HincII, SmaI-C and BsmI-C. The mutant N-PstI failed to bind. Therefore deleting to the HincII site from the C-terminus of BZLF1 does not effect the DNA binding function under these conditions whereas further deletion to the PstI site abolishes this activity. The protein could be deleted from the N-terminus to the BsmI site without affecting DNA binding. These results show that sequence specific DNA binding requires amino-acids between the BsmI and HincII sites. This region of 58 amino-acids spans the region of homology with the basic DNA binding domain of c-fos, and additional sequences which lie C-terminal of this structure (see figures 1.3 A and 6.1). It was noted that several of the mutants, e.g. HindIII delta gave multiple shifted bands. The significance of this observation is unclear since it is difficult to predict the behaviour of complexes in this gel system. The presence of multiple complexes was not consistent; compare the complexes obtained with BZLF1 in figure 4.4 and figure 3.2. This may be the result of subtle differences in the binding temperature used or the concentration of protein in the lysates. Alternatively it may represent complexing with additional factors in the lysates.

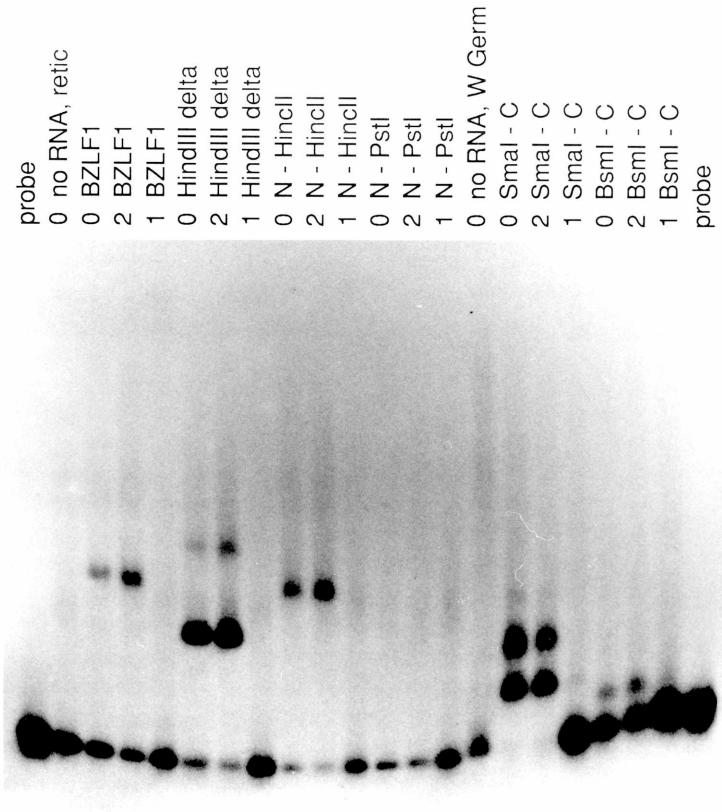


Figure 4.4 Gel retardation assay for BZLF1 deletion mutants binding to DNA

The BZLF1 deletion mutants were made by in vitro translation in the rabbit reticulocyte lysate or wheat germ extract systems in the absence of a radiochemical. Lysates containing the proteins and lysates with no added RNA (3µl each) were complexed with approximately 1ng of a 261bp radiolabelled probe derived from the promoter for the BSLF2+BMLF1 gene which spans a BZLF1 binding site. The binding was competed where indicated with (0) no additions, (2) an unrelated double-stranded oligonucleotide (sequence GGGTACCC) or (1) the oligonucleotide 292/293 which spans the binding site. Complexes were resolved on a 1% agarose gel in 0.5x TBE and detected by autoradiography.

Since the deletion analysis showed that the region of BZLF1 required for DNA binding is comparatively small it might be possible to synthesise this part of the protein in vitro using a peptide synthesiser and analyse its structure and interactions with DNA using X-ray crystallography. For such experiments it would therefore be important first to demonstrate that this region exhibits the same DNA binding properties in vitro as the wild type protein. To address this question the mutant BZLF1 BsmI-HincII was constructed which contains only the sequences shown to be necessary for DNA binding and could be made by in vitro transcription and translation as before. A portion of the translation reaction included [^{35}S]methionine to demonstrate translation of the protein (figure 4.5 A).

The protein generated was tested for its ability to bind oligonucleotide 292/293 (M site) in a gel retardation assay (figure 4.5 B). The BsmI-HincII protein did not bind to DNA with high affinity (upper panel, lane 11; a very weak signal could be seen on the original autoradiograph) although BZLF1 could bind efficiently in the same experiment (upper panel, lane 12). Therefore the mutant protein would not be a satisfactory structure to analyse by X-ray crystallography.

4.3 Salt sensitivity of DNA binding by BZLF1 deletion mutants

The salt sensitivity of the DNA binding of mutants which approach the boundaries of the DNA binding domain i.e. BsmI-C and N-HincII was investigated using gel retardation assays. The proteins and DNA targets were complexed as before and then analysed by electrophoresis in two different buffers. The electrophoresis buffers were either 0.5x TBE (low salt) or buffer G (higher salt). The complexes form as before but their stabilities are different during electrophoresis in the two buffer systems.

The SmaI-C and the BsmI-C mutants were complexed with radiolabelled oligonucleotide 292/293 and the complexes were analysed by gel electrophoresis in the two buffer systems (figure 4.5 B). Although the SmaI-C protein binds well to DNA under both salt conditions (lane 9, upper and lower panels), the BsmI-C protein does not bind DNA in the higher salt buffer (compare lane 10, upper and lower panels).

The binding activity of the N-HincII mutant was analysed in an analogous manner though using a slightly different DNA binding assay. Full length BZLF1, and the N-HincII, HindIII delta and N-StyI mutants were made by in vitro translation in the presence of [^{35}S]cysteine to radiolabel the products. The proteins were diluted into the same binding

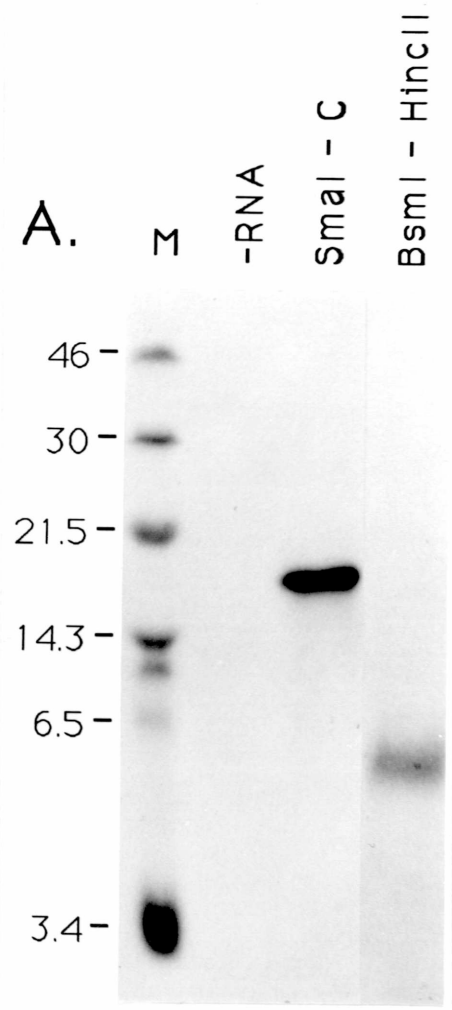


Figure 4.5 Gel retardation assay showing that deletion mutant BsmI-HincII does not bind DNA and that DNA binding by BsmI-C is sensitive to salt concentration

The deletion mutants SmaI-C, BsmI-C and BsmI-HincII were made by in vitro translation in the wheat germ extract system with or without [^{35}S]cysteine.

Panel A; The radiolabelled translation products (SmaI-C and BsmI-HincII) were resolved on a 20% SDS polyacrylamide gel and detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD.

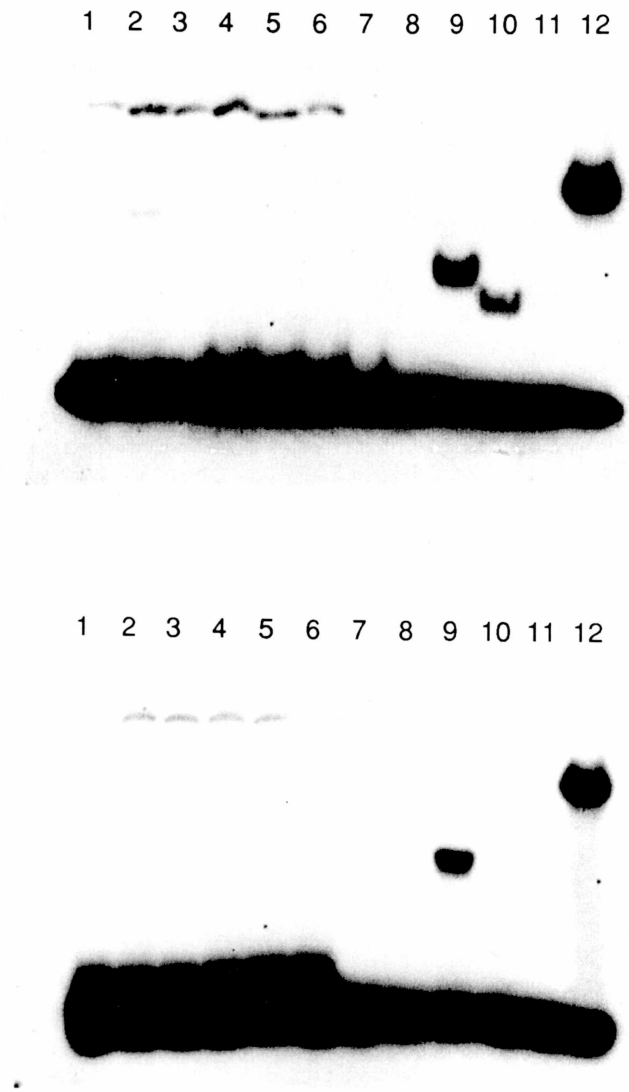


Figure 4.5 Panel B; Lysates containing the BZLF1 deletion mutants or lysate with no added RNA (3µl each) were complexed with 10ng of radiolabelled oligonucleotide 292/293 (lanes 7-12) or oligonucleotide ds458 as a control (lanes 1-6). Lanes 1 and 7, no added protein; lanes 2 and 8, lysate with no added RNA; lanes 3 and 9, lysate containing SmaI-C; lanes 4 and 10, lysate containing BsmI-C; lanes 5 and 11, lysate containing BsmI-HincII and lanes 6 and 12, pREX-SmaΔ fusion protein (a bacterially expressed BZLF1 SmaI-C fusion protein; as a positive control). Complexes were resolved on polyacrylamide gels in 0.5x TBE (upper panel) and buffer G (lower panel) and detected by autoradiography.

buffer as used in previous gel retardation assays (i.e. BC100 with 120μg/μl poly(dIC)) and various unlabelled oligonucleotides were added. After incubation for 25 min at room temperature the protein complexes were resolved on a polyacrylamide gel in the higher salt buffer G (figure 4.6). In this assay BZLF1 does not appear on the gel (it presumably binds weakly to high molecular weight poly(dIC) forming a complex which is too large to enter the gel). Addition of an unrelated oligonucleotide which will not bind BZLF1 has no effect. When an oligonucleotide spanning a BZLF1 binding site is added it presumably binds BZLF1 with a higher affinity than poly(dIC) and forms a smaller complex which can then be resolved. The protein N-StyI cannot bind DNA and therefore enters the gel regardless of the addition of competing oligonucleotides. In this way it can be seen that the mutant HindIII delta retains its ability to bind DNA while the mutant N-HincII no longer binds DNA under these conditions.

Together these results show that the DNA binding activity of the BsmI-C and N-HincII proteins is sensitive to salt concentration.

4.4 Deletion of the basic motif abrogates DNA binding

The sequences shown to be sufficient for DNA binding include the region of homology with the DNA binding structure of c-fos as well as additional sequences which lie C-terminal of this motif. By comparison with other members of the c-fos family it is likely that the basic motif of BZLF1 is responsible for interacting with DNA directly (Agre et al., 1989). To investigate this the BZLF1 mutant BsmI-PstI delta, which lacks almost exactly the basic motif, was generated. This protein could be made by in vitro transcription and translation as before. A portion of the translation reaction contained [^{35}S]methionine to demonstrate translation of the protein (figure 4.7 A). The protein generated was assayed for its ability to bind oligonucleotide 292/293 (M binding site) in a gel retardation assay (figure 4.7 B). This mutant is unable to bind DNA. Although this does not prove that protein sequences between the BsmI and PstI sites interact with DNA directly, it is consistent with this model.

4.5 Identification of BZLF1 dimerisation domain

The DNA binding structure of BZLF1 apparently consists of a basic motif which contacts DNA directly as well as additional sequences which lie C-terminal of this structure. In c-fos these sequences form the leucine

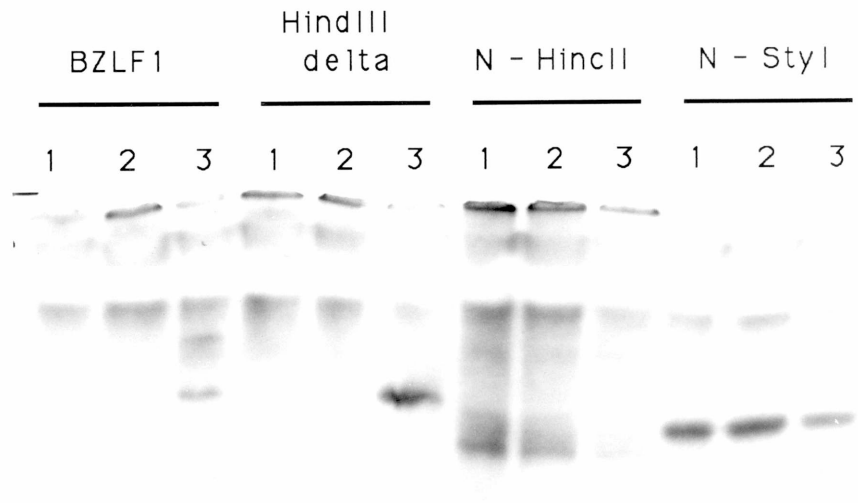


Figure 4.6 DNA binding by deletion mutant N-HincII is sensitive to salt concentration

BZLF1 and the deletion mutants HindIII delta, N-HincII and N-StyI were made by in vitro translation in the rabbit reticulocyte lysate system with [^{35}S]cysteine. Lysates containing the radiolabelled proteins (3µl) were complexed in BC100 with 120µg/µl poly (dIC) with (1) no additions, (2) an unrelated double-stranded oligonucleotide (sequence GGGTACCC) or (3) the oligonucleotide 292/293 which spans the M BZLF1 binding site. Complexes were resolved on a 4% polyacrylamide gel in buffer G (electrophoresis was towards the anode) and detected by fluorography.

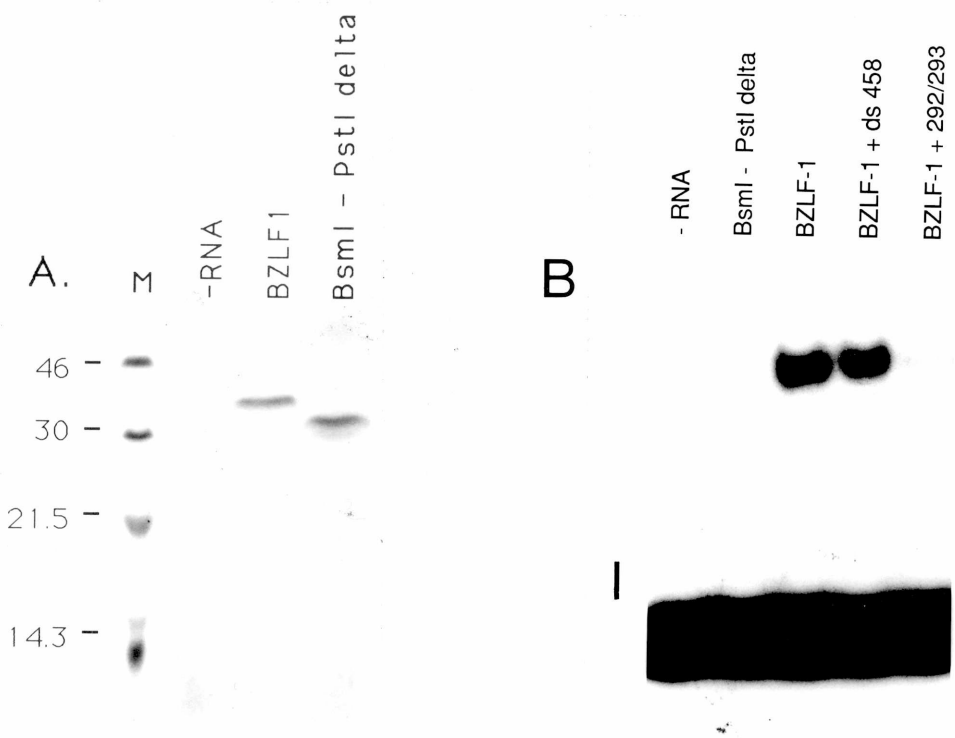


Figure 4.7 Deletion mutant BsmI-PstI delta does not bind DNA

BZLF1 and the deletion mutant BsmI-PstI delta were made by in vitro translation in the rabbit reticulocyte lysate system with or without [^{35}S]methionine.

Panel A; The radiolabelled translation products were resolved on a 12.5% SDS polyacrylamide gel and detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD.

Panel B; Lysates containing BZLF1, BsmI-PstI delta and lysate with no added RNA (2µl each) were complexed with 1.25ng radiolabelled 292/293 (M site) oligonucleotide. Binding was competed with a 10 fold molar excess of the oligonucleotides as indicated. Complexes were resolved on a 4% polyacrylamide gel in 0.5x TBE and detected by autoradiography.

zipper which functions as a dimerisation domain. BZLF1 was assayed for the ability to form dimers using an indirect immunoprecipitation assay.

This assay uses the rabbit serum, anti-TrpE-BZLF1 (gift of G. Miller) raised against the bacterially expressed BZLF1 fusion protein TrpE-BZLF1 which essentially contains protein sequences derived from the first N-terminal part of BZLF1 and is unable to imunoprecipitate the BsmI-C mutant (see section 5.3) which includes the putative dimerisation domain. The anti-serum will recognise and immunoprecitate the full length BZLF1 protein and mutants which include the N-terminal epitopes, e.g. BsmI-PstI delta, N-HincII and N-StyI. When the full length protein and the BsmI-C proteins are co-translated and then immunoprecipitated with the anti-serum the shorter protein is indirectly immunoprecipitated through its interaction with full length BZLF1 (figure 4.8). This demonstrates that BZLF1 is capable of forming homodimers in solution, i.e. when not bound to DNA.

This assay can be extended to define the part of BZLF1 required for dimerisation by using deletion mutants which lie entirely within the C-terminal part of the protein. The BsmI-C protein is indirectly immunoprecipitated with BsmI-PstI delta whereas deletions which remove C-terminal parts of the protein are no longer active in this assay (figure 4.8). This assay demonstrates that the sequences which lie C-terminal of the basic motif and which are required for DNA binding, direct dimerisation of BZLF1. It also shows that the basic motif itself plays no role in dimerisation.

