

STRUCTURE AND FUNCTION
OF THE
EPSTEIN-BARR VIRUS LEADER PROTEIN

Thesis submitted by

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Abstract

The protein structure of EBNA-LP is highly repetitive as it is almost entirely encoded within the major internal repeat region (IR-1) of Epstein-Barr virus. The size of this region was determined in a wide variety of EBV strains by running large DNA fragments containing IR-1 on field inversion gels. The mean size of IR-1 corresponds to a repeat copy number of 6.6; the number of repeats in the B95-8 viral strain is 8.6. Comparison of IR-1 size reveals that the BamHI W/Y deletion in the P3HR-1 viral strain removes 6806 bp of sequence from the parental Jijoye virus. Change in repeat copy number is common during virus induction and reinfection, whereas the size of IR-1 in latently infected cells is relatively stable. There is no clear correlation between the number of repeats in IR-1 and the reported size of LP in the different viral strains.

EBNA-LP can be expressed from either the EBV W or C promoters. Analysis of LP cDNA molecules from the B95-8 viral strain has revealed that the formation of an initiator methionine codon for LP is dependent upon certain splicing events, irrespective of which promoter is active. Initiation of LP translation cannot occur from CUG codons that are present in the non-coding C2 exon. EBNA-LP alone is unable to transactivate the C promoter, but may cooperate with EBNA 2 in doing so.

EBNA-LP is implicated in the immortalisation potential of Epstein-Barr virus. LP is unable to cooperate with the ras oncogene, but it might be able to substitute for SV40 large T antigen to immortalise fibroblasts. There is no direct interaction between LP and the retinoblastoma gene product. An assay for EBNA-LP function

was set up that is based on the LP defective 633 EB virus. This assay compares the growth characteristics between EBV immortalised B cells containing either truncated or full-length LP. The results imply that EBNA-LP may have a role at the G1/S boundary of the cell cycle.

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Declaration

All the work presented in this thesis was performed entirely by myself and in no way forms part of any other thesis. The work was carried out while I was a Graduate 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 Robert Williamson (University of London), Dr Paul Farrell and Dr David Rowe (Ludwig Institute for Cancer Research).

A handwritten signature in cursive script, reading "G. J. Allan".

Gordon J. Allan

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Abbreviations

Arg:	Arginine
BL:	Burkitt's Lymphoma
CAT:	Chloramphenicol Acetyl Transferase
Cp:	C promoter
Cys:	Cysteine
DTT:	Dithiothreitol
EBER:	Epstein-Barr virus Encoded RNA.
EBNA:	Epstein-Barr virus Nuclear Antigen
EBV:	Epstein-Barr virus
EDTA:	Ethylenediaminetetra-acetic acid
FIGE:	Field Inversion Gel Electrophoresis
Hepes:	2-Hydroxyethylpiperazine-2-ethanesulphonic acid
HPV:	Human Papilloma Virus
IM:	Infectious Mononucleosis
IR-1:	Major Internal Repeat
LCL:	Lymphoblastoid Cell Line
LMP:	Latent Membrane Protein
LP:	Leader Protein
Met:	Methionine
MSH:	Mercaptoethanol
NPC:	Nasopharyngeal Carcinoma
ORF:	Open Reading Frame
PAGE:	Polyacrylamide Gel Electrophoresis
PMA:	Phorbol-13 myristate
Rb:	Retinoblastoma susceptibility gene product
TCA:	Trichloroacetic acid
TP:	Terminal Protein
TPA:	12-o-tetradecanoyl phorbol-13 acetate
Wp:	W promoter

Chapter 1

INTRODUCTION

1.1 The Herpesviridae

The members of the family Herpesviridae can be identified by the presence of a double-stranded linear DNA genome packaged within the core of the virion, an icosahedral capsid containing 162 capsomeres assembled in the cell nucleus and an envelope derived from the nuclear membrane (Roizman, 1982). Herpesviruses vary greatly in their biological properties, such as host range, genome size and speed of replication; but a property common to all is their capacity to remain latent in the host in which they infect. Of approximately 80 herpesviruses which have been partially characterised, seven are known to infect humans; herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpesvirus type 6 (HHV-6) and type 7 (HHV-7).

The herpesviruses have been classified into three subfamilies: alpha-, beta-, and gamma herpesviridae. In vitro, all members of the latter subfamily replicate in lymphoid cells, and some also cause lytic infections in some types of epithelial and fibroblast cells. Epstein-Barr virus is the prototype of the gamma herpesviruses. The other well studied gamma herpesviruses are herpesvirus saimiri (HVS) and the Marek's Disease Virus (MDV), which are both T-lymotropic. HVS is not pathogenic in its natural host, which is the South American squirrel monkey. However, it has been shown to be extremely oncogenic in a number of other primate species that are presumably not infected in the wild (reviewed by Roizman, 1982). MDV infects T cells in the chicken (reviewed by Nonoyama, 1982), and Marek's

disease is characterised principally by T cell lymphoma formation. This was the first neoplastic disease shown to be caused by a herpesvirus, and it is also the only common naturally occurring lymphoma to be controlled by vaccination.

1.2 The Epstein-Barr Virus

Epstein-Barr virus (EBV) was first identified in a B-lymphocyte cell line, EB1, which had been established from Burkitt's lymphoma (BL) tumour biopsy material (Epstein and Barr, 1964; Epstein et al., 1964). Electron microscopy revealed virus particles with typical herpesvirus morphology, and further analysis demonstrated that this was a previously uncharacterised human herpesvirus. It is now known that the great majority of people in the world are infected with EBV (reviewed by Henle and Henle, 1979a).

a) The virus particle

The virus particle is 120nm in diameter and is composed of an outer membrane enclosing an icosahedral nucleocapsid (reviewed by Epstein and Achong, 1979). The outer membrane contains three glycoproteins (gp) of 350, 220 and 85 kDa in size, as well as a 140 kDa non-glycosylated protein (Qualtiere and Pearson, 1979; Thorley-Lawson and Edson, 1979; North et al., 1980). These four proteins are collectively referred to as Membrane Antigen (MA). Gp350 and gp220 are encoded by the same gene, (Hummel et al., 1984; Beisel et al., 1985; Biggin et al., 1985; Tosoni-Pittoni et al., 1989). These glycoproteins are expressed in late lytic infection and added to the virus particle as it is in transit through the cytoplasm (Gong and Kieff, 1990). Antibodies against these membrane antigens are capable of neutralising the infectivity of the virus (Hoffman et al., 1980; Thorley-Lawson and Geilinger, 1980; Thorley-Lawson and Poodry, 1982).

The nucleocapsid contains at least 33 proteins (Dolyniuk et al., 1976 a,b) most of which remain to be fully characterised. However, the major protein in the nucleocapsid is a 160 kDa polypeptide which is a component of the viral capsid antigen (VCA) (Vromen et al., 1985). The viral genome is packaged within the core of the virion.

b) The viral genome

The viral genome (reviewed by Farrell, 1989) is a double-stranded DNA molecule of about 170 Kbp in length. The genome is linear in the virus particle, but circularises inside the infected cell via joining of the terminal repeat sequences. The viral episome is then amplified by cellular DNA polymerase (Sixbey and Pagano, 1985). In Namalwa cells the viral DNA is integrated into the cellular chromosomes (Henderson et al., 1983; Matsuo et al., 1984) and some degree of integration may occur in many EBV infected lines.

The molecule is conventionally subdivided into four regions; the short and long unique regions, separated by the major internal repeat and the terminal repeat (Fig 1.1). Transcription units span all of the unique regions and the major internal repeat, as well as the terminal repeat of the episomal genome. There is only one relative arrangement of the unique and repetitive regions in EBV as compared with the four isomeric forms of HSV.

The EBV genome is too large to be cloned as a single piece in bacterial cloning vectors, but several groups have constructed libraries in phage λ , cosmid or plasmid vectors (Dambaugh et al., 1980; Skare and Stromenger, 1980; Arrand et al., 1981; Buell et al., 1981; Polack et al., 1984). The complete sequence of the B95-8 viral genome has been obtained using over 6,600 M13 clones and this has provided an excellent resource for further EBV research. The