Although the indirect immunoprecipitation assay clearly demonstrates that BZLF1 dimerises, the actual interaction appears to be quite weak. One possible explanation for this observation might be that additional interactions within other parts of the protein, for example, in the N-terminal part of BZLF1, stabilise dimer formation. Since the assay uses BsmI-C which has the N-terminal part deleted, this would result in relatively unstable dimer formation. A similar indirect immunoprecipitation assay was used, though employing a different anti-serum and a different deletion mutant to address this question. This assay uses the rabbit serum anti-pREX-SmaΔ (raised by D.T.Rowe against the bacterially expressed BZLF1 fusion protein pREX-SmaΔ) which recognises epitopes in the C-terminal part of the protein (see section 5.3). This antibody can immunoprecipitate full length BZLF1 but cannot immunoprecipitate the N-StyI mutant since this protein lacks the epitopes

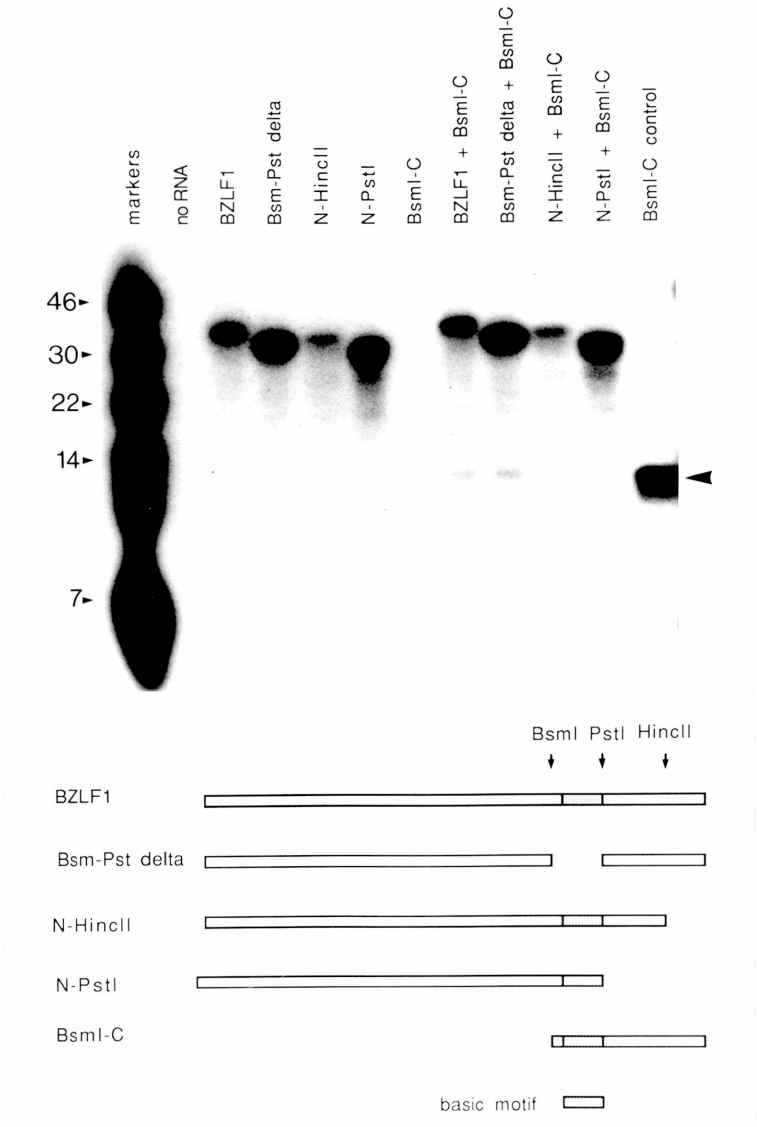


Figure 4.8 Indirect immunoprecipitation assay reveals dimerisation domain of BZLF1

BZLF1 and the deletion mutants BsmI-PstI delta, N-HincII, N-PstI and BsmI-C were made by in vitro translation in the rabbit reticulocyte lysate system with [^{35}S]cysteine. The proteins were translated alone or cotranslated with BsmI-C as indicated. The proteins (10µl) were immunoprecipitated with the anti-TrpE-BZLF1 antibody (2µl) except the BsmI-C control which was immunoprecipitated with anti-pREX-SmaΔ antibody (2µl). The proteins were resolved on a 17.5% SDS polyacrylamide gel and detected by fluorography. The sizes of the molecular weight markers are indicated in kD. The structures of the BZLF1 deletion mutants are shown below.

recognised by the antibody. The antibody cannot indirectly immunoprecipitate N-StyI with BZLF1 so there is no evidence for an interaction between these parts of BZLF1 (data not shown).

4.6 Transactivation of the promoters for the BMLF1+BSLF2 and the BZLF1 genes by BZLF1 deletion mutants

BZLF1 is capable of transactivating a number of viral promoters and this activity is thought to be central to the role of BZLF1 in the switch from viral latency to productive infection. The BZLF1 deletion mutants were tested for their ability to transactivate two BZLF1 responsive promoters in transient transfection assays.

In this assay cells were transfected with two plasmids. One plasmid enabled expression of the BZLF1 deletion mutants; the other consisted of a responsive promoter fused to a reporter gene. The increase in the amount of reporter gene product made relative to background levels was related to the ability of the mutant proteins to transactivate the promoter.

The mutants were assayed in the cell line Ramos which was derived from an EBV-negative Burkitt's lymphoma (Klein et al., 1975). This cell line was selected because it contains no other EBV genes, it approximates to a natural cell target for EBV infection in vivo and it could be transfected relatively easily and efficiently by electroporation.

The reporter constructs used were St.CAT (Rooney et al., 1989) and p48Z.CAT (Sinclair et al., 1991) which contain the promoters for the BMLF1+BSLF2 and the BZLF1 respectively; both promoters have previously been shown to be transactivated by BZLF1. The plasmids were constructed such that the promoters drive expression of the CAT gene (see section 2.5).

To express the BZLF1 deletion mutants in this cell line it was necessary to place them under the control of a eukaryotic promoter. The EBV latent promoter BWR1 was chosen since this is constitutively active in these cells and the BZLF1 deletion mutants were subcloned in to the eukaryotic expression vector, p3.1W (see section 2.5).

The plasmids were transfected into Ramos cells and 24 hours post-transfection cell extracts were prepared and analysed by Western immunoblotting (figure 4.9 panel A). The plasmid p3.1W was used as a control since that cannot direct expression of any protein. The immunoblot was probed with a human serum, EE which has high titres of antibodies specific for BZLF1 (gift of A.B.Rickinson). This demonstrates

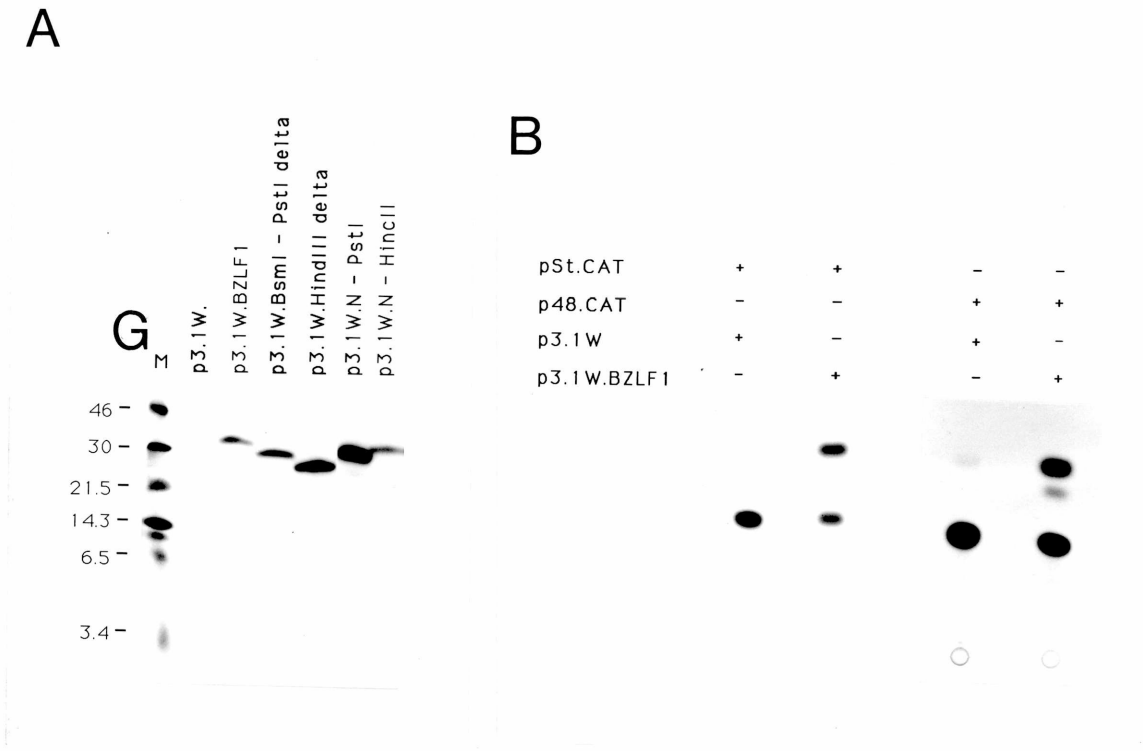


Figure 4.9 Transactivation of St.CAT and p48Z.CAT by BZLF1

Panel A; Ramos cells were electroporated with 10μg of each of the expression plasmids as indicated. The cells were harvested 24 hours post-transfection and the cell extracts resolved on a 17.5% SDS polyacrylamide gel. The proteins were transferred to nitrocellulose and the filter was probed with the human serum, EE. The sizes of the molecular weight markers (M) are indicated in kD.

Panel B; Ramos cells were electroporated with St.CAT (7.5μg), p48Z.CAT (10μg), p3.1W (10μg) and p3.1W.BZLF1 (10μg) where indicated. Cells were harvested 24 hours post-transfection and the CAT activity in the cell extracts was determined.

that each of the proteins are stable in Ramos cells and that the proteins are of the expected size. The immunoblot also gives an indication of the amount of each protein in the cells. However, the polyclonal serum used might be expected to demonstrate different titres against different regions of BZLF1 which would effect the detection of individual mutants.

The plasmids were then cotransfected into Ramos cells separately with either of the reporter constructs. The cells were harvested 24 hours post-transfection and the CAT activity in the cell extracts was determined.

The cotransfection experiments demonstrate that wild type BZLF1 can activate both reporter constructs (figure 4.9 panel B). This effect is due to expression of BZLF1 and not artefactual promoter competition effects since control transfections (i.e. p3.1W) contain the same quantity of promoter sequences.

The CAT activity for each transfection (p3.1W, p3.1W.BZLF1 and the deletion mutants) was quantitated by removing the regions of the TLC plate containing the chloramphenicol and its acetylated derivatives and counting the amount of radioactivity in each region. The fold transactivation of the reporter constructs for each protein was calculated and is presented as a histogram (figure 4.10).

The activation of the St.CAT and p48Z.CAT constructs by BZLF1 results in a 13 and 21 fold increase in CAT activity respectively. BZLF1 and the mutant N-HincII transactivate while the mutants BsmI-PstI delta, HindIII delta and N-PstI have a very low activity in this assay. The mutant N-HincII transactivates approximately half as well as wild type BZLF1. The ability of individual mutants to transactivate is not influenced by the target promoter used.

4.7 Discussion

Sequences in the C-terminal half of BZLF1 have previously been shown to be sufficient for sequence specific DNA binding but not for full transcriptional activity of the protein. Experiments presented constitute a more extensive deletion analysis and have defined sequences required for DNA binding, dimerisation and for the transcriptional activity of BZLF1. Sequences influencing the stability of various functions have also been identified.

The mutations were assayed for their ability to bind DNA in a gel retardation assay. This analysis demonstrated that sequence specific DNA binding requires protein sequences between the BsmI-C and HincII

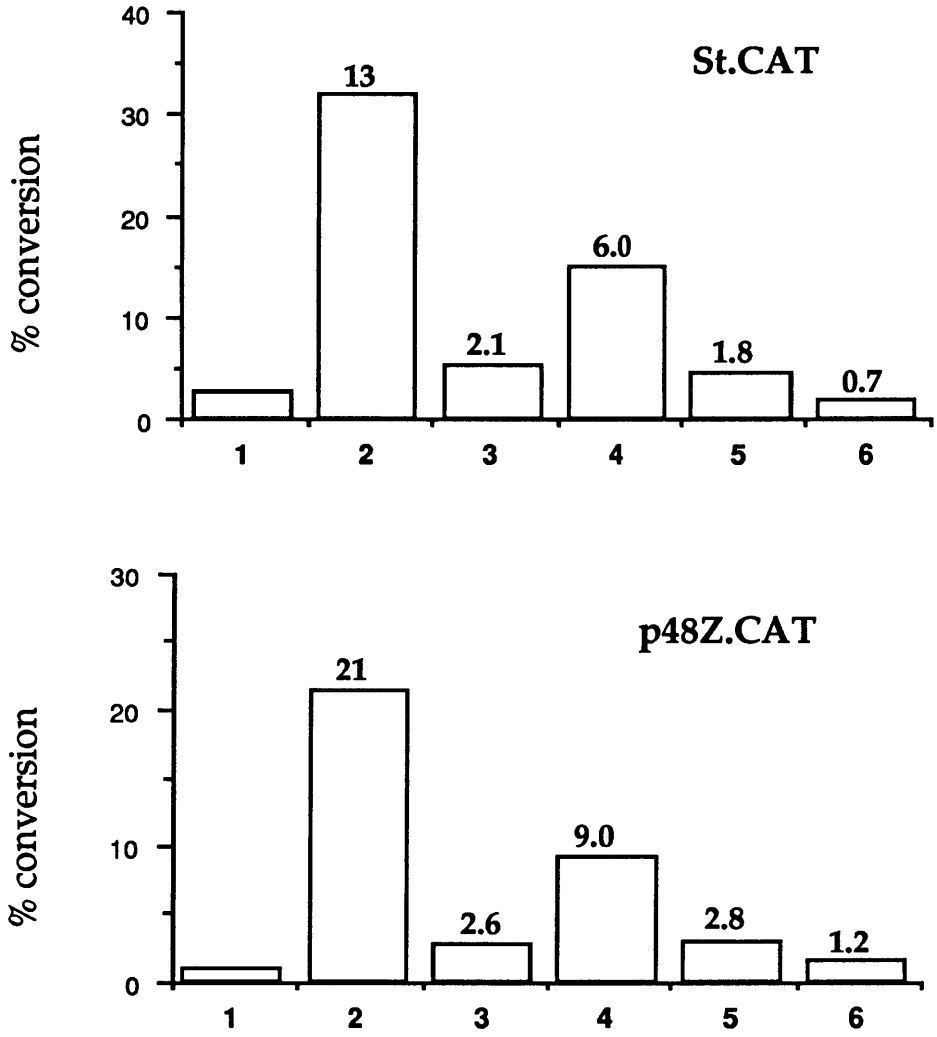


Figure 4.10 Transactivation of St.CAT and p48Z.CAT by BZLF1 deletion mutants

Ramos cells were electroporated with 10μg of each of the expression plasmids (1, p3.1W; 2, p3.1W.BZLF1; 3, p3.1W.BsmI-PstI delta; 4, p3.1W.HincII; 5. p3.1W.HindIII delta and 6, p3.1W.N-PstI) and with either St.CAT (7.5μg) or p48Z.CAT (10μg). Cells were harvested 24 hours post-transfection and the CAT activity in the cell extracts determined. The percentage conversion for each protein was calculated and the results are presented as a histogram. The fold conversion is given for each deletion mutant.

sites. This region of 58 amino-acids includes the region of homology with the basic DNA binding structure of c-fos as well as additional sequences which lie C-terminal of this structure.

Although the BsmI-C and N-HincII proteins can bind to DNA, the protein-DNA complex is less stable to the effects of salt concentration than SmaI-C or wild type BZLF1. This was illustrated by resolving the complexes formed with each protein in gels in a lower and a higher salt electrophoresis buffer. The actual DNA-protein interaction is evidently stable in both conditions since SmaI-C is stable in both buffers. Therefore the salt concentration must influence the stability of the protein structure itself. This observation implies that ionic interactions are important in maintaining the structure of the DNA binding domain and that protein sequences which lie between the SmaI and the BsmI sites and HincII site and the C-terminus are involved in this interaction. In fact there are several acidic residues in these sections which may potentially interact with residues in the basic motif region to stabilise this structure. This effect has been assigned to the differences in the salt concentration between the gel electrophoresis buffers though it should be noted that the buffers also differ with respect to their pH.

The protein can tolerate deletion of one of the two regions required for stabilisation and still bind DNA, albeit with increased salt sensitivity. The absence of a retarded complex with the BsmI-HincII protein even in the lower salt buffer is presumably a consequence of the instability of the folding of the DNA binding domain when both of the sequences required for stabilisation are deleted.

The sequences shown to be essential for DNA binding span the basic motif and additional sequences C-terminal of this domain. In c-fos, and other members of this family of DNA binding proteins sequences found in this position form a dimerisation domain. An indirect immunoprecipitation assay demonstrates that BZLF1 forms multimers in solution. This experiment does not formally prove that BZLF1 forms homodimers although other experiments have subsequently shown that BZLF1 binds DNA as a dimer (Lieberman and Berk, 1990; Chang et al., 1990; Flemington and Speck, 1990a; Kouzarides et al., 1991).

The sequences required for this function have been defined using the deletion mutants and indicate that protein sequences which lie between the PstI site and the C-terminal are required for this activity. In c-fos the dimerisation structure which is found in this position is characterised by a

heptad repeat of leucine residues. Such a structure is not apparent in BZLF1; the structure of the dimerisation domain is investigated in Chapter 6.

Protein sequences which lie between the HincII and the C-terminus have been shown to be dispensable for DNA binding under some conditions. These sequences are essential for dimerisation in this assay which suggests that the conditions used i.e. buffer ELB are equivalent to the higher salt buffer G used in gel retardation assays. Therefore protein sequences between the SmaI and the BsmI sites are involved in stabilising the BZLF1 basic motif and not the dimerisation domain since the BsmI-C protein is able to dimerise under these conditions. Sequences C-terminal to the HincII site appear to affect DNA binding by stabilising functions required for dimerisation.

The BsmI-PstI delta protein is able to dimerise but cannot bind to DNA. Therefore, the BZLF1 DNA binding domain can be considered to be bipartite. It has a basic motif required for DNA binding and an adjacent dimerisation domain required for dimerisation and DNA binding. Chimaeric proteins containing the fos basic motif and the BZLF1 dimerisation domain or the GCN4 dimerisation domain and the BZLF1 basic motif retain DNA binding activity supporting this conclusion (Kouzarides et al., 1991).

Transient transfections have analysed the ability of the deletion mutants to activate transcription from BZLF1 responsive promoters. Previously the BZLF1 deletion mutant SmaI-C has been shown to bind DNA but not to transactivate (Rooney et al., 1989). In these experiments wild type BZLF1 and the mutant N-HincII were able to transactivate the BZLF1 and BSLF2+BMLF1 promoter; the mutants BsmI-PstI delta, HindIII delta and N-PstI have little or no activity in this assay. Analysis of the amounts of the proteins in the cells by Western immunoblotting showed that their levels were approximately equivalent. However, the sub-cellular localisation of these proteins has not been investigated. Recent analysis has identified two nuclear localisation signals within BZLF1 which act in HeLa cells (Giot et al., 1991). One of these signals lies within the basic motif, the other lies within protein sequences between the SmaI and BsmI sites. If these sequences are functional in B-cells then the protein BsmI-PstI delta is unlikely to accumulate to high levels within the nucleus of the cell. The other proteins, BZLF1, N-HincII, HindIII delta and N-PstI should all be localised in the nucleus although this has not been

demonstrated directly in B-cells. The mutant N-PstI, which does not bind DNA has also lost the ability to transactivate, despite its stable production in cells. This observation is consistent with the idea that sequence specific DNA binding is part of the mechanism by which BZLF1 transactivates promoters. The mutant HindIII delta is also stably produced in cells yet has a very low transactivation activity. This protein apparently binds DNA as well as wild type BZLF1; this indicates that sequences which lie between the HindIII sites are part of a transactivation domain since these sequences are required for transcriptional activity without an obvious role in DNA binding or nuclear localisation. Extensive analysis of this domain has confirmed that this region has activating properties (Giot et al., 1991).

The mutant N-HincII retains the ability to transactivate. This is not suprising since the protein is capable of binding DNA and has an intact N-terminal transactivation domain. However, this protein does not dimerise under higher salt conditions which suggests that conditions within the cell are relatively mild or that dimer formation is stabilised by other interactions in the cell. The protein exhibits only 50% of the activity of the wild type protein despite similar levels of expression which may reflect some reduced stability of the basic motif in cells. The effect of mutations on the ability of BZLF1 to transactivate did not depend on the reporter construct used which suggests BZLF1 transactivates transcription from these two promoters by essentially similar mechanisms.

Chapter 5
Serological Responses to BZLF1

The serological responses directed against EBV antigens are an important marker of disease in a number of EBV associated malignancies. The ability of sera from NPC patients to recognise different parts of BZLF1 using the in vitro translated mutants in an immunoprecipitation assay was investigated. This approach was extended to two rabbit anti-sera and a monoclonal antibody specific for BZLF1, to identify the regions of the protein recognised by these antibodies.

5.1 Immunoprecipitation of BZLF1 made by in vitro translation

Experiments presented in this chapter and elsewhere in this thesis involve the immunoprecipitation of radiolabelled proteins made by in vitro translation. Often only one radiolabelled protein is immunoprecipitated in each experiment and it is not clear that the immunoprecipitation is specific and does not simply represent binding or trapping of target proteins in antibody complexes. To demonstrate the specificity of the antibody interaction under the conditions used, several rabbit anti-sera were tested for their ability to immunoprecipitate BZLF1 in the presence of EBNA-LP. EBNA-LP is an unrelated protein that would not be expected to cross react with the anti-BZLF1 sera. If the immunoprecipitation is specific for BZLF1 the unrelated protein would not be recognised.

Radiolabelled BZLF1 and EBNA-LP were both made by in vitro translation. Two rabbit anti-sera were then tested for their ability to immunprecipitate BZLF1 specifically from a mixture of the radiolabelled proteins (figure 5.1). The sera used were anti-TrpE-BZLF1 (gift of G.Miller) and anti-pREX-SmaΔ (gift of D.T.Rowe) which were raised against the bacterially expressed BZLF1 fusion proteins TrpE-BZLF1 and pREX-SmaΔ respectively. Preimmune serum from a control rabbit was also tested as a negative control. The results show that anti-TrpE-BZLF1 (lane 2) and anti-pREX-SmaΔ (lane 3) are capable of immunprecipitating BZLF1. This interaction is specific since EBNA-LP is not recognised. As expected the preimmune serum (lane 1) shows no reactivity towards BZLF1.