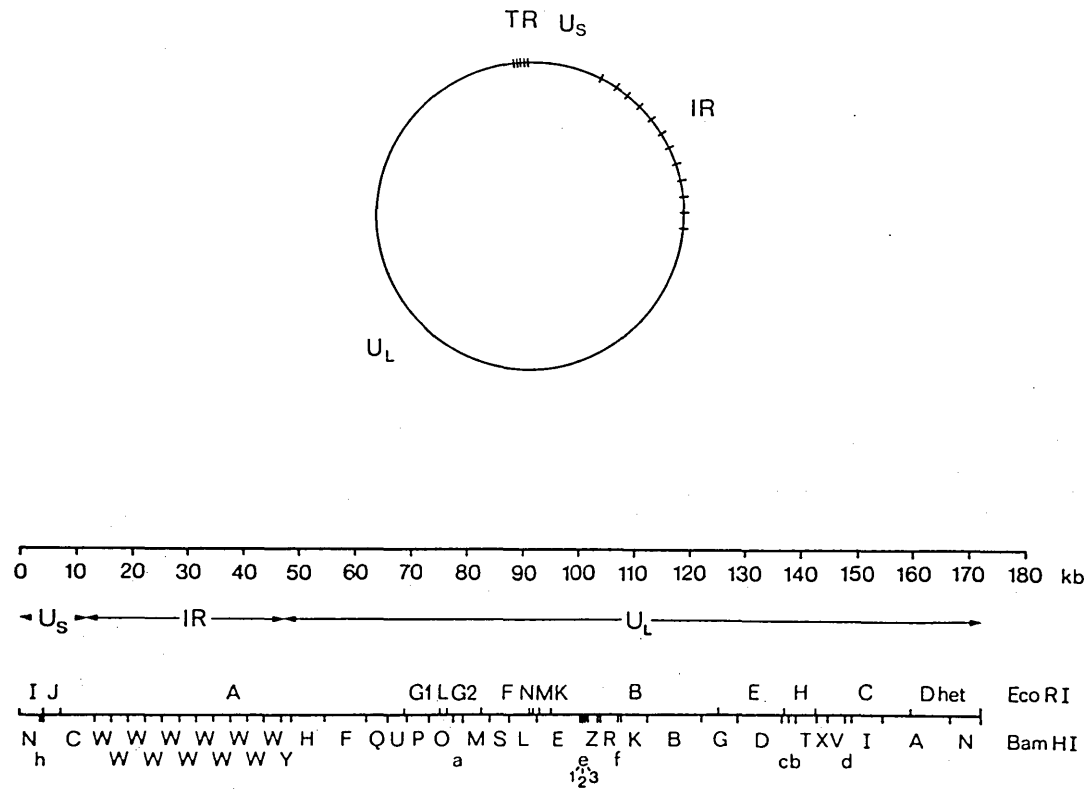


Figure 1.1 The standard circular and linear representation of the B95-8 Epstein-Barr virus genome are illustrated; the short unique (U_S), long unique (U_L), major internal repeat (IR-1), and terminal repeat (TR) are indicated. A restriction map for the enzymes EcoRI and BamHI is also shown. Principal open reading frames of EBV are named according to the BamHI fragment in which they begin, followed by L or R indicating whether they are to the left or right on the standard map and then a number.

sequencing revealed an excess of G and C content (59.9% GC) in the genome (Baer et al., 1984), confirming previous work which had determined the buoyant density of EBV DNA in CsCl (Schulte-Holthausen and zur Hausen, 1970).

Restriction maps are broadly similar throughout all EBV strains and when equivalent regions of B95-8 and Raji EBV were sequenced, they were found to be very similar at the nucleotide level (Hatfull et al., 1988). Significant variation has been reported in certain genes. In particular, two DNA hybridisation types (A and B) of the EBNA 2 gene (Dambaugh et al., 1984; Adldinger et al., 1985) have been identified and this results in recognition of two distinct serotypes (X and J) (Dambaugh et al., 1984; Rowe, D.T. et al., 1985; Rowe, M. et al., 1985). Since this discovery, EBV isolates can be broadly classed as A or B type depending on their EBNA 2 sequences. These types have also been called 1 and 2. The common and distinct epitopes on the EBNA 2A and 2B have been more closely identified (Rowe and Clarke, 1989) and it is now established that the proteins cause different degrees of induction of cell surface markers and induce different growth patterns in the cells (Dambaugh et al., 1986; Rickinson et al., 1987; Wang et al., 1987). Distinction between type A and B virus has now been extended to the EBNA 3 family (Rowe et al., 1989; Sculley et al., 1989), the EBER sequences (Arrand et al., 1989), and the 3' sequences of EBNA LP (Huen et al., 1988).

Some common EBV laboratory strains have substantial deletions with respect to each other. B95-8 has a 13.6 kbp deletion in the EcoRI C region, which has no apparent biological effect (Raab-Traub et al., 1980; Skare et al., 1982; Baer et al., 1984; Parker et al., 1990). Both Daudi and P3HR-1 harbour a roughly similar deletion in the BamHI W/Y region, and it has now been verified that it is this deletion

which renders P3HR-1 viruses non-immortalising (Bornkamm et al., 1982; Jones et al., 1984; Allan and Rowe, 1989; Hammerschmidt and Sugden, 1989). Raji virus contains two deletions relative to most other strains, and this virus is defective in part of the replicative cycle of EBV (Polack et al., 1984).

c) Cell Tropism

EBV is known to infect both B-lymphocytes and certain epithelial cells in the oropharynx and cervix, although only the former are readily infected in vitro (Jondal and Klein, 1973; Sixbey et al., 1986). Until recently, there was no simple epithelial system in which to study virus biology, and, therefore, much of our knowledge of EBV comes from studying infected lymphocyte lines. The EBV receptor on B-cells has been shown to be Cluster Determinant 21, (CD21), which is also the receptor for the C3d component of complement (complement receptor 2, CR2) (Fingerth et al., 1984; Frade et al., 1985; Nemerow et al., 1985; Weis et al., 1988). The presence of this 145 kDa surface glycoprotein on a cell confers EBV infectibility, as it binds to gp350/220 in the outer membrane of the EBV particle. CD21 has been shown to have a common domain for binding both gp350/220 and the C3d fragment (Lowell et al., 1989). Also, there are two regions of amino acid homology between C3d and gp350/220, although it appears that it is only the N-terminal portion of gp350/220 which binds to CD21 and thus mediates virus infection (Nemerow et al., 1989). Following complexing of these glycoproteins, the virus enters the cell via receptor-mediated endocytosis (Tanner et al., 1987). Earlier it was established that antibodies against either gp350/220 or CD21 were capable of neutralising the infectivity of the virus and inhibiting viral release (Hoffman et al., 1980; Thorley-Lawson and Geilinger, 1980; Thorley-Lawson and Poodry, 1982; Sairenji et al., 1988). More recently, it was found that both soluble CD21 and soluble

gp350/220 or only an N-terminal peptide of gp350/220, could block nearly 100% of EBV infection in vitro (Tanner et al., 1988; Nemerow et al., 1989; Nemerow et al., 1990). Clearly, this research has important implications for the development of an EBV vaccine (Emini et al., 1988; Morgan et al., 1988; Morgan et al., 1989). Once the virus has infected B-cells it usually remains latent, expresses only a limited number of genes, and does not replicate until induced to do so by unknown factors in vivo or by various agents in vitro.