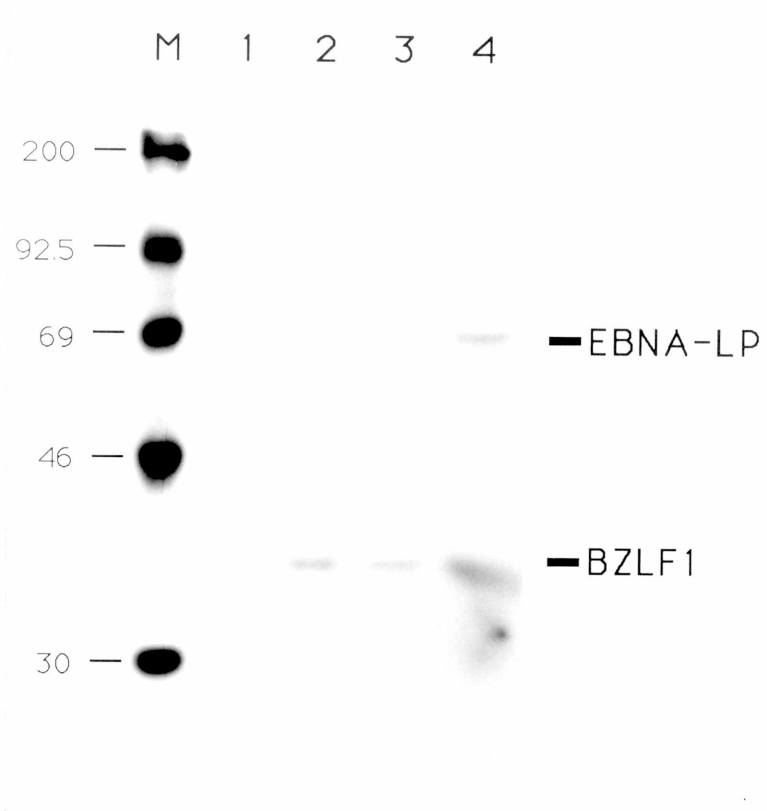


Figure 5.1 Immunoprecipitation of BZLF1 made by in vitro translation

Radiolabelled BZLF1 and EBNA-LP were made by in vitro translation in the rabbit reticulocyte system. Lysates containing the proteins were mixed (total volume 20µl) and were immunoprecipitated with the following rabbit sera; lane 1, preimmune sera; lane 2, anti-TrpE-BZLF1; lane 3, anti-pREX-SmaΔ. All sera were used at a 1/125 dilution. Lane 4 is a small portion of the mixed in vitro translation products before immunoprecipitation. The proteins were resolved on a 10% SDS polyacrylamide gel and visualised by fluorography. The sizes of the molecular weight markers (M) are indicated in kD. The position of migration of BZLF1 and EBNA-LP is shown.

5.2 Serological responses against BZLF1 of sera from NPC patients

The serological responses directed against a number of EBV antigens are important indicators in NPC. It has recently been determined that raised titres of antibodies specific for BZLF1 may also be important prognostic and diagnostic indicators (pers. comm., Yi Zeng; Joab et al., 1991). The ability of sera from NPC patients to recognise full length BZLF1 and various deletion mutants was investigated. A panel of 20 Chinese NPC sera (gift of Yi Zeng) was assayed for their ability to immunoprecipitate radiolabelled proteins made by in vitro translation. The immunoprecipitation procedure employs protein A sepharose which primarily recognises IgG class antibodies. Serum from an American EBV negative donor was used as a negative control.

The sera were initially assayed for their ability to immunoprecipitate full length BZLF1 (figure 5.2). The results demonstrate that although all sera recognise BZLF1 there is a wide range in titre and sera 438, 786, 405 and 435 clearly have low IgG anti-BZLF1 titres. So of this small sample approximately 80% of NPC patients exhibit high IgG anti-BZLF1 titres. As expected the control serum does not contain anti-BZLF1 antibodies.

The sera were then tested for their ability to immunoprecipitate N-StyI (which can be considered to represent the N-terminal half of BZLF1) and SmaI-C (which represents the C-terminal half) (figure 5.2). In fact the two proteins overlap slightly and may be expected to contain some common epitopes. Although the titres of these sera against these two halves of BZLF1 generally reflect the titre of the sera against the full length protein there are clear exceptions. For example, serum 771 recognises most strongly the N-terminal half whereas serum 365 recognises most strongly the C-terminal half.

The sera were also tested for their ability to immunoprecipitate BsmI-HincII which contains sequences entirely found within SmaI-C (figure 5.2). Once again the titre of the sera against BsmI-HincII generally reflects the titre against SmaI-C, though again exceptions do exist. For example, sera 365 and 779 recognise SmaI-C with approximately the same titre, though serum 779 clearly has a higher titre for BsmI-HincII than does 365.

These results indicate that individual sera differ in their ability to recognise different regions of BZLF1.

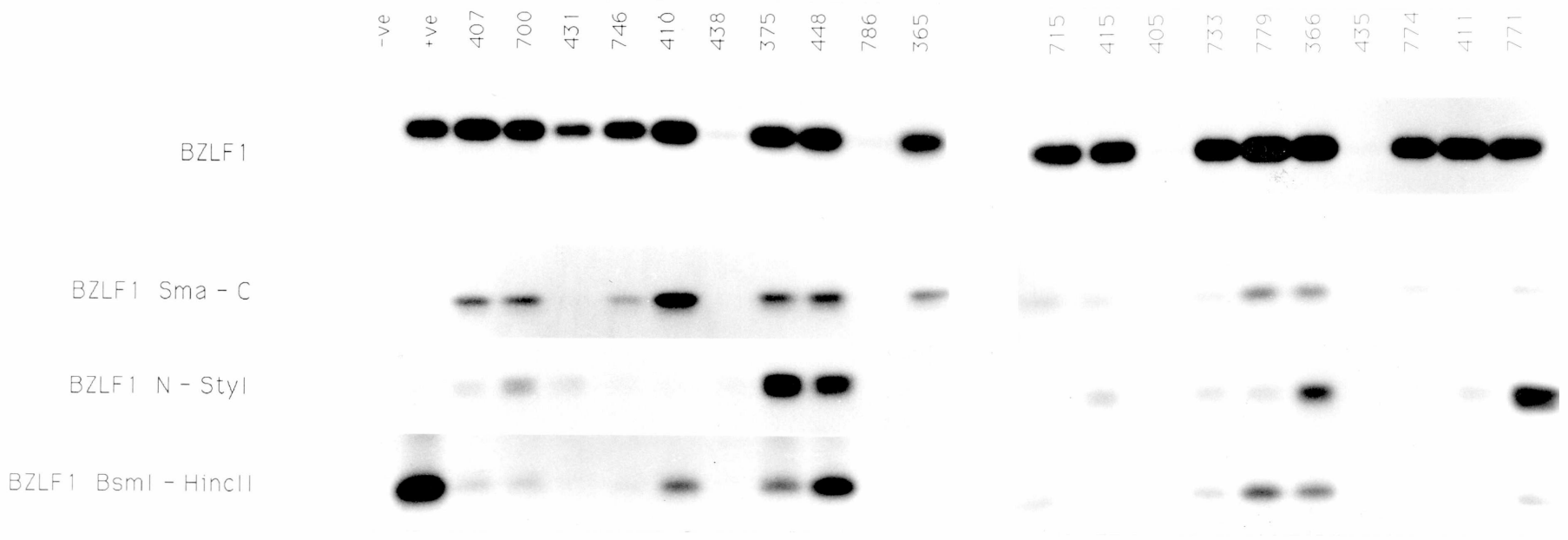


Figure 5.2 Immunoprecipitation of BZLF1 deletion mutants by NPC sera.

BZLF1 and the deletion mutants SmaI-C, N-StyI and BsmI-HincII were made by in vitro translation in the rabbit reticulocyte lysate system with [^{35}S]methionine. The proteins were immunprecipitated with each of a panel of 20 NPC sera and control sera. The NPC sera were used at a 1/50 dilution. The proteins were analysed on SDS polyacrylamide gels and were visualised by fluorography.

5.3 Identification of the parts of BZLF1 recognised by two rabbit antisera

The approach described above was extended to identify the regions of BZLF1 recognised by the two rabbit anti-sera which had been shown to immunoprecipitate BZLF1, anti-TrpE-BZLF1 and anti-pREX-SmaΔ. As before, radiolabelled deletion mutant proteins were made by in vitro translation and were immunoprecipitated using the rabbit anti-sera. The anti-TrpE-BZLF1 serum was raised against the bacterially expressed BZLF1 fusion protein TrpE-BZLF1 which essentially contains the first exon of BZLF1. This serum recognised all of the mutants assayed except BsmI-C (figure 5.3 A) and therefore recognises epitopes within approximately the N-terminal half of BZLF1. The anti-pREX-SmaΔ serum was raised against the bacterially expressed BZLF1 fusion protein pREX-SmaΔ which is equivalent to the SmaI-C deletion mutant. This serum recognised all of the mutants assayed except N-StyI (figure 5.3 B) and therefore recognises epitopes within approximately the C-terminal half of BZLF1.

5.4 The monoclonal antibody BZ1 recognises an epitope in the dimerisation domain of BZLF1

A monoclonal antibody, BZ1, specific for BZLF1 has recently been generated (Young et al., 1991). The antibody was raised against a bacterially expressed BZLF1 fusion protein pREX-SmaΔ which is equivalent to the SmaI-C deletion mutant. The region of the protein in which the epitope for this antibody lies was identified using the approach described above. BZ1 recognised all of the mutants tested except N-PstI (figure 5.4). Therefore deletion from the HincII site to the PstI site removes or destroys the epitope recognised by BZ1. This indicates that the epitope lies within or directly adjacent to the BZLF1 dimerisation domain.

In Chapter 6 the structure of the BZLF1 dimerisation domain is investigated by creating a series of single and double point mutants within this domain (see section 6.1 for full description). These proteins could also be radiolabelled by in vitro translation in the presence of a radiochemical. Since these point mutations lie within the region of BZLF1 shown to contain the epitope recognised by BZ1 it was decided to assess whether any of these mutants had any effect on recognition of BZLF1 by the antibody. All of the point mutants were recognised (figure 5.4). The ability of the antibody to recognise the mutant M appears to be reduced in one assay but this was not a consistent observation and the reduction was probably the result of experimental error. So BZ1 recognises an epitope within or

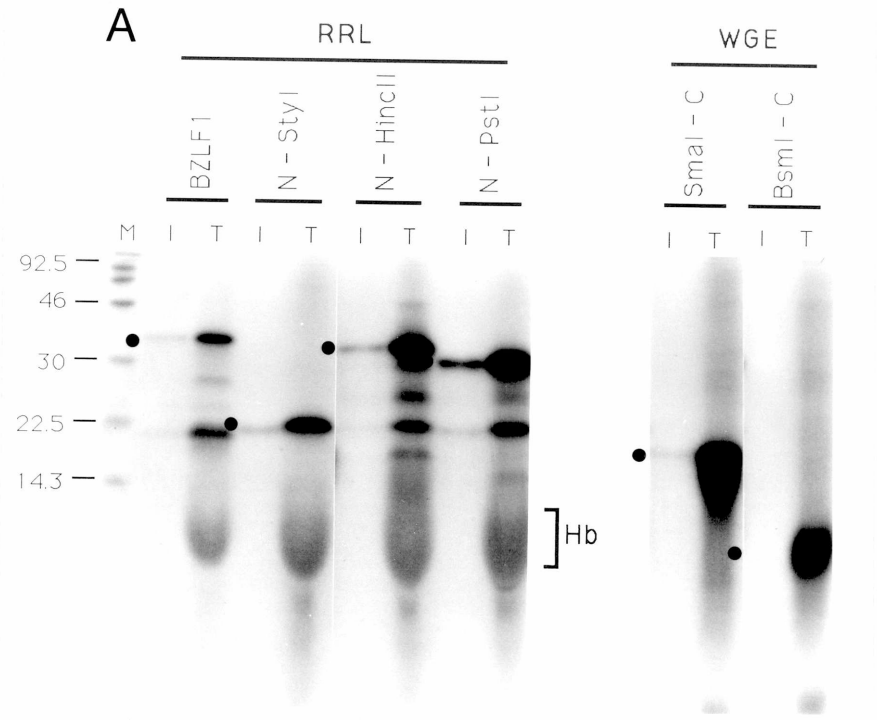


Figure 5.3 Identification of parts of BZLF1 recognised by rabbit anti-sera

Panel A; Radiolabelled proteins (BZLF1, N-StyI, N-HincII, N-PstI, SmaI-C and BsmI-C) were made by in vitro translation in the rabbit reticulocyte lysate system (RRL) or the wheat germ extract system (WGE) as indicated. Half (3µl) of the translation products were immunprecipitated with the rabbit sera anti-TrpE-BZLF1. The serum was used at a 1/125 dilution. The immunoprecipitated proteins (I) and a portion of the translation products (T, 3µl) were resolved on 15% SDS polyacrylamide gels and the proteins visualised by fluorography. The sizes of the molecular weights markers (M) are indicated in kD. The translations in rabbit reticulocyte lysate contained some radiolabelled haemoglobin as indicated.

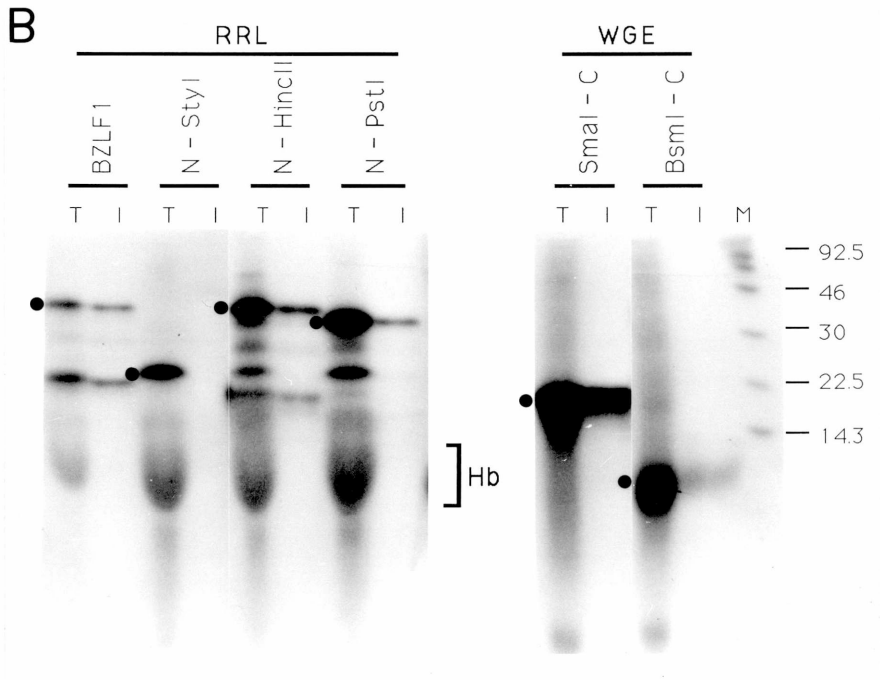


Figure 5.3 Panel B; legend as in A but serum anti-pREX-SmaΔ was used for immunoprecipitations.

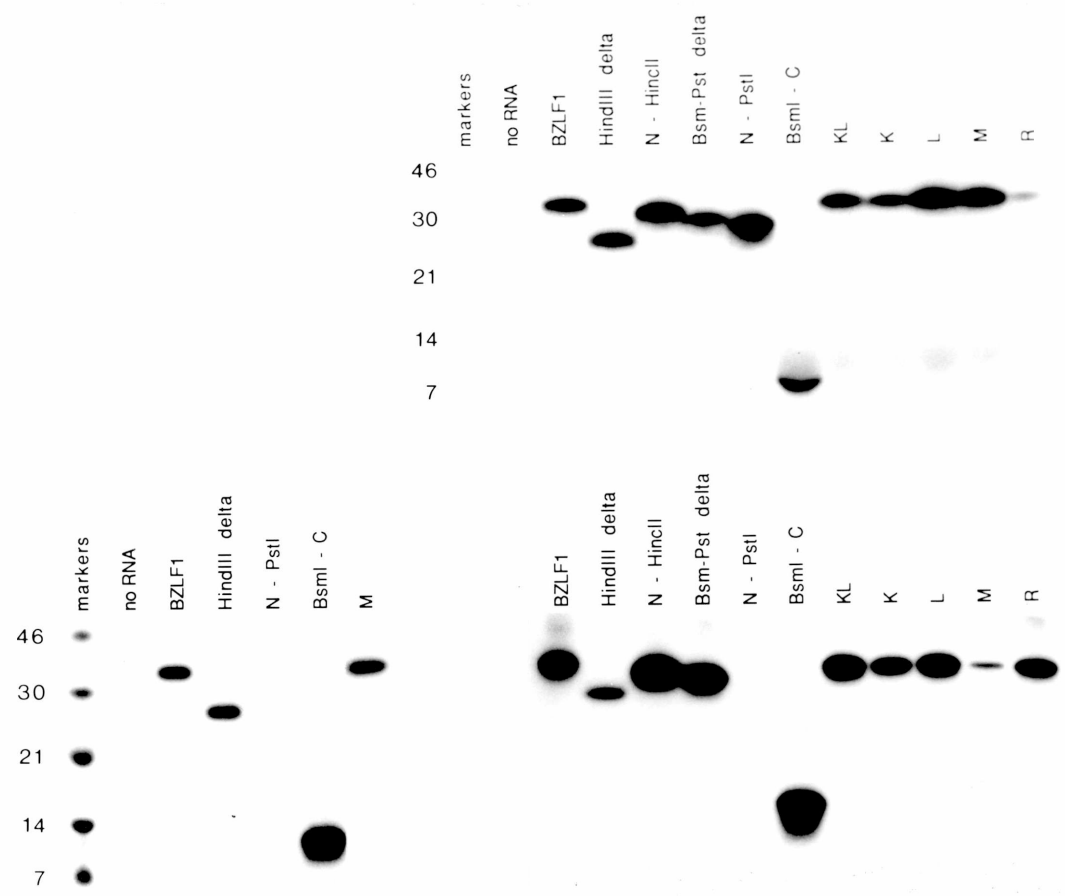


Figure 5.4 The monoclonal antibody BZ1 recognises an epitope in the BZLF1 dimerisation domain

Radiolabelled proteins were made by in vitro translation in the rabbit reticulocyte lysate system. Aliquots (2µl) of the translation products were analysed before immunoprecipitation (top panel). The remaining proteins (20µl) were then immunoprecipitated using the BZ1 monoclonal antibody (1/10 dilution of a culture supernatant). The results of two immunoprecipitation experiments are shown (bottom panel). The proteins were resolved on a 17.5% SDS polyacrylamide gel and visualised by fluorography. The sizes of the molecular weight markers are indicated in kD.

adjacent to the dimerisation domain of BZLF1 and this epitope does not include any of the residues which were mutated. An alternative explanation would be that the point mutations introduced (all to alanine) are conservative with respect to antibody recognition. It was interesting that the antibody was capable of recognising the KL mutant since under these conditions this mutant is unable to dimerise (see section 6.1). Therefore the antibody is able to recognise the monomeric form of the protein.

5.5 The monoclonal antibody BZ1 recognises the dimeric form of BZLF1

The experiment presented above shows that the antibody can recognise the monomeric form of BZLF1. If the epitope were masked when the protein dimerises, the antibody would fail to recognise the dimeric form of BZLF1. To address this question a 'supershift' experiment was performed. BZLF1 binds to DNA as a dimer. If the antibody is added to BZLF1 in a gel retardation assay and it binds the dimeric form, then the BZ1-BZLF1-DNA complex will migrate more slowly than the smaller BZLF1-DNA complex and the complex will appear to be 'supershifted'. The result of such an experiment is shown in figure 5.5. This experiment uses the bacterially expressed BZLF1 fusion protein, pGEX-SmaI-C to bind DNA (lanes 4-6). A control fusion protein, pGEX-EBNA-LP does not bind to the target DNA (lanes 1-3). The binding produced by BZLF1 is supershifted on addition of the BZ1 antibody (lane 5). The supershift is not seen when a monoclonal antibody, PE2 specific for EBNA2 is added (lane 6) showing that this effect is specific for BZ1.

Together these experiments demonstrate that the BZ1 recognises an epitope in the dimerisation domain of BZLF1 and that this epitope is present on both the monomeric and dimeric forms of BZLF1.

5.6 Discussion

Raised titres of antibodies specific for BZLF1 have been recognised as important indicators in NPC (Joab et al., 1991). Experiments presented in this chapter have measured the levels of IgG class antibodies specific for BZLF1 in the sera from NPC patients.

The results show that 80% (16/20) of sera from NPC patients exhibit high levels of IgG class anti-BZLF1 antibodies. A recent study (Joab et al., 1991) has proposed that IgG anti-BZLF1 antibodies may be used as a diagnostic indicator in NPC since the frequency of detection and titre



Figure 5.5 The monoclonal antibody BZ1 recognises the dimeric form of BZLF1

Radiolabelled 292/293 oligonucleotide (3ng) was complexed with; lanes 1-3, pGEX-EBNA-LP, (EBNA-LP fusion protein, 1µl); lanes 4-6, pGEX-SmaI-C, (BZLF1 fusion protein, 1µl) in a gel retardation assay. Lanes 2 and 5 are reactions incubated with the BZ1 monoclonal antibody and lanes 3 and 6 reactions incubated with the PE2 anti-EBNA 2 monoclonal antibody (each 1µl of ascites fluid). The complexes formed were resolved on a 4% polyacrylamide gel in 0.5x TBE and were detected by autoradiography.

of these anti-BZLF1 specific antibodies are raised in the sera from NPC patients relative to normal controls. The conclusions which can be drawn from this study are limited due to the lack of appropriate normal controls though the frequency of raised titres of BZLF1 specific antibodies is similar (87% (36/41); Joab et al., 1991).

The ability of the sera to recognise different parts of the protein was also investigated. No single region of the protein is likely to prove dominantly immunogenic and the results demonstrate that individual sera differ in their ability to recognise different regions of BZLF1. These findings may have some significance for the development of ELISA tests for measurement of BZLF1 titres and suggest that the best target in such an assay would be the whole protein as opposed to oligopeptides spanning seroreactive parts of the protein. Oligopeptides may prove to be unsatisfactory since individuals differ in their ability to recognise individual parts of the protein.