Monoclonal antibodies against different determinants on the 145 kDa EBV/C3d receptor of B-cells were shown to also react with cervical epithelia (Young et al., 1986). The antigen on the epithelial cells is a 200 kDa protein, but polymorphic variations in the CD21 coding sequence have been described and these may result in the generation of distinct forms of CD21 on different cell types (Toothaker et al., 1989), including a receptor on T-lymphocytes (Fingerroth et al., 1988). Further research has shown unequivocally that EBV can infect and replicate in epithelial cells in vivo (Young and Sixbey, 1988). But, how the virus enters such cells and the possible involvement of the 200 kDa protein as a virus receptor has yet to be elucidated.

Several observations suggest that EBV infection of epithelia may be central both to the events of primary infection and to the mechanism of virus persistence in the host (Wolf et al., 1984; Rickinson et al., 1985). In this model, infection of receptor-positive epithelium, followed by virus replication in progeny cells as they approach terminal differentiation could produce a self-sustaining reservoir of infection in the pharynx. Secondary infection of B-cells in transit through that reservoir would then serve to maintain the apparent virus carrier status of these lymphocytes. More recent evidence, however, shows that the level of virus in the blood of EBV positive

individuals is stably maintained for at least 4 weeks despite acyclovir mediated inhibition of virus replication in the oropharynx (Yao et al., 1989). The bone marrow is directly implicated as a virus reservoir, following a report of a seropositive patient who became seronegative following a bone marrow transplant, and then seroconverted again for a different subtype of EBV (Gratama et al., 1988; Gratama et al., 1989). The transit of virus between the epithelium and lymphocytes remains a poorly understood phenomenon.

1.3 Immortalisation by EBV and Associated Diseases

a) EBV immortalisation of B cells

EBV has been implicated in the development of several types of cell proliferative disorders in humans. The virus is very efficient at immortalising B-lymphocytes in vitro, where infection ultimately results in cells having the capacity for indefinite proliferation.

Virus binding and internalisation per se is sufficient for an initial activation of the B-cells out of G_0 into the G_1 phase of the cell cycle (Gordon et al., 1986). After exit from G_0 , cellular RNA synthesis starts and viral episomes are replicated in unison with the cellular DNA for 1 to 2 weeks (Hurley and Thorley-Lawson, 1988). Within 24 hours after infection, the first EBV Nuclear Antigens, EBNA 2 and multiple species of EBNA-LP, can be detected (Kallin et al., 1986; Moss et al., 1986; Dillner et al., 1986; Finke et al., 1987; Rooney et al., 1989a). After about 48 hours the other EBNA's are being expressed, and these are followed by the EBV latent membrane protein (LMP), which is synthesised at 48-72 hours post infection, when the cells undergo blast transformation and division (Robson and Smith, 1981; Mann et al., 1985). After 3-4 days, small foci of

EBV-transformed cells are visible microscopically and these expand to form EBV-positive lymphoblastoid cell lines (LCL), which are immortalised.

Activation of cellular genes by EBV-infection may also contribute to the immortalisation process. CD23 is one of the earliest B-cell activation antigens induced upon infection by transforming strains of EBV (Kinter and Sugden, 1981; Thorley-Lawson and Mann, 1985; Calender et al., 1987). The appearance of this 45 kDa protein on the cell surface is normally a prerequisite for immortalisation (Thorley-Lawson and Mann, 1985), and this can also be induced by the addition of B-cell growth factor (Azim et al., 1990). The membrane bound form of CD23 can function as a low affinity IgE Fc receptor (Yukawa et al., 1987), but it can also be cleaved to give a soluble form, s-CD23, which acts as an autocrine growth factor and enables the EBV infected cells to continue to proliferate (Swendeman and Thorley-Lawson, 1987). It is now established that CD23 is cooperatively induced by two viral latent cycle genes, EBNA 2 and LMP (Wang et al., 1987; Wang et al., 1990). The cellular proto-oncogenes c-fgr (Cheah et al., 1986) and c-myc (Lacy et al., 1987; Wennberg et al., 1987) have also been reported to be expressed at elevated levels in EBV-infected cells (deregulation of c-myc will be discussed later in relation to Burkitt's lymphoma). The elevated levels of c-fgr in some cell lines from human myelo- or lympho-proliferative diseases correlate with the presence or absence of EBV, and in vitro infection of EBV-negative cell lines results in a 50-fold increase in the steady state level of both the c-fgr transcript (Cheah et al., 1986) and protein (Katamine et al., 1988). It now appears that this elevation requires the expression of the viral EBNA 2 gene (Knutson, 1990), although the precise mechanism by which this induction takes place is not known. The c-fgr gene is the cellular homologue of the

oncogene of the Gardner-Rasheed feline sarcoma virus (Tronick et al., 1985; Nishizawa et al., 1986). It is related in sequence to c-src and c-yes and its product has a tyrosine kinase activity (Inoue et al., 1987; Katamine et al., 1988). Although its function is unknown, proteins with tyrosine kinase activity often play important roles in the regulation of cell growth, and their over expression can result in cellular transformation and proliferation.

b) Control of EBV-infected cells by immunosurveillance

In a normal EBV-infected host it is estimated that less than 1 in 10^5 - 10^6 of circulating B-cells contain EBV (Rocchi et al., 1977). This means that EBV infection in the majority of cases is asymptomatic or is unrecognised. Antibodies against the viral antigens EA, MA and VCA are produced during primary infection (reviewed by Rickinson et al., 1985; Rickinson, 1986). With the exception of anti-EA, antibodies against these antigens and those specific for the EBV nuclear antigens (EBNAs) are maintained for life (Henle and Henle, 1979a). Cell mediated responses are required to prevent the outgrowth of EBV immortalised B cells in vivo. Major histocompatibility complex (MHC) restricted cytotoxic T-lymphocytes (CTL) develop (Rickinson et al., 1979; Chen et al., 1989a) that recognise antigens on EBV-infected cells which have been termed Lymphocyte Detected Membrane Antigen, or LYDMA (Svedmyr and Jondal, 1975). LYDMA does not correspond to any single EBV gene product, but is a strictly functional term (Rowe et al., 1986). However, cytotoxic T-cells do recognise epitopes derived from certain latent gene products such as the latent membrane protein (Sulitzeanu et al., 1986; Thorley-Lawson and Israelsohn, 1987; Reiss et al., 1987; Murray et al., 1988a). It was shown that cytotoxic T-cells could discriminate between A- and B-type EBV transformed lines, and it was originally believed that this must be due to different epitopes on

the two EBNA 2 types (Wallace et al., 1987; Moss et al., 1988). This conclusion was premature since it is now known that the type variation can be extended to other latent cycle products, which may also be involved in the A and B type specific T-cell response. Cytotoxic T cell clones have been identified which specifically recognise EBNA 2 and EBNA 3A from A type virus only (Murray et al., 1990; Burrows et al., 1990). Perturbation of the cellular immune system, as seen in transplant recipients on immunosuppressive therapy or in AIDS patients, can result in the proliferation of immortalised B-cells.

c) Infectious Mononucleosis

EBV has been shown to be the causative agent of infectious mononucleosis (IM) (Henle et al., 1968; reviewed by Sumaya, 1989). Most EBV carriers periodically shed virus particles into the buccal cavity (Yao et al., 1985), so that the observed horizontal transmission of the virus is most likely a consequence of close salivary contact. Primary infection usually occurs in childhood, but where it is delayed until adolescence or later, infectious mononucleosis commonly results (reviewed by Henle and Henle, 1979b).

There is a far greater proportion of circulating B-cells infected with EBV in an IM patient than in the normal individual (Robinson et al., 1980). This elicits an abnormally vigorous cytotoxic T-cell response, which, during the acute phase of the disease, results in an approximately 2-fold increase in the number of leukocytes in the blood. The disease typically presents with sore throat, fever and lymphadenopathy; the latter two being the basis for its more commonly known name, Glandular Fever. The symptoms usually last for 1-3 weeks, but in older patients the illness may be chronic, lasting for several months or longer (Sumaya, 1988). IM is most prevalent in the

developed countries, particularly in the upper socio-economic groups. It is essentially unheard of in countries like Africa, where primary infection almost always occurs before the age of 4 years.

d) Burkitt's Lymphoma

Burkitt's lymphoma (BL) was first identified in the late 1950's in Uganda (Burkitt, 1958; reviewed by Ernberg and Kallin, 1984; Richtsmeier, 1987). BL is an undifferentiated B-cell lymphoma, which occurs sporadically worldwide, but has a much higher incidence in central Africa. It is the commonest cancer of African children (Magrath, 1983) occurring in 5 to 15 children/100,000/year and the incidence is 20- to 100-fold lower in western countries (de The, 1985).

Epstein-Barr virus is implicated in the development of endemic BL, as the great majority (>95%) of patients have EBV DNA and nuclear antigens (EBNA) within the tumour cells (zur Hausen et al., 1970; Lindahl et al., 1974). EBV-infected cells from a normal individual show sufficient heterogeneity in their EBV termini to allow use of terminal fragment size as a clonal marker (Brown et al., 1988). Analysis of cellular immunoglobulin rearrangements and terminal repeat copy number of resident EB virus has demonstrated that BL is a monoclonal cancer with respect to the virus and the cell (Preud'homme et al., 1985; Raab-Traub and Flynn, 1986; Brown et al., 1988). There is, however, the possibility that the passage of BL cells in tissue culture may clone the virus. The clonality of the cell and virus is taken to imply that, in EBV positive tumours, the virus was present in the original cell that underwent malignant transformation. The hypothesis is that, as less than 1% of circulating B cells contain virus, but over 95% of endemic BL tumours are EBV positive, then the

chances of malignant transformation must be much greater in EBV infected cells.