The part of BZLF1 recognised by two rabbit anti-sera has also been identified. As expected the sera recognised the parts of the protein against which they had been raised. The epitope for the monoclonal antibody, BZ1 has been demonstrated to lie within or adjacent to the BZLF1 dimerisation domain. Furthermore, the antibody is capable of interacting with BZLF1 without disrupting the active dimeric form of the protein.

Chapter 6
Analysis of the BZLF1 dimerisation domain

Deletion analysis has shown that, like c-fos, BZLF1 has a bipartite DNA binding domain consisting of a basic motif required for DNA binding but not for dimerisation and an adjacent structure required for dimerisation as well as DNA binding. In c-fos, and other related proteins, the dimerisation domain is termed a leucine zipper (see section 1.5; alignment of sequences figure 6.1). This structure exhibits several characteristic features,

1. The leucine zipper is found in a region of protein which adopts an α-helical secondary structure.

2. A heptad repeat of conserved leucine residues is found along one side of the helix (position (d) in figure 6.1).

3. Residues found along the same face and between the conserved leucines (positions (a)) are also hydrophobic.

4. The heptad repeat of leucines is found at a conserved distance from the basic motif of these proteins.

Dimerisation is thought to occur by the formation of a coiled-coil structure facilitated by the leucine zipper which places the adjacent basic motif in the precise position required for sequence specific interactions with DNA.

Protein sequences required for dimerisation of BZLF1, although found in a position occupied by the leucine zipper of c-fos do not show the characteristic heptad repeat of leucine residues. However, computer-aided prediction of secondary structures (Garnier et al., 1978) predicts that this region of BZLF1 has the propensity to form an α-helix. The predicted α-helix would extend from the alanine residue at position 185 and be disrupted by the proline residue at position 223 and would span approximately 10 helix turns.

Domain swap experiments have shown that the BZLF1 dimerisation domain can functionally replace the leucine zipper domain of c-fos (Kouzarides et al., 1991). This demonstrates that the dimerisation domain of BZLF1 can place the basic motif of c-fos in the correct position to bind DNA, a property essential for the leucine zippers of DNA binding proteins. In addition, the dimerisation domain of BZLF1 can be replaced by the leucine zipper of GCN4 (Kouzarides et al., 1991).

Despite the absence of a heptad repeat of leucines in BZLF1 further sequence inspection shows that BZLF1 has features compatible with

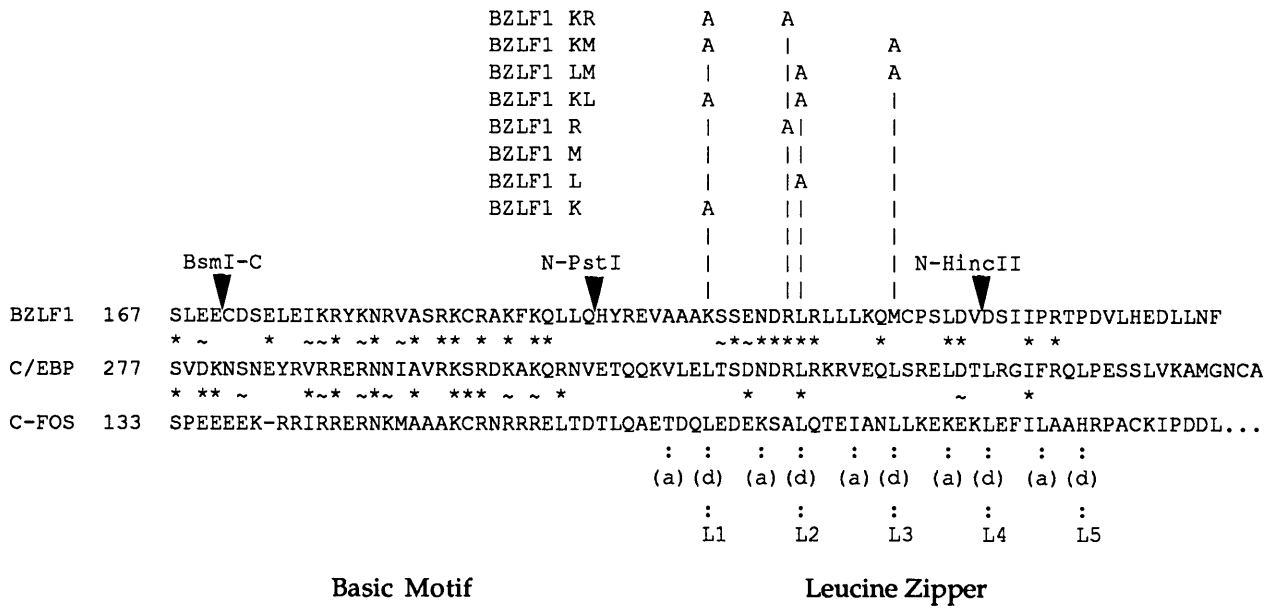


Figure 6.1 Alignment of parts of the protein sequences of BZLF1, C/EBP and c-fos. Amino-acid homologies to BZLF1 are shown for the basic and leucine zipper regions. Amino-acid are shown using the one letter code; identities are shown as stars, conservative substitutions (S-T, R-K, I-V, E-D) are shown as (~). The numbers indicate the residues of each protein illustrated. The positions of the conserved leucine residues in the C/EBP and c-fos leucine zippers are shown as L1 to L5 and the (a) and (d) positions of the helix are indicated. The boundaries of the BsmI-C, N-PstI and N-HincII BZLF1 deletion mutants are shown by arrows. The positions of the alanine mutants introduced into the BZLF1 dimerisation domain is indicated above the sequence (adapted from Farrell et al., 1989; Kouzarides et al., 1991).

coiled-coil formation (figures 1.3 B and 6.1). Considering the (d) positions of BZLF1, there is a leucine at the position of C/EBP L2 and a methionine at position L3. A methionine residue is predicted to be most suited to replace leucine in a coiled-coil structure (Landschulz et al., 1988b). At the position C/EBP L1 there is a lysine residue in BZLF1 which, although not ideal, can be accommodated in coiled-coil structures considering the abundance of lysine residues in the rod domain of myosin (McLachlan and Karn, 1983). In the (a) positions of the helix BZLF1 has alanine, asparagine and leucine residues. These hydrophobic residues are also compatible with a coiled-coil structure; indeed, the asparagine residue is highly conserved in this position in other members of the leucine zipper family.

Hence the structure of BZLF1 has features compatible with coiled-coil formation and functional features characteristic of leucine zippers. Furthermore, these features are in an exact register, with respect to the basic motif, when compared to the leucine zipper of C/EBP and c-fos. To investigate the structure of the dimerisation domain of BZLF1, point mutagenesis was performed.

6.1 Point mutagenesis of the BZLF1 dimerisation domain

A series of single and double mutants of residues within the BZLF1 dimerisation domain were obtained by site directed mutagenesis of the BZLF1 cDNA (figure 6.1; gift of T.Kouzarides). Mutations were introduced at the C/EBP L1, L2 and L3 positions which would result in substitution by alanine, a small hydrophobic amino-acid which should not disrupt helix formation. Three single mutants were made K, L and M which have mutations at the L1, L2 and L3 positions respectively. Three double mutants were also generated, KL, LM and KM. As a control the arginine residue located directly N-terminal of the leucine residue of BZLF1 (C/EBP position L2) was mutated (mutant R). This residue would not be predicted to play a role in the formation of a coiled-coil structure. The double mutant KR was made as a control for the double mutants.

All of the mutant proteins were made by in vitro transcription and translation. Since the BZLF1 point mutants include the epitope recognised by the anti-TrpE-BZLF1 antibody the mutants could be assayed in an indirect immunoprecipitation assay as before (figure 6.2; see also section 4.5). This assay tests the ability of each mutant protein to

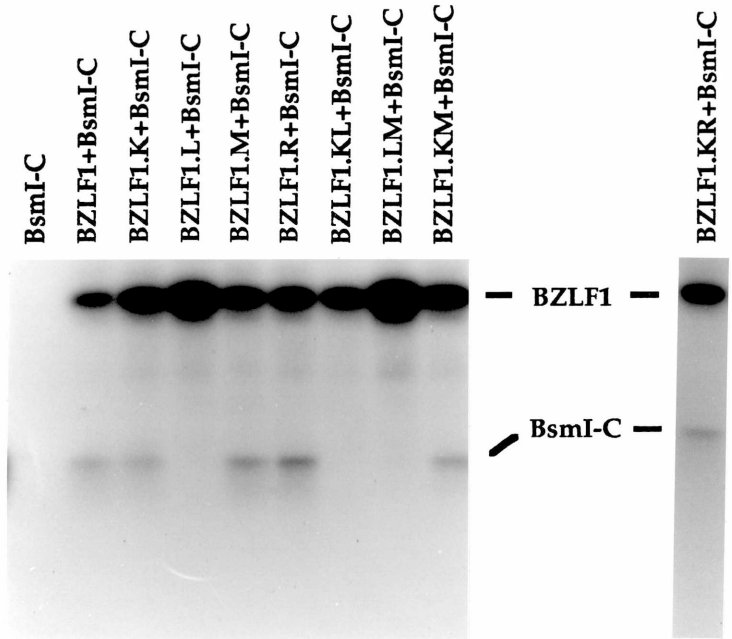


Figure 6.2 Point mutagenesis of BZLF1 dimerisation domain

BZLF1, the deletion mutant BsmI-C and the BZLF1 point mutants were made by in vitro translation in the rabbit reticulocyte lysate system with [^{35}S]cysteine. The proteins were translated alone or cotranslated with BsmI-C as indicated. The translation products (20µl) were immunoprecipitated with the anti-TrpE-BZLF1 antibody at a 1/250 dilution. The proteins were resolved on a 17.5% SDS polyacrylamide gel and detected by fluorography.

dimerise and indirectly immunoprecipitate the BZLF1 mutant protein BsmI-C independently of DNA binding.

The single mutants K, M and the control R and the double mutant KM and the control KR could dimerise in this assay since they could all indirectly immunoprecipitate BsmI-C. The mutants L, KL and LM have lost this function. Therefore, mutation of the leucine residue (C/EBP position L2) abolishes dimerisation while mutations at the L1 and L3 C/EBP positions are tolerated.

6.2 BZLF1 does not heterodimerise with BRLF1, c-jun, c-fos or C/EBP

The leucine zipper has been shown to direct protein heterodimerisation, as well as homodimerisation. For example, between c-fos and c-jun related proteins (Nakabeppu et al., 1988), between c-jun and CRE-BP1 (a member of the CREB family; Benbrook and Jones, 1990; MacGregor et al., 1990), and between c-jun and fra 1 (Cohen et al., 1989a). Since the dimerisation domain of BZLF1 is to some extent both functionally and structurally related to leucine zippers, BZLF1 might heterodimerise with other viral or cell proteins. These possible interactions might greatly influence the specificity and affinity of DNA binding by BZLF1 and its transcriptional activity.

One approach used to address this question is to attempt to demonstrate interactions between BZLF1 and other proteins using indirect immunoprecipitation experiments. In these experiments proteins were made by in vitro translation as before. The proteins were made by translation together where necessary to allow the nascent proteins to interact and lysates containing the two proteins were then immunoprecipitated using an antibody shown to recognise only one of the proteins. The presence of an indirectly immunoprecipitated protein would indicate an interaction between the proteins. Using this approach BZLF1 was assayed for its ability to heterodimerise with the EBV encoded transcription factor BRLF1 and the cell proteins c-jun, c-fos and C/EBP. The proteins c-jun, c-fos and C/EBP all contain leucine zippers which may allow interaction with the coiled-coil structure of BZLF1. BRLF1 and BZLF1 have been shown to synergistically activate transcription from the BSLF2+BMLF1 promoter and this activity may be due to a direct interaction between the two proteins (Kenney et al., 1989a; Buisson et al., 1989; Giot et al., 1991).

The plasmid used to generate BRLF1 RNA by in vitro transcription was pGEM.4Z.BRLF1 which was constructed by cloning a B95-8 virus genomic fragment containing the complete BRLF1 coding sequence into the EcoRI site of pGEM.4Z (gift of A.Sinclair). The plasmid was linearised using HindIII prior to transcription using SP6 RNA polymerase. BZLF1 and BRLF1 were made by in vitro translation and lysates containing mixtures of the two proteins were immunoprecipitated in two experiments with the anti-TrpE-BZLF1 (figure 6.3) or the BZ1 (figure 6.5) BZLF1 specific antibodies and analysed by SDS-PAGE. BRLF1 was not indirectly immunoprecipitated by BZLF1 in either experiment. One possible explanation for this observation is that antibody binding disrupts the putative heterodimer formation. However, since the antibodies used recognise epitopes which lie in different regions of the protein (anti-TrpE-BZLF1 recognises epitopes in the N-terminus and BZ1 recognises epitopes in the dimerisation domain) this would not occur with both antibodies. Therefore, it is unlikely that a stable interaction occurs between BRLF1 and BZLF1 under these conditions.

BZLF1 was then tested for its ability to heterodimerise with the leucine zippers of the c-fos and C/EBP proteins. Truncated c-fos and C/EBP proteins were used. The c-fos protein was generated from the plasmid pSP65.fos-core which contains part of the c-fos coding sequence cloned into SP65 (gift of T.Kouzarides; Kouzarides and Ziff, 1988). The fos-core translation product was a 92 amino-acid protein which includes the basic motif and the leucine zipper and is sufficient for DNA binding. The C/EBP C-terminal protein (C/EBP.Ct) was generated from the plasmid pGEM1.C/EBP (gift of T.Kouzarides) which contains part of a C/EBP cDNA (Landschulz et al., 1988a) cloned into pGEM1. The C/EBP.Ct translation product contains protein sequences shown to be sufficient for DNA binding (Landschulz et al., 1989).

The proteins were made alone or together with BZLF1 by in vitro translation and were immunoprecipitated with the anti-TrpE-BZLF1 or the BZ1 antibodies (figure 6.4). The anti-TrpE-BZLF1 antibody would not be expected to disrupt dimer formation since it recognises epitopes outside of the dimerisation domain. Also the BZ1 antibody has been demonstrated to interact with BZLF1 without disrupting dimerisation (see section 5.5). The immunoprecipitated proteins were analysed by SDS-PAGE and show that there is no interaction between BZLF1 and fos-core or C/EBP.Ct under these conditions. The low molecular weight proteins in lanes with large

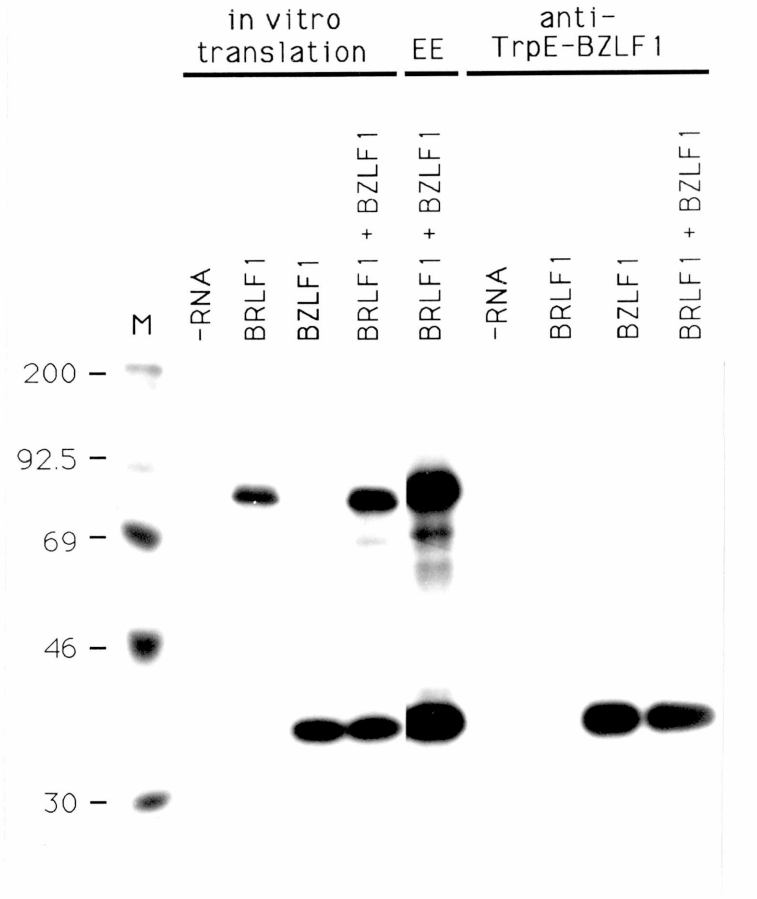


Figure 6.3 Indirect immunoprecipitation assay fails to demonstrate interaction between BZLF1 and BRLF1

BZLF1 and BRLF1 proteins were made by in vitro translation in the rabbit reticulocyte lysate system with [^{35}S]methionine. The proteins were cotranslated where indicated. The translation products (15µl) were immunoprecipitated with the human serum EE or with the anti-TrpE-BZLF1 antibody as indicated. Sera were used at a 1/250 dilution. A portion of the translation products was analysed before immunoprecipitation. The proteins were resolved on a 10% SDS polyacrylamide gel and detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD.

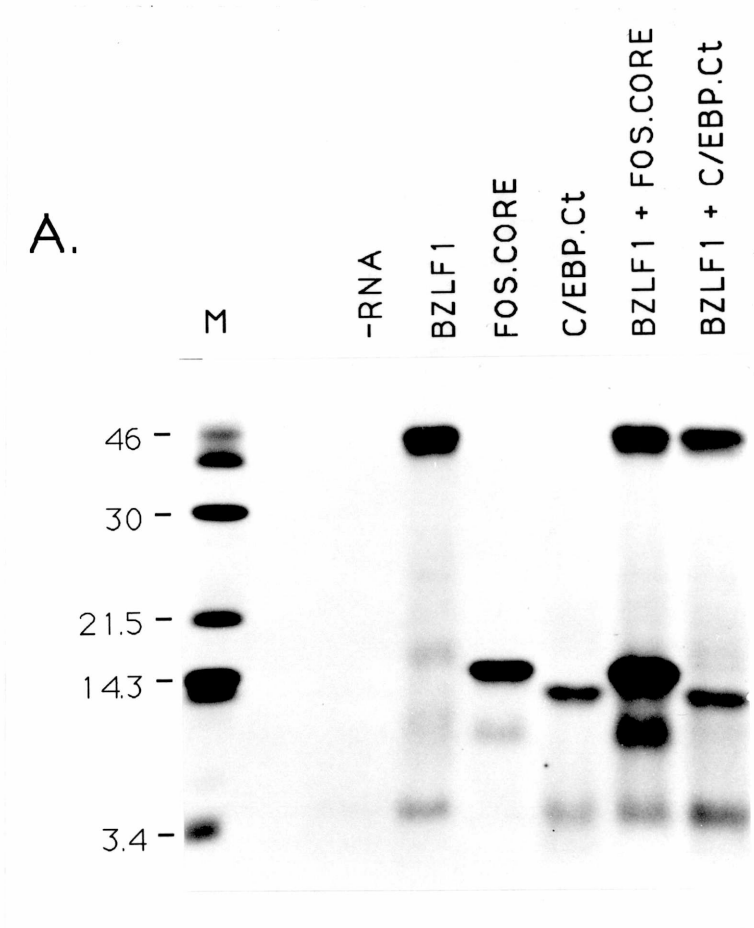


Figure 6.4 Indirect immunoprecipitation assays fail to demonstrate interaction between BZLF1 and fos.core or C/EBP.Ct

Panel A; BZLF1, fos.core and C/EBP.Ct were made by in vitro translation in the wheat germ extract system with [^{35}S]methionine. The proteins were cotranslated where indicated. Aliquots of the translation products (2µl) were analysed before immunoprecipitation. The proteins were resolved on a 17.5% SDS polyacrylamide gel and detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD.

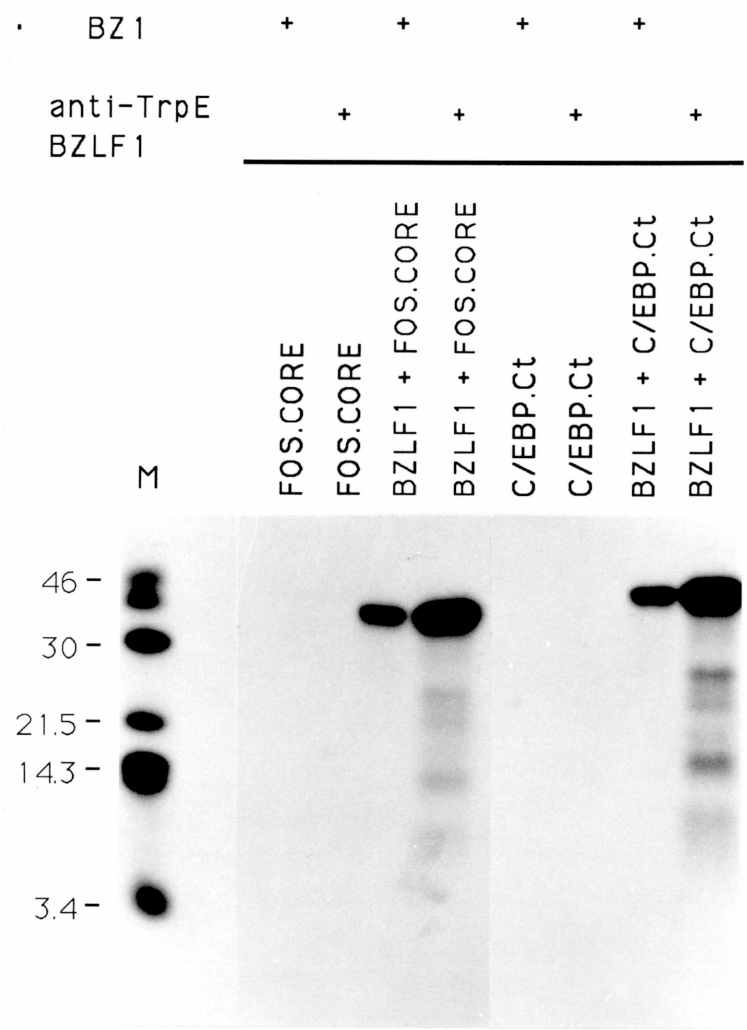


Figure 6.4 Panel B; BZLF1, fos.core and C/EBP.Ct were made by in vitro translation in the wheat germ extract system with [^{35}S]methionine. The proteins were cotranslated where indicated. The remaining translation products (20µl) were immunoprecipitated with the anti-TrpE-BZLF1 antibody (a 1/125 dilution) or BZ1 (a 1/16 dilution of a culture supernatant) as indicated. The proteins were resolved on a 17.5% SDS polyacrylamide gel and detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD.

amounts of BZLF1 are degradation products of BZLF1 (see figure 6.4 A) and not indirectly immunoprecipitated proteins.