It is important to differentiate here between endemic and sporadic BL. Sporadic BL occurs in low incidence areas such as Europe and the United States, and viral markers are not present in up to 85% of these tumours (Lenoir et al., 1984). In fact, the incidence of EBV negative BL in equatorial Africa is very like the overall incidence in Europe and North America, suggesting that sporadic cases occur at a similar frequency throughout the world.

A common feature in all BL cells is the presence of a reciprocal translocation between chromosomes 8 and 14 or, less frequently, between 8 and 2 or between 8 and 22 (reviewed by Lenoir, 1987). These translocations always involve the c-myc proto-oncogene on chromosome 8 (Dalla-Favera et al., 1982) and the various immunoglobulin loci on the other chromosomes. In the case of the most common, 8:14 translocation, c-myc moves from chromosome 8 to chromosome 14. The translocation fuses the c-myc gene to an Ig heavy-chain constant gene on chromosome 14. In the other types, c-myc remains on chromosome 8 and Ig light-chain loci move to there from chromosomes 2 or 22, so that c-myc is again located 5' of an Ig constant region. In all cases, the breakpoints on the Ig genes are usually located at or close to sites that are used for physiological Ig rearrangement (Cory et al., 1986).

The c-myc gene was originally defined as the cellular homologue of the gene transduced by avian myelocytomatosis virus (Sheiness and Bishop, 1979). Transcriptional expression of c-myc is controlled with respect to the cell cycle in many cell types (Kelly and Siebenlist, 1986), including normal human B cells (Lacy et al., 1986). c-myc RNA

and c-myc protein are extremely short lived (15-30 min) (Dani et al., 1984; Ramsay et al., 1984). The c-myc protein is a 60 kDa nuclear protein which is believed to be involved in regulating cell cycling (Kelly et al., 1983; Campisi et al., 1984; Kelly and Siebenlist, 1986; Studzinski et al., 1986; Iguchi-Arigo, 1987). Cells from a wide range of species produce two myc proteins and it has been shown that these are derived from alternative translational initiations in the same reading frame in exons 1 and 2 (Hann et al., 1988). The ways in which the aforementioned translocations may contribute to the deregulation or activation of c-myc expression are as follows; loss of 5' regulatory sequences that removes transcriptional feedback control (Battey et al., 1983; Adams et al., 1983); suppression of the larger myc protein due to the loss of exon 1 (Hann et al., 1988) and a possible corresponding increase in the expression of the smaller protein due to the removal of a block to transcriptional elongation (Bentley and Groudine, 1986); enhanced transcription due to close proximity to Ig regulatory and enhancer sequences (Hayday et al., 1984), increased stability of c-myc RNA due to structural alteration of the truncated message (Rabbitts et al., 1985), alteration of the c-myc protein by somatic mutation (Rabbitts et al., 1983), or a combination of all of these. It is also conceivable that EBV may contribute to altered c-myc expression in BL cells. There is no conclusive evidence whether the chromosomal translocation occurs before or after infection with EBV (reviewed by Lenoir and Bornkamm, 1987; Klein, 1987), but EBV cannot be the direct cause of the translocations, because of the existence of EBV negative BLs. The virus can cause deregulation of c-myc in human B cells even in the absence of a chromosomal translocation (Lacy et al., 1987; Wennberg et al., 1987). This may be a result of a post-transcriptional increase in stability of mature c-myc mRNA in the presence of the virus (Lacy et al., 1989). In BL there is an alteration in

utilisation of the two c-myc promoters, with the first one being favoured in tumour cells (Taub et al., 1984; Yang et al., 1985). More recently, it has been shown that the EBV early gene product, BRLF1, can either directly or indirectly transactivate the P1 c-myc promoter (pers. comm. Shannon Kenney).

The geographical distribution of endemic BL is closely linked to the incidence of infection by Plasmodium falciparum, the malarial parasite of humans. Malarial infections have long been recognised as having profound effects on general immune function, and it is now clear that, during the acute phase of malaria, cytotoxic T cell control of EBV-infected B cells is impaired (Whittle et al., 1984). The immune challenge against the disease will result in a proliferation of B cells and, because of the impairment to T cell control, a proportion of these will be immortalised by EBV. A greater number of immortalised B cells may then increase the possibility of the translocations taking place (reviewed by Klein, 1987).