BZLF1 was then tested for its ability to dimerise with the c-jun protein. The wild type c-jun protein was used in this assay and was generated from the plasmid pSP65.jun which contains a c-jun cDNA (derived from pBSSKc-jun; Turner and Tjian, 1989) cloned into plasmid SP65. The proteins were made together by in vitro translation and immunoprecipitated with the BZ1 antibody or an anti-jun antibody. The anti-jun antibody was a rabbit antiserum raised against a bacterially expressed fusion protein containing the C-terminal 133 amino-acids of c-jun (gift of R.Tjian). The immunoprecipitated proteins were analysed by SDS-PAGE (figure 6.5) and show that a stable interaction between BZLF1 and c-jun is unlikely to occur under these conditions.

6.3 Immunoprecipitation of BZLF1 from cells fails to demonstrate heterodimer formation

The experiments presented have failed to demonstrate an interaction between BZLF1 and BRLF1, c-jun, c-fos or C/EBP but the possibility remains that BZLF1 might interact with some other cell or viral protein. An approach to test this would be to immunoprecipitate BZLF1 from radiolabelled cell extracts made from EBV infected cells and to see if other proteins are specifically coprecipitated. Any single antibody might be expected to give a number of coprecipitated proteins (for example, due to cross-reactivity) and only coprecipitated proteins common to several BZLF1 specific antibodies should be considered to represent specific coprecipitations.

Two cell culture systems were used, both of which could be induced to enter the productive cycle to increase expression of BZLF1. The cell lines used were the marmoset LCL B95-8 which could be induced by treatment with TPA (zur Hausen et al., 1978) and the human cell line Akata which could be induced by treatment with anti-IgG antibodies (Takada and Ono, 1989).

B95-8 cells were treated overnight with TPA (40ng/ml) and then metabolically labelled for 3 hours with [^{35}S]cysteine. The cell extracts obtained were immunoprecipitated with the BZLF1 specific antibodies BZ1, anti-TrpE-BZLF1 and anti-pREX-SmaΔ and with rabbit preimmune and control sera. The immunoprecipitated proteins were analysed by SDS-PAGE (figure 6.6 A). BZLF1 was immunoprecipitated by the three

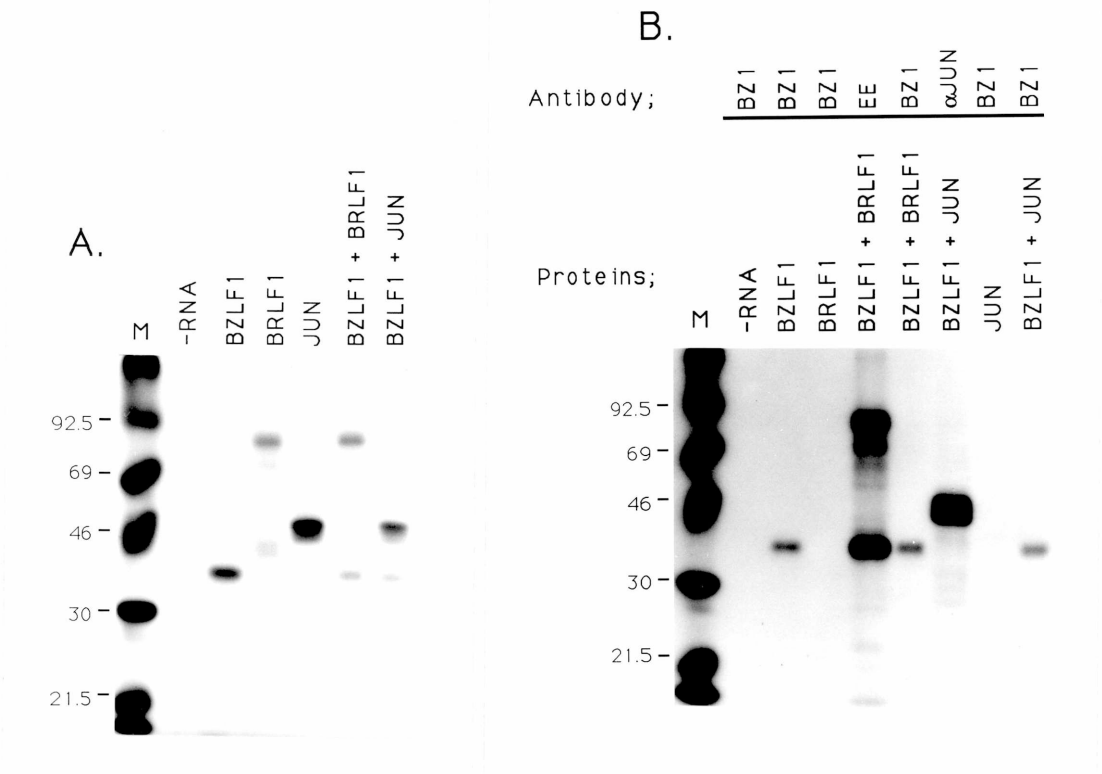


Figure 6.5 Indirect immunoprecipitation assays fail to demonstrate interaction between BZLF1 and BRLF1 or c-jun

BZLF1, BRLF1 and c-jun proteins were made by in vitro translation in the rabbit reticulocyte lysate system with [^{35}S]methionine. The proteins were cotranslated where indicated. A portion (1µl) of the translation products was analysed before immunoprecipitation (panel A). The remaining translation products (15µl) were immunoprecipitated with BZ1 (a 1/10 dilution of a culture supernatant), the human serum EE (a 1/125 dilution) or an anti-jun serum (a 1/60 dilution) as indicated (panel B). The proteins were resolved on a 10% SDS polyacrylamide gel and were detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD.

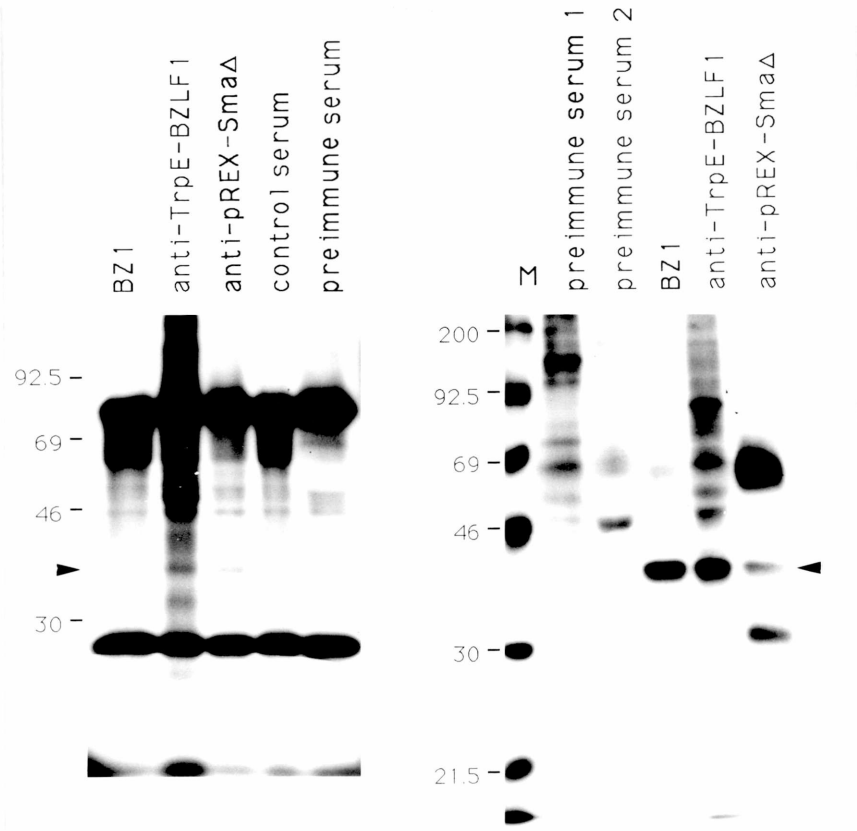


Figure 6.6 Immunoprecipitation of BZLF1 from metabolically labelled B95-8 and Akata cells

Panel A; B95.8 cells were induced to the productive cycle by treatment with TPA and radiolabelled with [^{35}S]cysteine. Cells were lysed in ELB and proteins were immunoprecipitated with BZ1, anti-TrpE-BZLF1, anti-pREX-SmaΔ or a rabbit control serum or preimmune serum. The sera were used at a 1/50 dilution, BZ1 was used at a 1/50 dilution of a culture supernatant.

Panel B; Akata cells were induced by treatment with 1% (v/v) anti-human IgG antibody and radiolabelled with [^{35}S]cysteine. Cells were lysed in ELB and the lysate precleared with protein A Sepharose beads before being immunoprecipitated with two rabbit preimmune sera, BZ1 and the anti-TrpE-BZLF1 and anti-pREX-SmaΔ antibodies. The sera were used at a 1/25 dilution, BZ1 was used at a 1/16 dilution of a culture supernatant.

The proteins were resolved on 12% SDS polyacrylamide gels and were detected by fluorography. The sizes of the molecular weight markers (M) are indicated in kD. The position of BZLF1 is indicated.

BZLF1 specific antibodies but not the control sera. However, two major bands were also present which obscured parts of the gel and made it difficult to identify coprecipitated proteins. These major bands of molecular weights approximately 80kD and 30kD are the heavy and light chains of immunoglobulins endogenous to the B95-8 cells which are immunoprecipitated directly by protein A sepharose.

The experiment was repeated using the cell line Akata. Akata cells were treated with 1% (v/v) anti-human IgG antibody for 6 hours and then metabolically labelled for 3 hours with [^{35}S]cysteine. Radiolabelled immunoglobulins were removed from the cell extracts before immunoprecipitation by incubation with protein A sepharose. The precleared extracts were immunoprecipitated with the BZLF1 specific antibodies BZ1, anti-TrpE-BZLF1 and anti-pREX-SmaΔ and two rabbit preimmune sera as controls. The immunoprecipitated proteins were analysed by SDS-PAGE (figure 6.6 B). The amount of immunoprecipitated BZLF1 was increased which presumably reflects that Akata is a more efficient system for induction of the productive cycle. The heavy and light chain had been almost completely removed by the preclearing process which enabled coprecipitated proteins to be identified. However, since no proteins coprecipitated consistently with BZLF1 and were also absent in control immunoprecipitations none of the coprecipitated proteins can be considered to represent specific interactions.

To increase the sensitivity of the assay immunoprecipitated proteins were analysed by Western immunoblotting. Unradiolabelled cell extracts for these experiments were made in a parallel fashion without addition of a radiochemical and were immunoprecipitated using the antibodies as above. The proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters which were probed with the following antibodies; EE, which is a human serum with high titres of antibodies specific for EBV replicative proteins (gift of A.B.Rickinson; EBV reciprocal titres of VCA: 80,000 and EA:20,000; pers. comm., A.B.Rickinson); no antibody as a control; an anti-jun rabbit serum; BP, which is a human serum from an EBV negative donor and an anti-fos serum. The anti-jun antiserum is that used in section 6.2, the anti-fos antibody is a rabbit antiserum raised against a synthetic peptide spanning N-terminal c-fos protein sequences (Hunt et al., 1987).

The results are shown in figure 6.7. The serum EE contains antibodies specific for EBV replicative proteins including BZLF1 and this

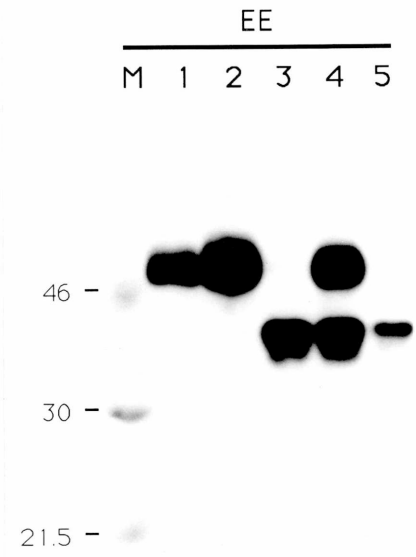


Figure 6.7 Western immunoblots of proteins immunoprecipitated from Akata cells

Akata cells were induced by treatment with 1% (v/v) anti-human IgG antibody. The cells were lysed in ELB and proteins immunoprecipitated with the antibodies described in figure 6.6; 1, preimmune serum 1; 2, preimmune serum 2; 3, BZ1; 4, anti-TrpE-BZLF1 antibody and 5, anti-pREX-SmaΔ antibody. The proteins were fractionated on 12% SDS polyacrylamide gels and transferred to nitrocellulose. The filters were Western immunoblotted using the following antibodies, dilutions are shown in brackets; EE (1/1500); no antibody; anti-jun (1/2000); BP (1/500); anti-fos (1/1000). The sizes of the molecular weight markers (M) are indicated in kD.

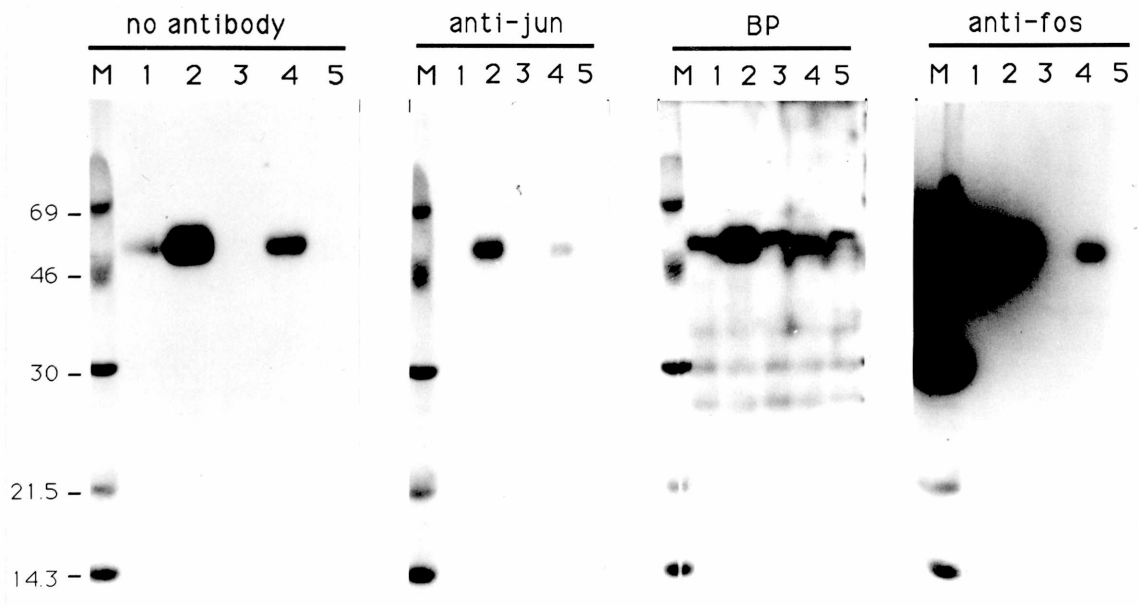


Figure 6.7 Western immunoblots of proteins immunoprecipitated from Akata cells (cont.) see previous page for legend.

immunoblot shows that BZLF1 was immunoprecipitated in this experiment by the BZ1, anti-TrpE-BZLF1 and anti-pREX-SmaΔ antibodies and not by the preimmune sera. This confirms that the protein identified in [^{35}S]cysteine labelled cells is in fact BZLF1. The higher molecular weight band (approximately 50kD) is presumably the residual immunoglobulin heavy chain not removed by the preclearing process. The immunoglobulin heavy chain differs in molecular weight between species due to differential glycosylation and is recognised directly by the protein A used in the immunoblotting since it is detected on the control immunoblot with no added antibody. Presumably protein A does not bind light chain on an immunoblot. It is not clear why the amount of heavy chain present is not equivalent for each individual immunoprecipitation though this observation was consistent. There are no proteins coprecipitated with BZLF1 which are recognised by EE. Serum EE contains antibodies specific for BRLF1 (see figure 6.3) which confirms lack of interaction between BZLF1 and BRLF1 or other replicative proteins. Filters probed with the anti-jun and anti-fos antibodies also showed no additional proteins. It should be noted that the anti-fos antibody cross-reacts with the 30kD molecular weight marker, carbonic anhydrase. The human serum BP detects some additional proteins put these are not specific for BZLF1 and are present in control immunoprecipitations.

Therefore this analysis fails to demonstrate interaction of BZLF1 with any other cell or viral protein.

6.4 Functional analysis of BZLF1 dimerisation domain

Sequence comparison of C/EBP and BZLF1 reveals that the two proteins share a region of primary sequence homology within the dimerisation domains of the proteins (figure 6.1; Kouzarides et al., 1991). Several of these residues are not predicted to be involved in formation or stabilisation of a coiled-coil structure and this homology may reflect the conservation of a common functional domain in the two proteins. BZLF1 and C/EBP do not appear to heterodimerise (see figure 6.4) so it is unlikely that this homology represents a conservation of residues involved in protein-protein interactions between BZLF1 and C/EBP. It is possible that BZLF1 and C/EBP interact with another factor, possibly a transcription factor, through this region. To test the significance of this region of homology the BZLF1 dimerisation domain was replaced by the leucine zipper region of GCN4, a protein which does not have primary sequence

homology with BZLF1 in this region. The resulting chimaeric protein, B_BG_{Zip} has been shown to bind an AP-1 site (Kouzarides et al., 1991) demonstrating that the GCN4 leucine zipper can place the basic motif of BZLF1 in the correct position to bind DNA. The BZLF1 cDNA and the B_BG_{Zip} derivative (a gift of T.Kouzarides) were subcloned into the eukaryotic expression vector, pCMV19 (gift of P.O'Hare; Grieves and O'Hare, 1989) so that their expression was driven by the CMV immediate early promoter. The proteins were tested for their ability to transactivate the BMLF1+BSLF2 promoter in a transient transfection assay. The expression plasmids and the control plasmid, pCMV19 were electroporated into the EBV negative BL line, Ramos and cells were analysed for expression of BZLF1 proteins (figure 6.8 A). The immunoblot demonstrates that both proteins are present in approximately equivalent amounts though, as before, the serum may not have exactly the same titre for each protein. The plasmids were then cotransfected with the BZLF1 responsive promoter construct, St.CAT (see section 4.6) and cells analysed for CAT expression (figure 6.8 B). The results show that both proteins transactivate and the assay fails to demonstrate a dramatic effect when the dimerisation domain of BZLF1 is replaced by the leucine zipper of GCN4. However, the B_BG_{Zip} protein is half as efficient as wild type BZLF1 in this assay despite approximately equivalent protein expression. Therefore the possiblility remains that this region of BZLF1 does interact with another factor and this interaction increases the transcriptional activity of BZLF1. However, the reduced activity of the B_BG_{Zip} protein compared with wild type BZLF1 might also be due to differences in DNA binding affinity and degree of nuclear localisation, aspects which have not been addressed directly.

6.5 Discussion

Deletion analysis has shown that BZLF1, like c-fos, has a dimerisation domain directly adjacent to a basic motif. Despite functional and structural similarities with the leucine zipper dimerisation domain of c-fos BZLF1 does not show a classic heptad repeat of leucine residues. However, sequence inspection shows that the protein sequence of BZLF1 in this region does conform to more general requirements for coiled-coil formation. To test whether the dimerisation domain of BZLF1 has characteristics consistent with those of a coiled-coil this domain was mutated.

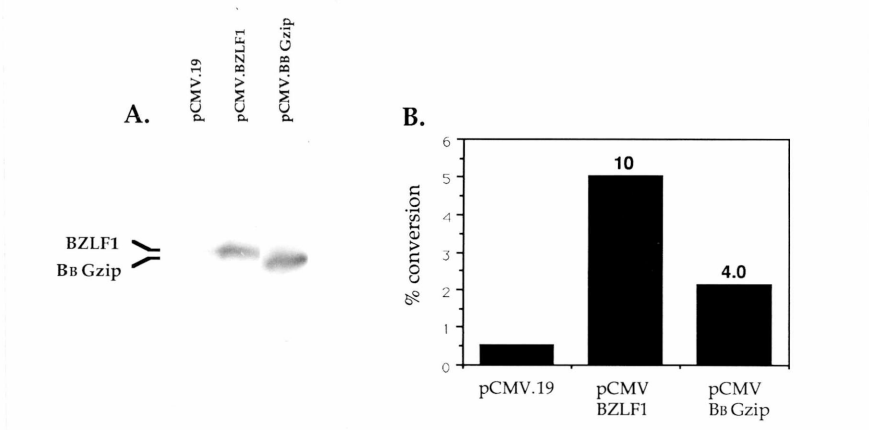


Figure 6.8 Transactivation of the BMLF1+BSLF2 promoter by BZLF1 and B_BG_{Zip}

Panel A; Ramos cells were electroporated with pCMV19, pCMVBZLF1 and pCMVB_BG_{Zip} (2μg of each) as indicated. The cells were harvested 24 hours post-transfection and the cell extracts resolved on a 10% SDS polyacrylamide gel. The proteins were transferred to nitrocellulose and the filter was probed with the human serum, EE.

Panel B; Ramos cells were electroporated with St.CAT (7.5μg) and with 2μg of each of the expression plasmids (1, pCMV19; 2, pCMV.BZLF1 and 3. pCMVB_BG_{Zip}). Electroporations were performed in duplicate. Cells were harvested 24 hours post-transfection and the CAT activity in the cell extracts determined. The percentage activity was calculated and the results are presented as a histogram. The average fold conversion is given for BZLF1 and B_BG_{Zip}.

BZLF1 was mutated at the three positions equivalent to leucines L1, L2 and L3 in C/EBP. The results show that mutation of the central leucine residue (C/EBP position L2) is sufficient to disrupt dimerisation. Mutations of residues found at the L1 and L3 C/EBP positions are tolerated, even when both residues are altered. This implies that the central leucine position is important in the structure of the BZLF1 dimerisation domain. The same BZLF1 mutants have been assayed for their ability to bind DNA in a gel retardation assay (Kouzarides et al., 1991). In this assay the single mutants, K, L, M and R were all able to bind DNA. The double mutants KM and the control KR could still bind DNA while the mutants KL and LM had lost this ability. In this assay mutations which abolish DNA binding are assumed to abolish dimerisation. The difference in results between the two assays is probably due to differences in the assay conditions. The gel retardation assay uses buffer BC100 for the binding reaction and electrophoresis in buffer G whilst buffer ELB was used in the immunoprecipitation assay. Buffer ELB, which contains 0.1% (v/v) NP40 and has a different salt concentration and pH probably destabilises the BZLF1 mutants relative to the gel retardation assay conditions. To test this directly the immunoprecipitation assay was performed using BC100 in place of ELB. In these conditions the immunoprecipitation reaction is no longer specific and all proteins are immunoprecipitated (data not shown).

In the gel retardation assay all single mutants retained DNA binding activity. This observation mirrors that obtained from mutagenesis of the leucine zippers of fos (Kouzarides and Ziff, 1988; Turner and Tjian, 1989) and C/EBP (Landschulz et al., 1989) where mutagenesis of at least two leucine residues is necessary to abolish DNA binding. The fact that mutation of the leucine residue in the immunoprecipitation assay presented here abolished dimerisation suggests that the leucine residue at the central L2 C/EBP position may be more critical for dimerisation than the adjacent L1 and L3 C/EBP positions. In the gel retardation assay double mutants containing the central leucine residue no longer dimerise, while the non-consecutive mutant KM retains some activity. This again indicates that the central leucine residue is probably most critical to the dimerisation function. Therefore, the residues found at C/EBP positions L1, L2 and L3 are important for the function of this domain which is consistent with the idea that part of the BZLF1 dimerisation domain has a structure compatible with coiled-coil formation. These results have been confirmed by a more extensive mutagenic analysis (Flemington and

Speck, 1990a) although unequivocal proof requires analysis of the structure of this domain by physical techniques.

Leucine zippers generally extend for 10 or more helical turns spanning 5 leucine repeats. The region of BZLF1 predicted to adopt an α-helical structure that is involved in coiled-coil formation is likely to extend for only 3 equivalent repeats since computer-aided structural analysis assumes that the helix will be disrupted by the proline residue at position 223. However, features consistent with coiled-coil formation extend beyond this proline. Amino-acid residues found at the following (a) positions of the helix, leucine and isoleucine are both hydrophobic. Since proline residues can be accommodated in an α-helix with only a small kink in the helix (Barlow and Thornton, 1988) it remains possible that the BZLF1 coiled-coil extends beyond this proline. Mutagenic analysis (Flemington and Speck, 1990a) has shown that the leucine residue at position 225 is important to the dimerisation domain which supports this idea.

Experiments were performed to determine whether BZLF1 was capable of heterodimerising with other cell or viral proteins using indirect immunoprecipitation assays. This approach has commonly been used to demonstrate protein-protein interactions. Immunoprecipitation reactions were carried out in buffer ELB. This buffer was used since it does not disrupt stable protein-protein interactions. For example, this buffer has been used to demonstrate the interaction between human papillomavirus-16 E7 protein and p105-RB in an indirect immunoprecipitation assay (Dyson et al., 1989).

Two approaches were employed. The first involved mixing radiolabelled proteins made by in vitro translation followed by immunoprecipitation with an antibody capable of recognising only one of the proteins. The second involved immunoprecipitation of BZLF1 from lysates from metabolically labelled cells in an attempt to identify specific coprecipitating proteins. Neither approach identified an interaction between BZLF1 and another protein.

No direct interaction of BZLF1 with BRLF1 could be demonstrated despite the ability of these proteins to synergistically activate transcription. However, direct physical interaction is only one possible explanation for synergistic activation and the possibility remains that BRLF1 and BZLF1 interact with some common factor involved in transcription. An alternative method for identifying proteins which interact with BZLF1

would be to screen a human cDNA library probing with radiolabelled or biotinylated BZLF1 protein.

BZLF1 is an example of a protein with a coiled-coil dimerisation domain without a leucine repeat that places an adjacent basic motif in the correct position for sequence specific DNA binding. Recently a GCN4 related protein, CPC1 has been reported to contain a dimerisation domain which forms a coiled-coil structure without a conserved heptad leucine repeat (Paluh and Yanofsky, 1991). Clearly the functions of a leucine zipper can be achieved by a related structure which lacks a heptad repeat of leucines so why are the leucine residues so highly conserved in the leucine zipper proteins? Leucine is the amino-acid residue most suited to coiled-coil formation so leucine zipper proteins may form relatively more stable homodimers than would the dimerisation domain of BZLF1. The presence of a heptad repeat of leucine residues may generate a conformation which allows heterodimerisation with a variety of (though not all) leucine zipper proteins. For example, the leucine zipper of c-jun can direct homodimerisation as well as heterodimerisation and in this way the DNA binding and transcriptional activity of a limited number of leucine zipper proteins is altered. This may permit a large number of different responses to be realised by differential expression of a limited number of leucine zipper proteins. BZLF1 function may require that the protein act independently of these complex regulatory influences and it is possible that the absence of a heptad repeat of leucines in the dimerisation domain of BZLF1 generates a conformation capable only of homodimerisation. In this respect BZLF1 does not appear to dimerise with other cell proteins and this may reflect a structural requirement enabling BZLF1 to interact with the cell transcriptional activation machinery while being independent of the cell leucine zipper proteins. However, it should also be noted that dimerisation domains apparently restricted in function to homodimerisation can also be achieved by leucine zipper structures, e.g. in the yeast transcription factor GCN4.

Chapter 7
Analysis of Early Events in Induction of the Epstein-Barr Virus Productive Cycle

Many experiments have now demonstrated a pivotal role for BZLF1 in the activation of the EBV productive cycle. However, the initial events in induction are unclear. The physiologically important stimulators of the productive cycle are unknown but it is presumed that viral gene expression is in some way sensitive to changes in the activities of host cell gene regulatory factors. Again the identity of these factors and the viral promoters which are sensitive to these changes are unknown. Experiments presented in this chapter identify viral promoters which are activated directly by the changes in cell factors associated with induction of the productive cycle.

The cell line used in these studies is the EBV-positive B-lymphocyte line, Akata (gift of K.Takada) which expresses surface IgG. The cells can be induced to enter the productive cycle by treatment with an anti-human IgG antibody, which cross-links the surface Ig and leads to B-cell activation (Takada and Ono, 1989; Daibata et al., 1990). The Akata virus does not contain any major deletions associated with some prototypic strains of virus and in this respect is similar to naturally occuring viral isolates (Takada et al., 1991). Akata cells are considered to be a good system in which to study viral transcription in productive infection since induction in response to B-cell activation may be physiologically relevant and is the most rapid EBV induction system known in culture. The cells have a phenotype representative of BL cells in vivo and respond rapidly to anti-IgG treatment; up to 50% of the cells express VCA within 12 hours.

Because of the central role that BZLF1 plays in induction, it is likely that the primary induction signal acts to activate expression of BZLF1 although other promoters may also be activated. BZLF1 is potentially encoded by two mRNA species, a 1.0kb monocistronic mRNA and a 2.8kb bicistronic mRNA which encodes BRLF1 and BZLF1. The transcripts are transcribed from distinct promoters and either of these promoters might be activated by cell factors. Experiments analysing the level of these two transcripts following induction of Akata cells demonstrates that both species are activated within one hour (Sinclair et al., 1991). Within two hours the level of the transcripts rises greatly due to autoactivation of the promoters by their products, BZLF1 and/or BRLF1. Because of the effects of

autoactivation it is not clear which transcript is expressed first. To circumvent the effects of autoregulation the level of these two transcripts, and other transcripts associated with productive infection, have been analysed after induction of Akata cells in the presence of an inhibitor of protein synthesis.

7.1 Inhibition of protein synthesis in Akata cells

To measure the initial response without autoactivation it is essential to completely inhibit protein synthesis since even low levels of expression of BZLF1 protein will stimulate viral promoters. The sensitivity of Akata cells to the inhibitors of protein synthesis, anisomycin (100μM, Sigma, USA) or cycloheximide (50μg/ml, Sigma, USA) was measured. The protein synthesis in treated cells was determined relative to untreated cells up to six hours after treatment (data not shown). Protein synthesis was measured by determining the incorporation of [^{35}S]methionine into TCA-precipitable material. It was determined that it takes six hours for either inhibitor at these concentrations to completely inhibit protein synthesis. In the following experiments Akata cells were treated with 100μM anisomycin for 6.5 hours prior to induction to ensure complete inhibition of protein synthesis.

7.2 Analysis of anisomycin resistant mRNAs in Akata cells induced to the productive cycle

Preliminary experiments investigating the induction of Akata cells which had been maintained under different growth conditions showed that the number of cells spontaneously producing virus (i.e. without treatment) was variable. Akata cells were therefore diluted in fresh medium every day and maintained at a cell density shown to result in a low number of spontaneously productive cells and low levels of productive cycle mRNAs in uninduced cell populations. However, it was noted that cells which were grown in this way induced more slowly and that the proportion of cells entering productive infection was also reduced. Cells maintained at higher cell densities and diluted less frequently showed higher numbers of spontaneously productive cells, induced more rapidly and the proportion of cells entering productive infection was also raised.

Akata cells were grown overnight at a cell density ($0.4x10^6$ cells/ml) shown to result in a minimal number of spontaneously productive cells.

The following day half of the cells were treated with anisomycin (100μM) to inhibit protein synthesis, the remaining cells were left untreated as a control. Six and a half hours after treatment with anisomycin, 1% (v/v) anti-human IgG antibody (Dakopatts, Denmark) was added to the growth medium of all cells to induce the productive cycle. Cells were taken at this time, and 1, 4 and 20 hours after induction for RNA preparation. Cells were also taken and analysed for protein expression by Western immunoblotting and indirect immunofluoresence.

The immunoblot (figure 7.1) was probed with the human serum, EE which has high titres against BZLF1 and viral early antigens (EA; gift of A.B.Rickinson). The monoclonal antibody BZ1, specific for BZLF1 was used in the indirect immunofluoresence anaysis (figure 7.2).

The data shows that the cells entered the productive cycle and confirmed that protein synthesis was inhibited. The number of cells expressing BZLF1 at the time when the cells were induced was very low (<0.5% by immunofluoresence) although expression of EA appears to be significant. This presumably reflects the higher level of expression of these antigens relative to BZLF1 in the small number of spontaneously producing cells or the higher titre of the anti-serum used against these antigens. It is unlikely that cells which do not express BZLF1 express EA, but this cannot be excluded. After induction, the number of cells expressing BZLF1 can be seen to increase slightly within one hour. Four hours post-induction the level of BZLF1 has risen greatly and the expression of EA is also increased. By immunofluoresence, the increase in the number of cells expressing BZLF1 four hours post-induction seems relatively small (approximately 5%). This is consistent with the observation that a smaller proportion of cells enter productive infection when the cells whichwere maintained in growth conditions where the number of spontaneously productive cells is very low. At twenty hours post-induction the expression of BZLF1 and EA is very high. As expected, cells which had been induced in the presence of anisomycin showed no increase in expression of BZLF1 or EA.

Total RNA was isolated using the 'RNAgents Total RNA Isolation Kit' (Promega, USA) which uses guanidine thiocyanate to inactivate endogenous ribonuclease activity. The level of specific transcripts was analysed using RNase protection assays (RPA). The RNA samples were hybridised with the riboprobes overnight and then digested with RNase A

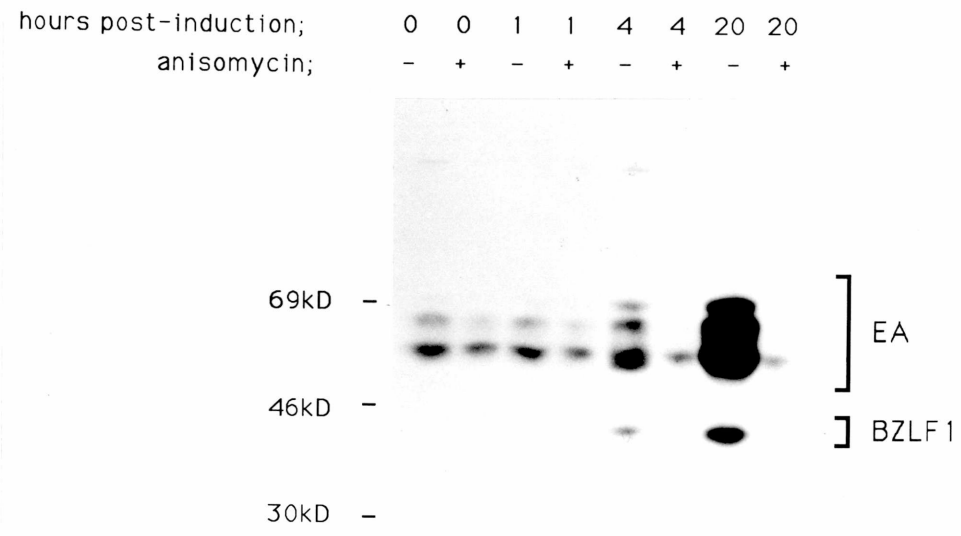


Figure 7.1 Western immunoblot showing induction of Akata productive cycle antigens

Akata cells were induced by treatment with 1% (v/v) anti-human IgG antibody in the presence or absence of 100μM anisomycin where shown. Cells ($1x10^6$ cells) were harvested 0, 1, 4 and 20 hours after treatment and proteins were electrophoretically separated on a 10% SDS polyacrylamide gel before being transferred to nitrocellulose. The filter was probed with a human serum, EE which has high titres of antibodies against EBV early antigens (EA, reciprocal titre of 20,000) and BZLF1.

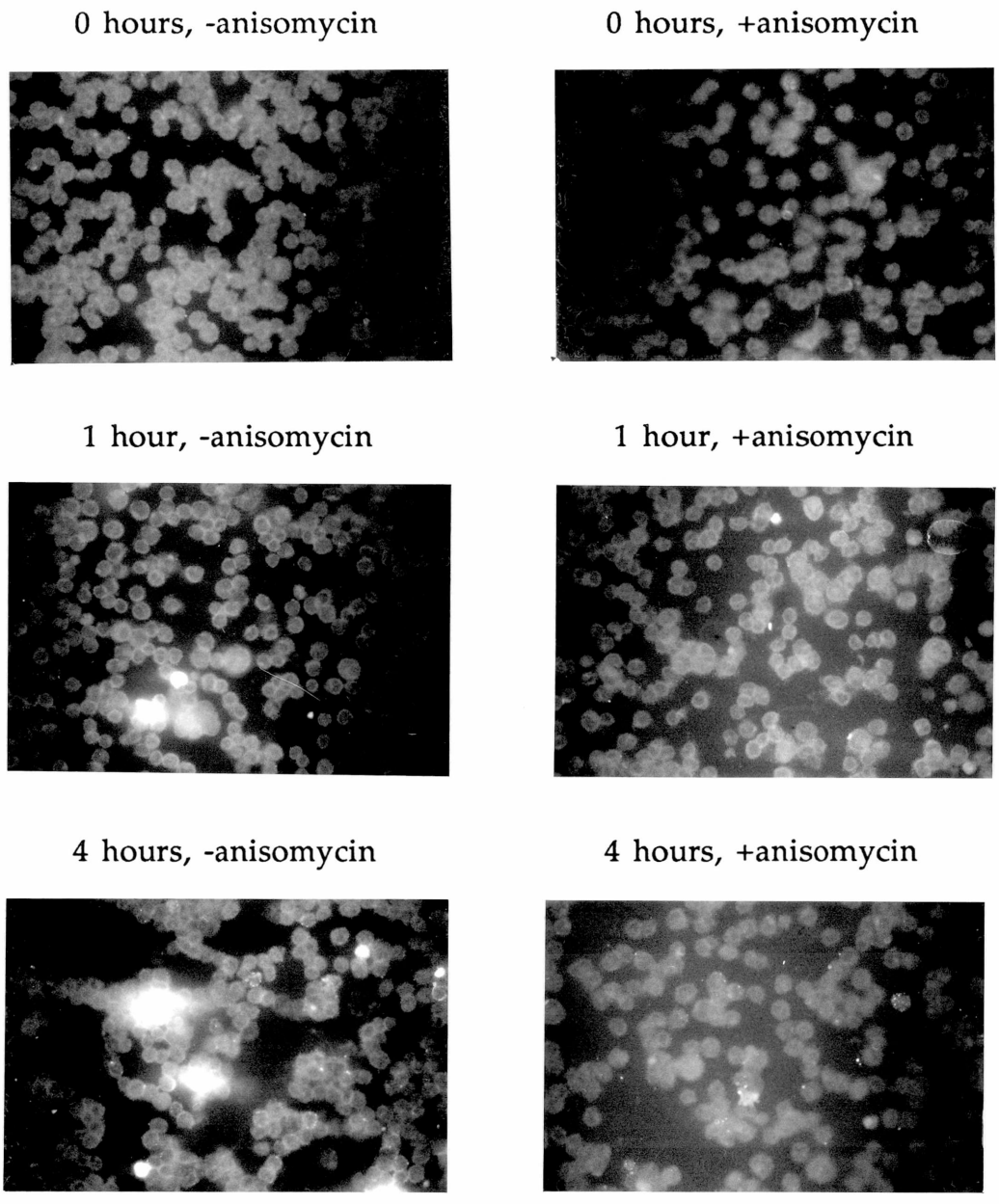


Figure 7.2 Indirect immunofluorescence of Akata cells following treatment with anti-immunoglobulin

Akata cells were induced by treatment with 1% (v/v) anti-human IgG antibody in the presence or absence of 100μM anisomycin where shown. Cells ($0.5x10^6$ cells) were taken 0, 1 and 4 hours post-induction and were fixed onto glass slides. The cells were analysed by indirect immunofluorescence using the BZ1 monoclonal antibody. The cells were photographed at 40x magnification and representative fields are shown.

and RNase T1. The protected species were resolved on denaturing polyacrylamide gels and detected by autoradiography. The transcripts analysed were the 1.0kb and 2.8kb mRNAs encoding BZLF1 and BRLF1+BZLF1 respectively, the mRNAs encoding BMLF1 and BSLF2+BMLF1 and the mRNA encoding BALF2.

The construction of the plasmids used to generate the riboprobes is given in section 2.5 and the EBV content, probe lengths and sizes of the protected species are shown in figure 7.3. The RNA samples were also analysed for the amount of actin mRNA as a control. The actin probe was supplied with the RPA kit and consisted of a 250bp β-actin gene fragment inserted downstream of the SP6 RNA polymerase promoter.

BZLF1 and BRLF1+BZLF1 mRNAs

BZLF1 is potentially encoded by two mRNA species, a 1.0kb monocistronic mRNA and a 2.8kb bicistronic mRNA which also encodes BRLF1. Both transcripts were detected using the Z44 probe. The level of these two transcripts following induction in the presence or absence of anisomycin is shown in figure 7.4. As expected, two protected species are seen in cells induced without inhibition of protein synthesis which correspond to the sizes predicted for protection by both the 1.0kb and 2.8kb mRNAs. There is a low level of these transcripts at the time when the cells were induced which presumably is present in the low number of spontaneously producing cells. Four hours after treatment the level of both mRNAs has increased as the number of productive cells increases. The level of expression of the two transcripts is still raised at 20 hours after treatment. In cells which have been induced in the presence of anisomycin expression of both mRNAs is still activated four hours post-induction though the levels are reduced when compared to uninhibited cells.