BL tumours have now been shown to have a restricted pattern of EBV latent gene expression, where LMP and EBNA 2 are frequently not expressed (Rowe, D. et al., 1986; Rowe, M. et al., 1987). There is also an altered surface phenotype compared to in vitro EBV immortalised B cells, with expression of BLA and CALLA (Rooney et al., 1986) and down regulation of cell adhesion molecules LFA-1 and ICAM-1 (Gregory et al., 1988). These effects underlie the ability of the malignant clone to evade EBV-specific T cell surveillance in vivo. Further research is required to understand how Epstein-Barr virus may be involved in the development of Burkitt's lymphoma.

e) Immunosuppression and Lymphoma

It is apparent that normal cytotoxic T cell suppression of EBV infected B cells is impaired in people who are immunosuppressed. This applies not only to children suffering from acute malaria, but also to transplant patients on immunosuppressive therapy and AIDS patients. Patients receiving immunosuppressive drugs in order to lower the risk of organ rejection have a 100-fold increased risk of developing cancer, and particularly lymphomas (reviewed by Cleary et al., 1986). These lymphomas are predominantly high grade B cell neoplasms classified as diffuse large-cell lymphomas, and they usually contain EBV DNA and EBNA (Crawford et al., 1980; Hanto et al., 1981; Saemundsen et al., 1982). Very similar polyclonal proliferations occur in AIDS patients (Kalter et al., 1985), and these people also have a high incidence of EBV positive BL-like lymphomas (Ziegler et al., 1982; Ahmed et al., 1987; Hamilton-Dutoit et al., 1989; Thomas and Crawford, 1989). Burkitt's lymphomas usually occur in those patients who have previously had persistent generalised lymphadenopathy and in whom the immune dysfunction is less severe (Kalter et al., 1985). AIDS-associated BLs are also aetiologically distinct from the endemic cases found in equatorial Africa, and it may be that, in the geographical zones where the traditional "BL belt of Africa" overlaps with areas of high seroincidence for HIV, there will be a move away from the childhood predominance of endemic BL towards AIDS-associated BL in adults. However, this observation may be confounded by other factors, such as high mortality of infantile AIDS and advanced Kaposi's sarcoma affecting the overall presentation and survival of AIDS cases in Africa.

f) Nasopharyngeal carcinoma

Undifferentiated nasopharyngeal carcinoma (NPC) is an epithelial tumour, which is also associated with Epstein-Barr virus infection (reviewed by Richtsmeier et al., 1987; Tarr and Glaser, 1989). Like Burkitt's lymphoma, NPC has a distinctive geographical distribution, being predominant in South-East Asia where it is the most common cancer in young to middle-aged men (17 per 100,000 per year in Shanghai; 84 per 100,000 per year in Hong Kong).

EBV has been closely associated with NPC by two main lines of evidence. NPC patients have unusual antibody patterns against several EBV encoded antigens (Henle et al., 1973), which characteristically include those of the IgA class (Henle and Henle, 1976). There are high antibody titres (IgA and IgG) against EBV EA, VCA and MA, and antibodies against the virus encoded enzymes, DNase and DNA polymerase (Liu et al., 1989). Secondly, undifferentiated NPC tumour cells invariably contain EBV DNA (zur Hausen et al., 1970; Wolf et al., 1973; Klein et al., 1974), whereas cells from NPC with squamous cell differentiation, seldom do (Andersson-Anvet et al., 1977). In addition, the EBV receptor, CD21, has been reported to be present on the surface of EBV positive cells derived from NPC (Takimoto and Umeda, 1989), while medium taken from NPC cultures has also been shown to contain the B cell activation marker CD23 (Billaud et al., 1989).

Analysis of NPC tumour biopsies and a nude mouse-passaged NPC cell line has revealed a novel type of EBV-cell interaction in NPC cells which is distinct from that seen in in vitro transformed B cell lines and in EBV-positive B cell lymphomas. Only one of the Epstein-Barr virus nuclear antigens, EBNA 1, appears to be expressed (Fahraeus et al., 1988; Young et al., 1988; Hitt et al., 1989). There is also

considerable variation in the degree of Latent Membrane Protein (LMP) expression in different tumour biopsies (Fahraeus et al., 1988; Young et al., 1988). Until recently, the study of the relationship of EBV to NPC, and the process whereby epithelial cells are infected and transformed has been hampered by the lack of an appropriate in vitro model. Transfection of immortalised, non-tumorigenic, human, keratinocyte cell lines with LMP can result in a change in surface phenotype and differentiation responsiveness (Dawson et al., 1990), as well as altered morphology and the down regulation of keratin expression (Fahraeus et al., 1990). Taken together, these results suggest that LMP expression may be an important causal factor in the development of NPC. Transfection of certain EBV sequences into primate kidney epithelial cells has been reported to result in immortalisation, and it was inferred that this may be relevant to NPC (Griffin and Karran, 1984). What is surprising about these results is that the sequences in question do not encode any of the identified latent cycle products of EBV. This would suggest a mechanism for immortalisation of epithelial cells by the virus different from that thought to act in lymphocytes.

Environmental and genetic factors also seem to play a role in the pathogenesis of NPC. Traditional life styles, especially consumption of Cantonese-style salted fish and other preserved foods, may be an important factor in the development of NPC. These food products contain relatively high levels of volatile N-nitrosamines (Poirier et al., 1987), and some have a strong EBV activation activity (Shao et al., 1988). Chinese medicinal herbs and plants contain naturally occurring phorbol esters (Duke and Ayensu, 1985), as well as other tumour-promoting agents capable of activating EBV replication (Zeng et al., 1983; reviewed by Ito, 1986).