BMLF1 and BSLF2+BMLF1 mRNAs

The levels of the transcripts encoding BMLF1 and BSLF2+BMLF1 were also analysed since the activation of expression of these transcripts has been described as being resistant to protein synthesis inhibitors (Takada and Ono, 1989). Two protected species were detected with the probe 374 which correspond to the spliced and unspliced transcripts (figure 7.5). The spliced BSLF2+BMLF1 mRNA is the most abundant mRNA. The levels of both mRNAs is low in uninduced cells and their

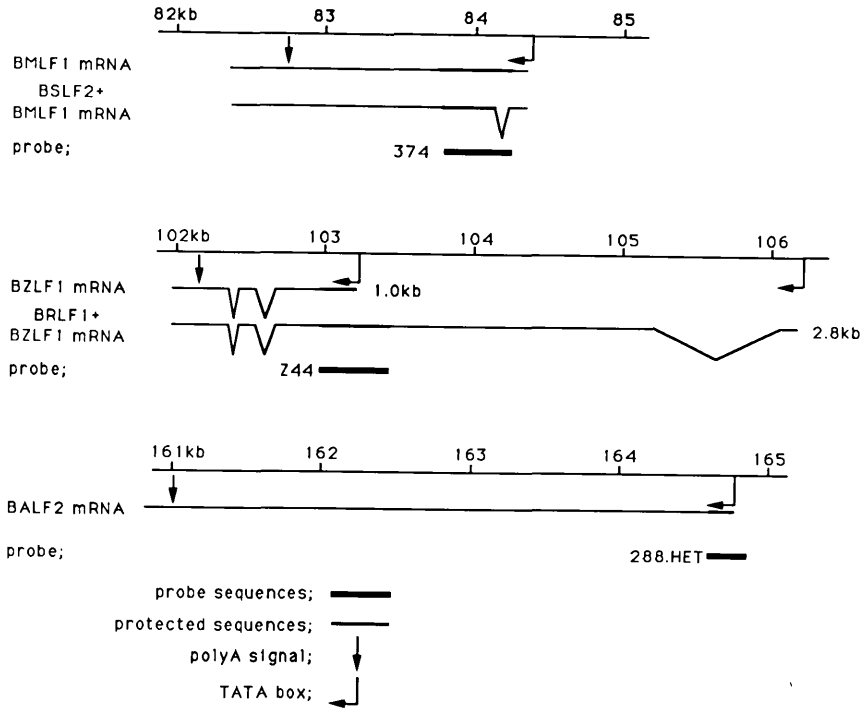


Figure 7.3 Structures of Epstein-Barr virus transcripts and the probes used for their detection

374; spans part of the BMLF1 mRNA and the splice acceptor (at EBV genome number 84122) used to generate the BSLF2+BMLF1 mRNA. The probe is about 450 nucleotides long and contains EBV sequences 84233 to about 83800. Hybridisation to the BSLF2+BMLF1 mRNA and the BMLF1 mRNA would result in protected species of about 350 nucleotides and about 430 nucleotides respectively.

Z44; spans the BZLF1 mRNA transcription initiation point (EBV genome number 103194). The probe is 473 nucleotides long and contains EBV sequences 103419 to 102984. Hybridisation to BZLF1 mRNA and to the BRLF1+BZLF1 mRNA would result in protected species of about 210 nucleotides and 435 nucleotides respectively.

288.HET; spans the presumed transcriptional start point of BALF2 mRNA (at about EBV genome number 164785). The probe is about 300 nucleotides long and contains EBV sequences 164608 to about 164838. Hybridisation to BALF2 mRNA would be expected to give a protected species of about 180 nucleotides.

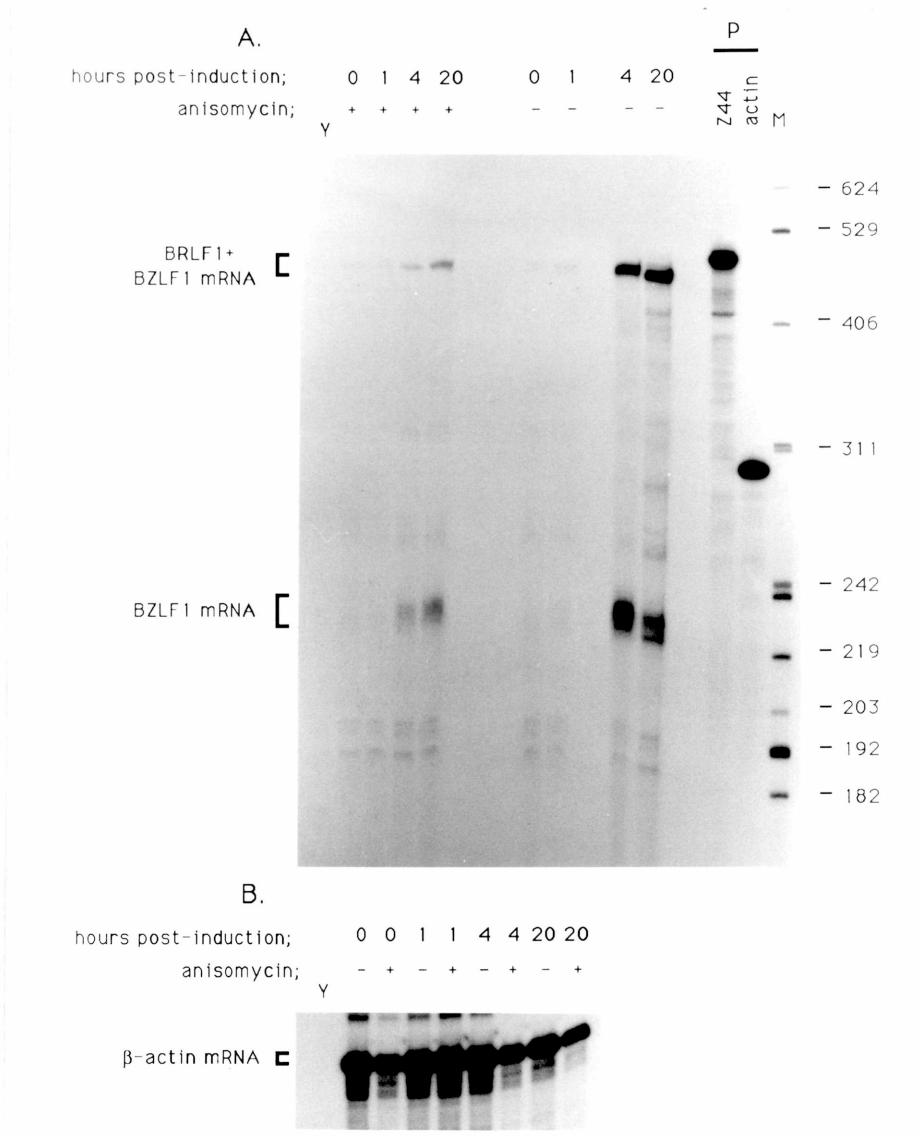


Figure 7.4 RNase mapping of 1.0 and 2.8kb BZLF1 mRNA in anisomycin treated Akata cells

Total RNA was prepared from Akata cells 0, 1, 4, and 20 hours after treatment with 1% (v/v) anti-human IgG antibody in the presence or absence of 100μM anisomycin.

Panel A; shows RNase mapping of Akata RNA and yeast RNA, Y (20μg each) with probe Z44. The protected species for the 1.0 and 2.8kb mRNAs are shown. Markers (M) are an MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in nucleotides are indicated.

Panel B; shows RNase mapping of Akata RNA and yeast RNA, Y (5μg each) with a β-actin probe. The undigested probes (P) are shown in panel A.

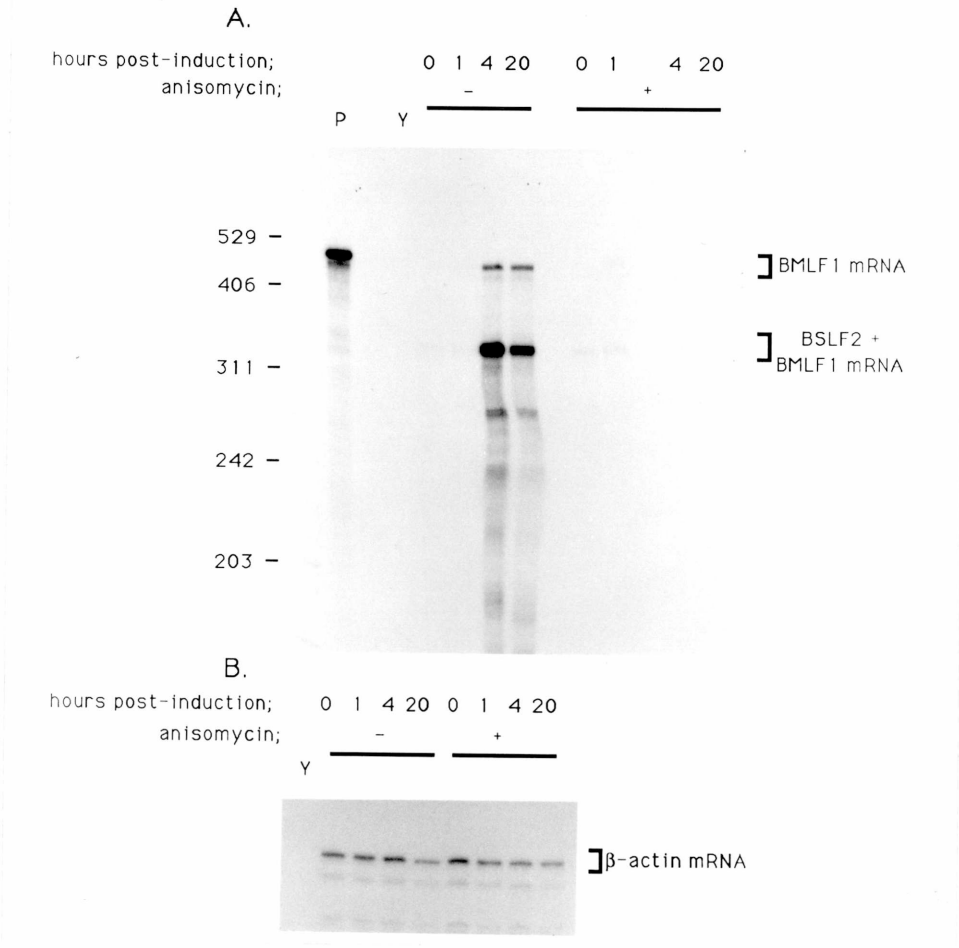


Figure 7.5 RNase mapping of BSLF2+BMLF1 and BMLF1 mRNA in anisomycin treated Akata cells

Total RNA was prepared from Akata cells 0, 1, 4 and 20 hours after treatment with 1% (v/v) anti-human IgG antibody in the presence or absence of 100μM anisomycin.

Panel A; shows RNase mapping of Akata RNA and yeast RNA, Y (20μg each) with probe 374 (undigested probe, P). The protected species for the BMLF1 and the BSLF2+BMLF1 transcripts are shown. Markers are an MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in nucleotides are indicated.

Panel B; shows RNase mapping of Akata RNA and yeast RNA, Y (5μg each) with a β-actin probe.

expression is greatly increased 4 and 20 hours after treatment. In cells which have been treated with anisomycin expression of both transcripts remains low.

BALF2 mRNA

The level of the mRNA for the BALF2 gene, which is considered to be a delayed early RNA was analysed. The precise transcription start for this mRNA has not been mapped but there is a TATA sequence at EBV genome number 164814 which is assumed to be part of the promoter for this transcript. The probe 288.HET spans the presumed transcription start site at about 164785 and the size of the transcript protected by this probe was determined using total RNA from Akata cells induced with anti-Ig treatment and from B95-8 cells induced with TPA (figures 7.6 and 7.7). This probe gives a protected species of about 180 nucleotides with both RNA samples which corresponds to a transcription initiation point at about EBV genome number 164787.

The level of this transcript was determined in Akata cells induced in the presence or absence of anisomycin (figure 7.7). The level of the transcript is low in uninduced cells and its expression is greatly increased in uninhibited cells 4 and 20 hours after induction. The level of the transcript does not change in anisomycin treated cells.

These experiments used Akata cells which were maintained in growth conditions which resulted in very few spontaneously producing cells, although the cells induced slowly. Preliminary experiments used Akata cells maintained in conditions which allowed a more rapid response to the inducing signal but with a higher number of spontaneously productive cells.

The levels of the BZLF1 mRNA, the BRLF1+BZLF1 mRNA, the BMLF1 mRNA and the BSLF2+BMLF1 mRNA were analysed in these cells after induction in the presence or absence of anisomycin (figure 7.8). In this experiment the levels of all of the transcripts was relatively high in uninduced cells due to the increased number of spontaneously productive cells. An increase in the levels of the BZLF1 and BRLF1+BZLF1 mRNAs could be detected within 1 hour, although the size of this effect is very modest because of the raised background levels (figure 7.8 A). Activation of expression of these transcripts in uninhibited cells preceded that of the BMLF1 and BSLF2+BMLF1 mRNAs, which increased 4 hours

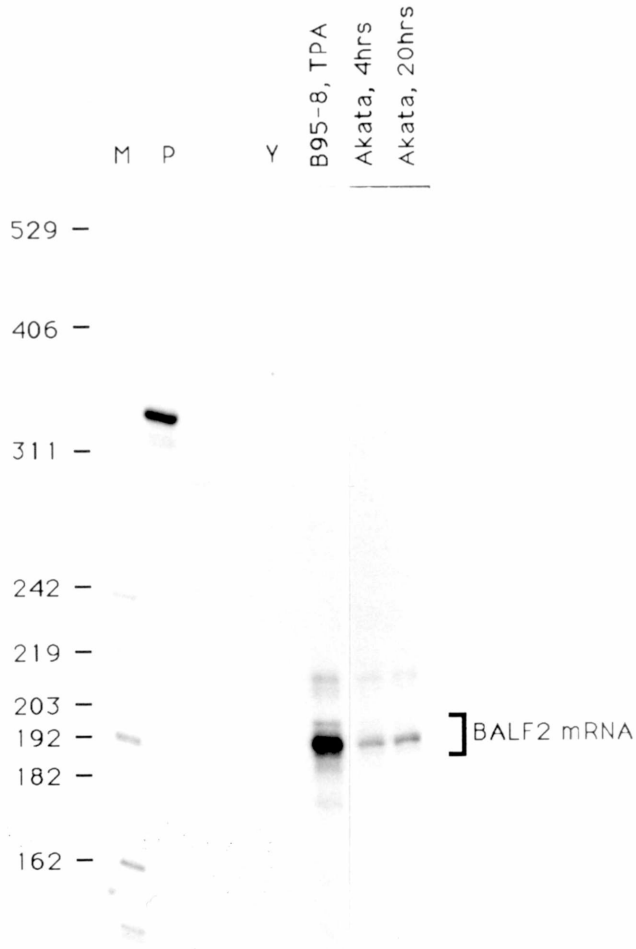


Figure 7.6 RNase mapping of BALF2 mRNA from B95-8 and Akata cells

Total RNA was prepared from B95-8 cells which had been induced with TPA and Akata cells 4 and 20 hours after treatment with 1% (v/v) anti-human IgG antibody. The figure shows RNase mapping of B95-8 RNA, Akata RNA and yeast RNA, Y (20µg each) with probe 288.HET (undigested probe, P). The protected species for the BALF2 mRNA is shown. Markers (M) are an MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in nucleotides are indicated.

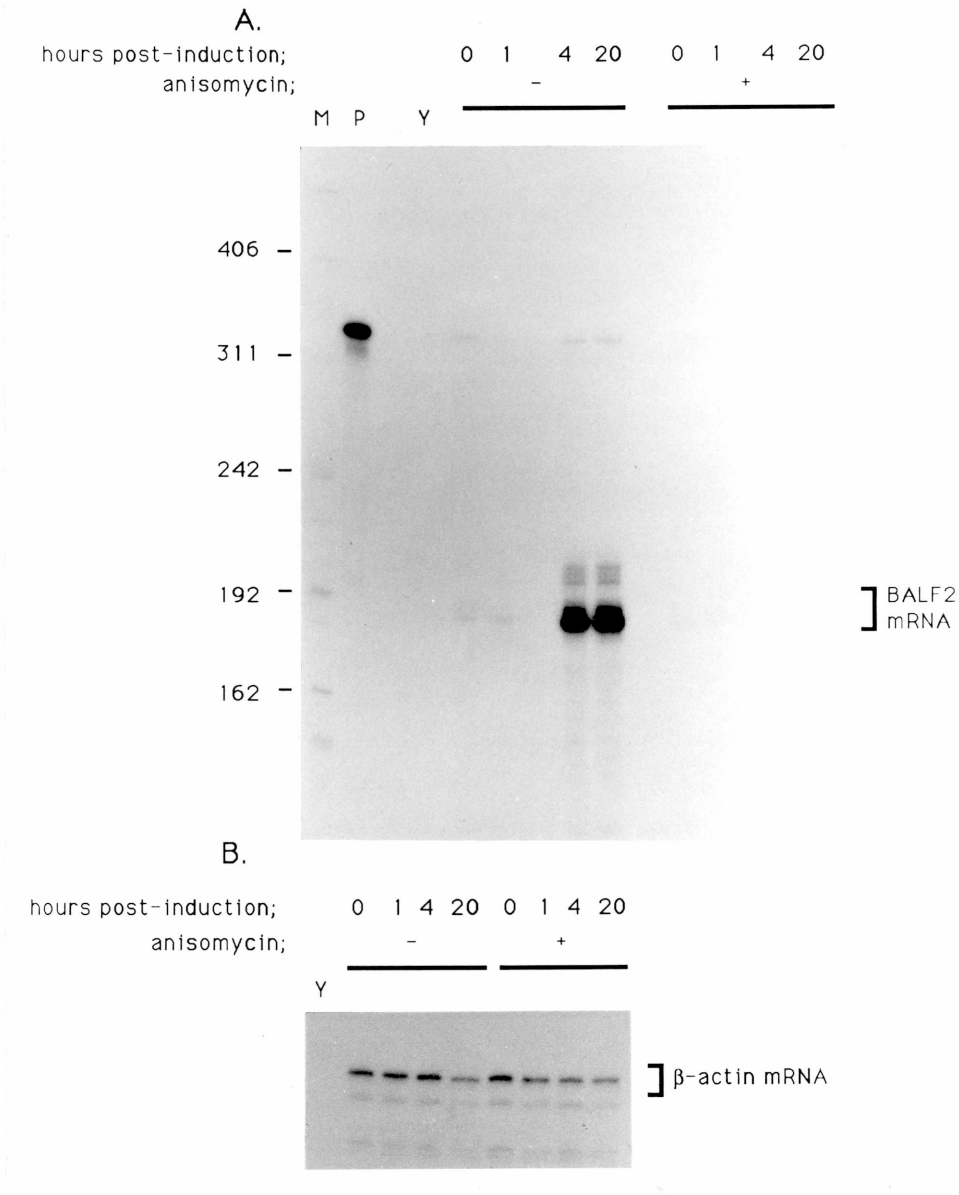


Figure 7.7 RNase mapping of BALF2 mRNA from anisomycin treated Akata cells

Total RNA was prepared from Akata cells 0, 1, 4 and 20 hours after treatment with 1% (v/v) anti-human IgG antibody in the presence or absence of 100μM anisomycin.

Panel A; shows RNase mapping of Akata RNA and yeast RNA, Y (20μg each) with probe 288.HET (undigested probe, P). The protected species for the BALF2 mRNA is shown. Markers (M) are an MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in nucleotides are indicated.

Panel B; shows RNase mapping of Akata RNA and yeast RNA (5μg each) with a β-actin probe.

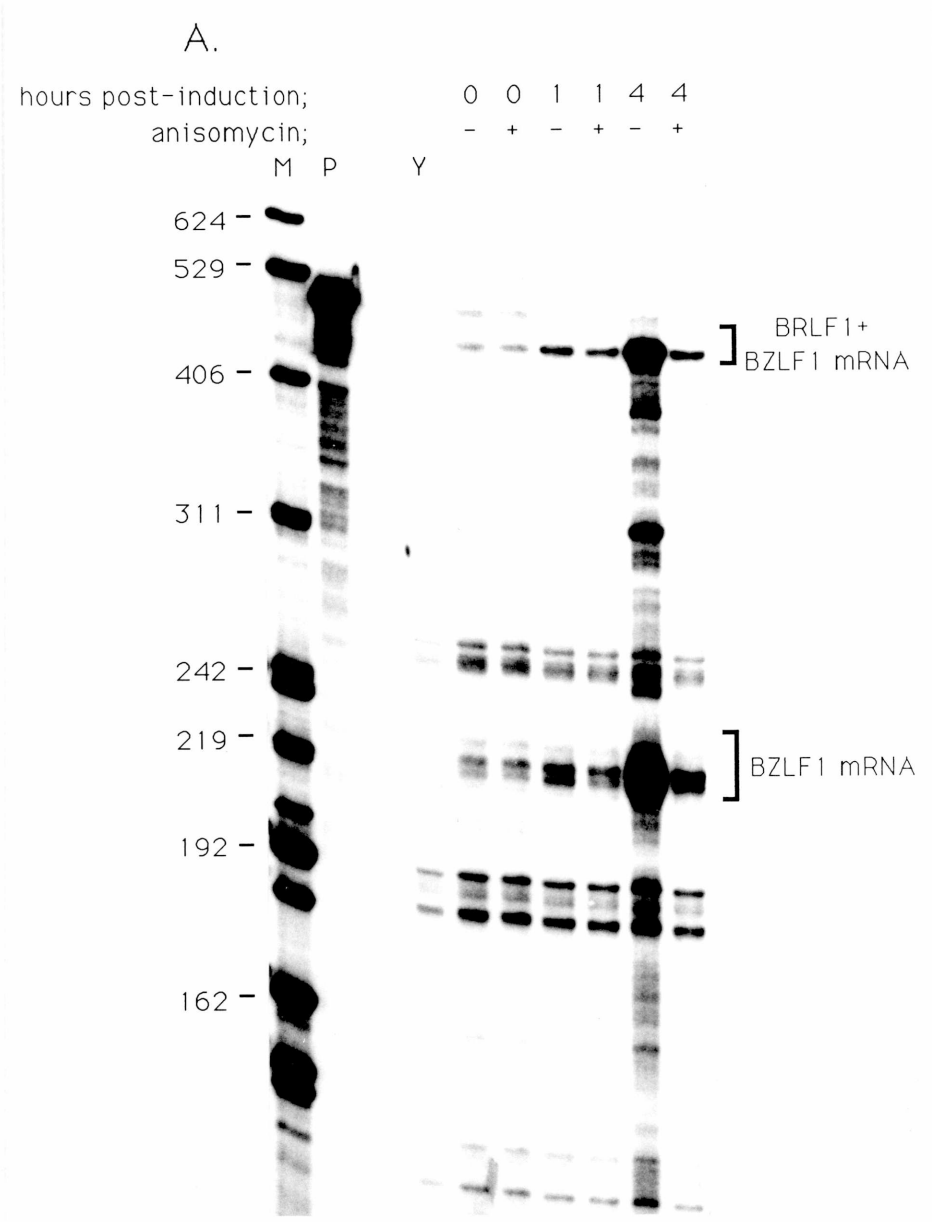


Figure 7.8 RNase mapping showing induction of expression of BZLF1 and BRLF1+BZLF1 mRNAs precedes BMLF1 and BSLF2+BMLF1 mRNAs

Total RNA was prepared from Akata cells 0, 1 and 4 hours after treatment with 1% (v/v) anti-human IgG antibody in the presence or absence of 100μM anisomycin.

Panel A; shows RNase mapping of Akata RNA and yeast RNA, Y (20μg each) with probe Z44 (undigested probe, P). The protected species for the 1.0 and 2.8kb mRNAs are shown.

Markers (M) are an MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in nucleotides are indicated.

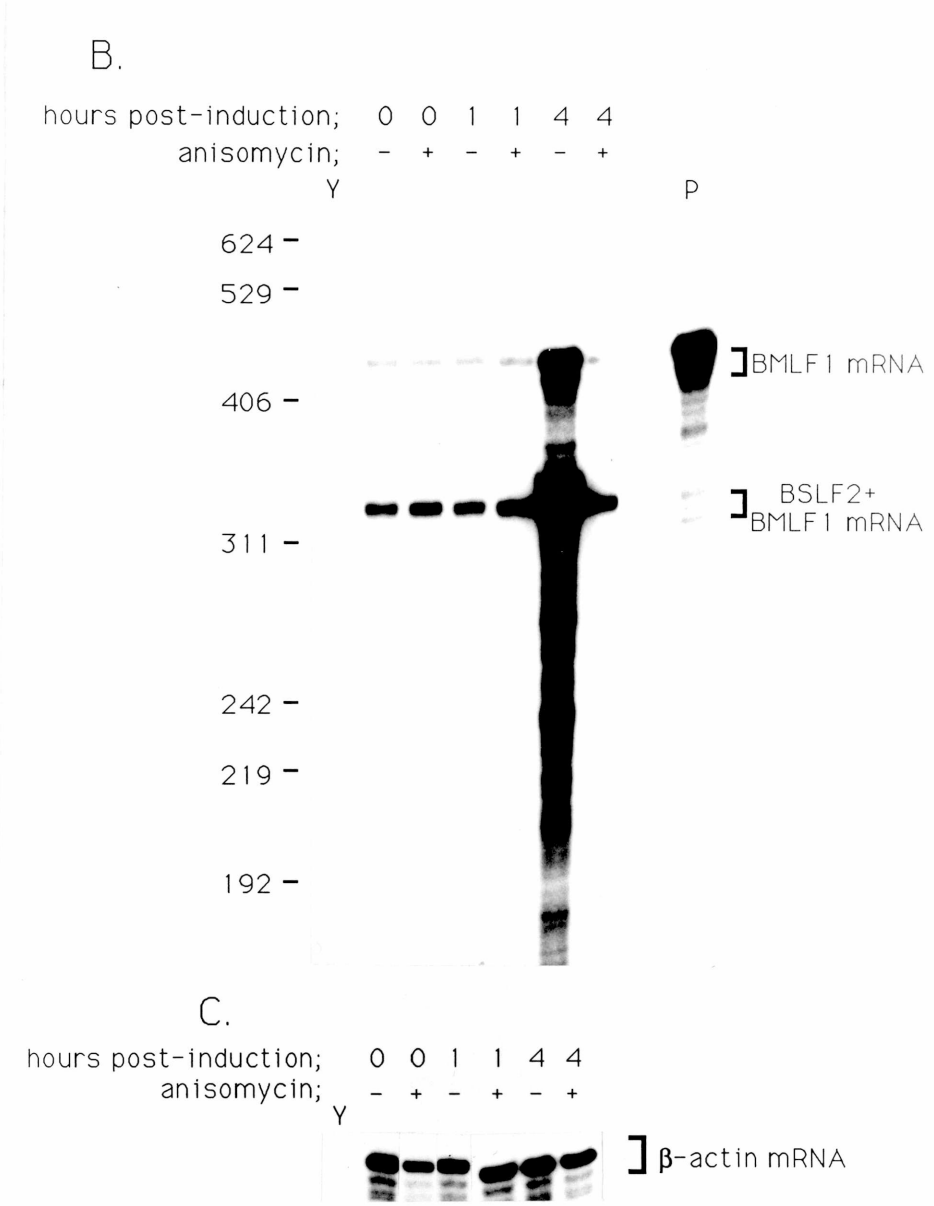


Figure 7.8 Panel B; shows RNase mapping of Akata RNA and yeast RNA, Y (20μg each) with probe 374 (undigested probe, P). The protected species for the BSLF2+BMLF1 and the BMLF1 mRNAs are shown.

Markers (M) are an MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in nucleotides are indicated.

Panel C; shows RNase mapping of Akata RNA and yeast RNA, Y (5μg each) with a β-actin probe.

post-induction (figure 7.8 B).

In another experiment using cells which induced relatively quickly it was possible to observe late downregulation of the BZLF1 mRNAs (figure 7.9). The levels of the BZLF1 and BRLF1+BZLF1 mRNAs were at their highest 4 hours after induction and both transcripts were downregulated to some extent 20 hours after induction. The 1.0kb BZLF1 transcript was completely downregulated while the 2.8kb transcript was only partially downregulated.

These results show that the BZLF1 and the BRLF1+BZLF1 transripts do not require any other EBV gene product for their expression in anti-IgG treated Akata cells, and that their promoters are directly sensitive to the changes in cell factors associated with B-cell activation.

7.3 Discussion

Experiments presented have analysed events associated with the early stages of induction of the productive cycle. Previous experiments analysing the levels of the 1.0kb BZLF1 mRNA and the 2.8kb BRLF1+BZLF1 mRNA have demonstrated that both transcripts are activated one hour after treatment of Akata cells with anti-IgG. However, it is not clear which promoters are responding to the initial activation signal or to the effects of autoactivation. These experiments have used anisomycin to ensure a complete inhibition of protein synthesis and have analysed the early events in induction of the productive cycle more clearly.

Complete inhibition of protein synthesis in these cells by anisomycin or cycloheximide requires at least six hours of treatment. Therefore Akata cells are relatively insensitive to these inhibitors; this may reflect differential metabolism or uptake of these inhibitors by different cell types.

It was noted that Akata cells which were maintained in different growth conditions showed variable numbers of spontaneously productive cells. More specifically, cells maintained at lower cell densities and which were fed every day showed fewer spontaneously productive cells. Cells maintained in this way induced more slowly and the number of cells entering productive infection was also reduced. This presumably reflects subtle changes in the levels of host cell transcription factors in response to different growth conditions.

The levels of a number of viral transcripts associated with the productive cycle have been analysed in anti-Ig treated Akata cells.

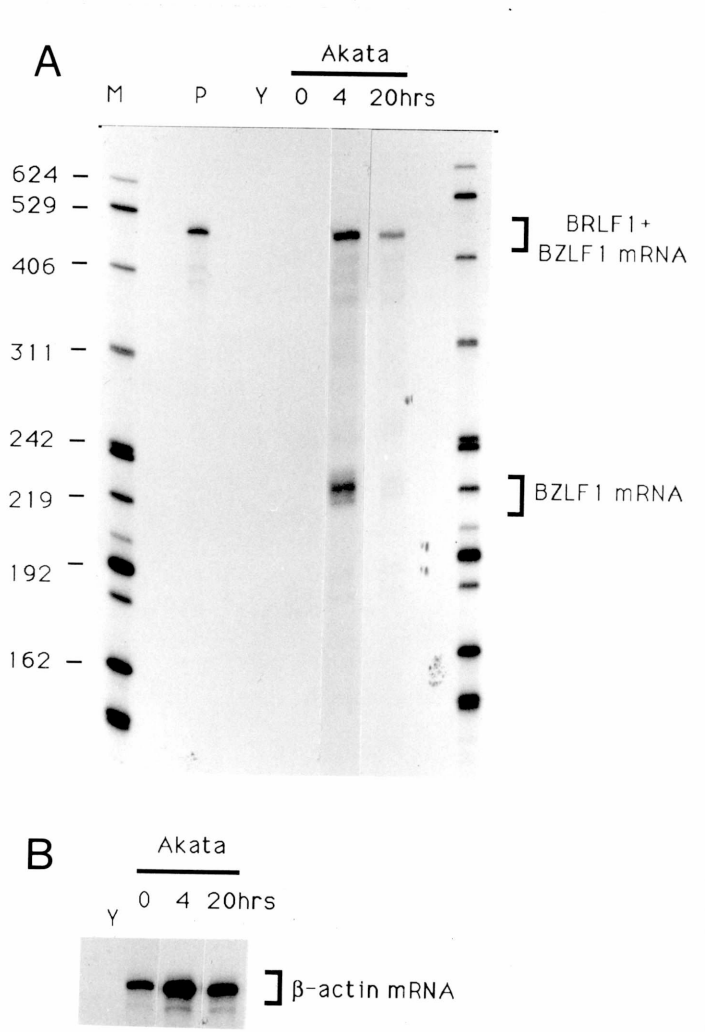


Figure 7.9 RNase mapping showing late downregulation of BZLF1 mRNAs in anti-immunoglobulin treated Akata cells

Total RNA was prepared from Akata cells 0, 4 and 20 hours after treatment with 1% (v/v) anti-human IgG antibody.

Panel A; shows RNase mapping of Akata RNA and yeast RNA, Y (20μg each) with probe Z44 (undigested probe, P). The protected species for the 1.0 and 2.8kb messages are shown. Markers (M) are an MspI digest of pBR322 DNA end-labelled with [α^{32}P]dCTP and Klenow DNA polymerase. The sizes of the DNA fragments in nucleotides are indicated.

Panel B; shows RNase mapping of Akata RNA and yeast RNA, Y (5μg each) with a β-actin probe.

The activation of expression of both the 1.0kb BZLF1 mRNA and the 2.8kb BRLF1+BZLF1 mRNA was resistant to inhibition of protein synthesis. The levels of the two transcripts was greatly reduced in anisomycin treated cells indicating that autoregulation of the promoters for both of these transcripts plays an important role in generating the levels of transcripts found in cells in the productive cycle.

Activation of expression of the BMLF1, BSLF2+BMLF1 and BALF2 mRNAs was completely inhibited by anisomycin treatment. Since the promoter for the BMLF1 and the BSLF2+BMLF1 transcripts is known to be activated by BZLF1 this confirms that protein synthesis has been completely inhibited.

The exact transcription start for the BALF2 mRNA had not been previously mapped although a putative TATA sequence for this mRNA is located at EBV genome number 164814. A probe spanning the presumed transcription start was used to analyse transcripts in Akata cells and cells containing the prototypic virus strain B95-8. An identical protected species of 180 nucleotides was identified in both RNA samples. This protection pattern does not prove that this is the 5' end of the BALF2 mRNA; it is possible that the protected fragment might not be the 5' end of the probe and that the probe protects a spliced mRNA. However, the presence of the TATA sequence strongly argues that the transcriptional start point for the BALF2 mRNA lies at about EBV genome number 164787.

These experiments used Akata cells which had been maintained to produce the lowest levels of productive cycle mRNAs in uninduced cells. Experiments using cells which induced more quickly demonstrated other aspects of early events in induction of the productive cycle. The results obtained are essentially similar, i.e. that activation of expression of the BZLF1 and the BRLF1+BZLF1 mRNAs is resistant to anisomycin while activation of expression of the BMLF1 and the BSLF2+BMLF1 mRNAs is completely sensitive. Activation of expression of the BZLF1 and the BRLF1+BZLF1 mRNAs occured within one hour of anti-Ig treatment while expression of the BMLF1 and BSLF2+BMLF1 mRNAs in uninhibited cells was activated four hours post-induction. Therefore activation of this promoter is delayed and presumably requires expression of BZLF1 and BRLF1 for its activation.

Analysis of the levels of the 1.0kb and 2.8kb mRNAs in uninhibited cells shows that both transcripts are downregulated at 20 hours post-induction. Interestingly the BZLF1 transcript is completely downregulated

while the BRLF1+BZLF1 transcript is partially downregulated. This differential downregulation may reflect a difference in stability between the transcripts or that their promoters are responding to different activities.

Therefore, the promoters for the BZLF1 and the BRLF1+BZLF1 transcripts are both directly sensitve to the changes in cell factors associated with induction. The activation of the promoters for BMLF1, BSLF2+BMLF1 and BALF2 are secondary events and they are probably activated by BZLF1 and BRLF1. There may be other promoters which respond directly to changes in cell factors.

These results are somewhat different to those previously reported for cycloheximide resistant genes in response to treatment of Akata cells with anti-IgG (Takada and Ono, 1989). Takada reported the activation of expression of five mRNAs (those for BZLF1, BRLF1+BZLF1, BMLF1, BSLF2+BMLF1 and BALF2) in cycloheximide treated cells. However, the cells were treated with cycloheximide for 4 hours, an incubation time that would result in only partial inhibition of protein synthesis. It is likely that expression of BZLF1 and/or BRLF1 because of the partial block accounts for the number of transcripts seen in apparently inhibited cells and does not truly show the induction of expression of any of the transcripts to be resistant to inhibitors of protein synthesis.

These experiments demonstrate that the switch from latency to productive cycle is, at least partly, brought about by changes in the activities of cell factors which do not have to be synthesised de novo. It is likely that the promoters for BALF2 and BMLF1 and BSLF2+BMLF1 are activated by BZLF1 and BRLF1 although cell factors synthesised in response to the inducing signal may also play a role in induction of the productive cycle. Autoactivation of the BZLF1 and BRLF1+BZLF1 promoters is also an important, though delayed event. Induction of the productive cycle in TPA induced Raji cells has been shown to require protein synthesis (Laux et al., 1988a) and therefore the two systems are mechanistically distinct.

The promoters for BZLF1 and BRLF1+BZLF1 can be activated in the absence of expression of other EBV productive cycle gene products. It may therefore be possible to activate these promoters in EBV-negative cells when they are activated in an equivalent manner, i.e. cross-linking of sIg. Future experiments will involve transient transfection of plasmid constructs containing the promoters for the 1.0kb and 2.8kb mRNAs fused

to the CAT reporter gene into EBV negative cell lines. The cells would then be treated with anti-Ig and cell extracts assayed for CAT expression. If the cells still respond to the inducing signal after transfection then CAT activity in these cells should increase following activation of the reporter constructs. If this system works then it may form a basis for a mutagenic study of the promoters to define sequence elements required for this response. This may help to define the molecular basis of reactivation.

Chapter 8
General Conclusions

BZLF1 is a sequence specific DNA binding protein capable of transactivating other EBV early genes and is thought to play a central role in the switch from latent to productive EB viral infection. Experiments presented in this thesis have investigated the structure and function of this important viral regulatory molecule. Homology between BZLF1 and the c-fos and C/EBP proteins has been identified (Farrell et al., 1989; Kouzarides et al., 1991) and experiments presented in this thesis extend the structural and functional similarities between these proteins.

BZLF1 has been demonstrated to have a DNA binding specificity which includes both consensus C/EBP binding sites and some but not all AP-1 (c-fos/c-jun) binding sites. Mutational analysis of a BZLF1 binding site has confirmed these observations. The structure of BZLF1 has been shown to be related to the c-fos and C/EBP proteins since it has a basic motif required for DNA binding but not for dimerisation and a directly adjacent coiled-coil structure required for dimerisation as well as DNA binding. The observation that BZLF1 can transactivate the promoter for the c-fos gene itself (Flemington and Speck, 1990d) further extends these functional relationships. So in BZLF1 EBV appears to have captured parts of a cell gene regulation pathway as part of its mechanism of reactivation from B-cells. The basic motifs of BZLF1 and c-fos are encoded with almost identically sized exons and this argues a common evolutionary origin for these proteins (Farrell et al., 1989). Furthermore, BZLF1 may interact directly with the gene regulatory pathways controlled by c-fos and C/EBP as part of its role in induction.

BZLF1 has been proposed to be a member of the bZIP family of DNA binding proteins (Chang et al., 1991; Kouzarides et al., 1991). Experiments presented here support this conclusion. Deletion analysis has demonstrated that BZLF1 possesses a bipartite DNA binding structure. The basic motif is homologous to other bZIP proteins (Farrell et al., 1989) and BZLF1 has been shown to possess a coiled-coil dimerisation domain analogous to the leucine zipper but without a heptad repeat of leucine residues (chapter 6; Kouzarides et al., 1991; Flemington and Speck, 1990a). Deletion analysis of BZLF1 identified sequences which lie outside of the basic motif and coiled-coil dimerisation domain which help to stabilise the DNA binding structure. Sequences which lie directly N-terminal of the

basic motif appear to be involved in stabilising the folding of the basic motif structure. Protein sequences which lie C-terminal of the dimerisation domain are required, under some conditions, to stabilise the dimerisation function. Such stabilisation functions have also been recognised in the c-fos and c-jun proteins (Cohen and Curran, 1990).

The possibility that BZLF1 might heterodimerise with other viral or cell proteins has been investigated extensively. Immunopreciptitation assays failed to demonstrate a direct interaction between BZLF1 and BRLF1, C/EBP, c-fos or c-jun. Furthermore, immunoprecipitation of BZLF1 from EBV positive cells that were induced to the productive cycle did not identify proteins interacting with BZLF1. Presumably BZLF1 does interact with other components of the transcription complex at a promoter (for example TFIID; pers. comm., P.Lieberman) but there is at present no evidence for such an interaction in the dimerisation domain. The apparent inability of BZLF1 to heterodimerise perhaps indicates that BZLF1 contains a coiled-coil dimerisation interface capable only of homodimerising. The lack of a heptad repeat of leucines in this structure may have some bearing on this observation.

The EBV positive BL line Akata has been used to analyse early events following induction of the productive cycle. Akata cells can be induced to enter the productive cycle when treated with anti-immunoglobulin and this system represents an attractive in vitro system for analysing reactivation. Experiments presented in this thesis analyse the levels and kinetics of appearance of productive cycle mRNAs and proteins following anti-immunoglobulin treatment in the presence or absence of an inhibitor of protein synthesis. Previous experiments have suggested that induction of the expression of five productive cycle mRNAs is resistant to protein synthesis inhibitors (Takada et al., 1989). Experiments presented here, where a complete inhibition of protein synthesis is achieved, show that induction of the expression of mRNAs for BZLF1 and BRLF+BZLF1 is resistant to inhibition of protein synthesis. Induction of the expression of mRNAs for BMLF1, BSLF2+BMLF1 and BALF2 is completely sensitive to this inhibition and their activation is clearly a secondary event.

These results indicate that the initial response of the virus to the induction signal can occur in the absence of protein synthesis. This is different to the situation in TPA induced Raji cells (Laux et al., 1988a) where induction of productive cycle gene expression has an absolute

requirement for protein synthesis and these two systems for studying EBV reactivation are therefore mechanistically distinct.

The promoters for the 1.0kb BZLF1 and the 2.8kb BRLF1+BZLF1 bicistronic mRNAs are directly sensitive to the changes in cell factors associated with induction. Future experiments will aim to identify sequence elements which confer anti-immunoglobulin inducibility in an attempt to understand regulation of these promoters. The differential downregulation of each promoter that is observed in some experiments perhaps shows that the two promoters can respond to different regulation. However, the overlapping arrangement of the genes and their coordinate induction in Akata cells imply that they can also be coregulated.

BZLF1 plays a central role in the switch from latent to productive viral infection and the results presented in this thesis increase our understanding of reactivation. In the cell line Akata, the virus enters the productive cycle by responding initially to changes in the activity of host cell factor(s). The promoters for the BRLF1+BZLF1 and BZLF1 mRNAs are directly sensitive to this change in activity. However, subsequent autoregulation of these promoters is clearly an important step in achieving the levels of BZLF1 and BRLF1 seen in cells induced to the productive cycle. The other productive cycle promoters respond directly or indirectly to BZLF1 and/or BRLF1 though it is possible that cell factors made de novo in response to induction of the productive cycle also play a role in transactivating viral promoters. BZLF1 appears to be related to a number of cell proteins, especially c-fos and C/EBP, and interaction with the gene regulation systems controlled by these transcription factors may be a central feature of BZLF1 activity. However, the identity of the cell factors which initiate these changes remains unknown. The characterisation of the two responsive viral promoters and the realisation that their initial activation is resistant to protein synthesis inhibitors will help in the identification of these factors.

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