It was known previously that possession of certain HLA phenotypes (A2 and BW46) could predispose people towards the development of NPC (Chan et al., 1983). Recent linkage analysis supports the idea of a disease susceptibility gene (or genes) closely linked to the HLA locus, which confers a greatly increased risk of nasopharyngeal carcinoma (Sheng-jing et al., 1990). The identification of this gene(s) may help to clarify the respective role of EBV and environmental co-factors in the development of NPC.

1.4 EBV Gene Expression

The infection of appropriate B lymphocytes by EBV normally results in a latent (non-productive) infection (Hurley and Thorley-Lawson, 1988). In this case EBV immortalises the host lymphocyte (Sugden, 1989; Klein, 1989), and the latent genes that are expressed in this process will be discussed later. Latently infected lymphocytes can often be induced at a rather low efficiency to the productive cycle by treatment of the cells with various chemical or biological inducers.

1.4.1 The Switch between Latency and Virus Replication

The mechanism(s) whereby viral latency is disrupted and viral replication occurs is not known. The most consistently effective inducers of replicative infection are a tumour-promoting phorbol ester, 12-o-tetradecanoyl phorbol-13 acetate (TPA) (also known as phorbol-13 myristate, PMA), sodium butyrate, iodo deoxyuridine and TGF β (zur Hausen et al., 1978; Kallin et al., 1979; Bauer et al.,

1980). There is continuous maturation of cells within a lymphoblastoid line, where the more differentiated cells, which are in the minority, no longer express EBNA. This loss of the immortalised status of the cell would allow completion of the lytic cycle with the production of virus particles and cell death (Crawford and Ando, 1986; Wendel-Hansen et al., 1987). Therefore, the aforementioned inducers of replicative infection may operate via their ability to promote cell differentiation. Many other chemicals, including inhibitors of cellular DNA, RNA and protein synthesis, hormones, cyclic nucleotides and antibody against IgM on the cell surface, have some inducing activity (Gerber, 1972; Hamper et al., 1972, 1974, 1976; Joncas et al., 1973; Tovey et al., 1978; Takada, 1984). As some of these agents are growth inhibitory, it may be that they promote an unidentified cell signal(s) when the long-term viability of the host cell is threatened, that tells the virus to replicate. It is important to note that these agents, when combined, exhibit considerable synergy. This may suggest multiple points at which viral replication can be induced (Bauer, 1983).

Superinfection of some latently infected cultures, including Raji, with the virus produced by the P3HR-1 cell line results in a more permissive state of viral infection than can be induced by TPA (Yajima and Nonoyama, 1976). P3HR-1 virus harbours a defective form of EBV which is a rearranged, partially deleted version of the EBV genome that has remained infectious (Heller et al., 1981; Cho et al., 1984; Miller et al., 1984). Comparison of P3HR-1 clones which did or did not produce defective virus (called het virus, for heterogeneous DNA) demonstrated that it was the het virus that was critical for induction during a superinfection (Rabson et al., 1983; Miller et al., 1984). More detailed analysis of het virus activity revealed that a single small DNA region of about 3kb was sufficient when

transfected to induce early antigen expression in an otherwise latent recipient cell (Countryman and Miller, 1985). This region contains the BZLF1 reading frame and it was finally established that it was this gene in the het virus which causes the induction of the productive cycle (Countryman et al., 1987). The protein product of BZLF1 is also known as EB1 (Chevallier-Greco et al., 1986) and ZEBRA (Countryman and Miller, 1985). It is now known that the BZLF1 protein is a sequence specific DNA binding protein (Farrell et al., 1989) that can act as a transcription factor to upregulate other early EBV genes, including BRLF1 and BMLF1 (Chevallier-Greco, et al., 1986; Rooney et al., 1989b; Packham et al., 1990). The protein has a sequence homologous to the basic DNA binding motif found in many DNA binding proteins of the bZip family (Packham et al., 1990). Two other early productive cycle genes, BRLF1 and BMLF1, are thought to have important roles in the switch from latency to virus replication. The BRLF1 protein was shown to be a powerful transactivator of transcription (Hardwick et al., 1988), whereas, in experiments using the CAT reporter gene, the BMLF1 protein appears to stabilize CAT mRNA or promote translation of CAT, rather than have a direct effect on transcription (Kenney et al., 1989; Buisson et al., 1989). It is thought that BRLF1 and BMLF1 may be involved in the activation of the EBV lytic cycle by cooperating with BZLF1 to switch on other early genes.

1.4.2 The Replicative Cycle of EBV

When EBV is induced to enter the replicative cycle, the majority of the 80 or so viral genes are expressed (reviewed by Farrell, 1989). The identity and function of the majority of the replicative cycle proteins is not known, but some have been identified through their homology with genes encoded by other herpesviruses. Gene expression

during viral replication has been divided into two temporal classes, early and late, and these are separated by viral lytic DNA synthesis.

a) Early Gene Expression

The viral proteins expressed in the early phase of the lytic cycle have been mapped (Farrell, 1989). Originally, many of these were grouped together as members of the early antigen (EA) complex, which can be visualised by immunofluorescence (Henle et al., 1970). The appearance of EA in a cell indicates that that cell has entered the replicative phase of the viral cycle.

Early EBV gene expression is not dependent on viral DNA synthesis, which it precedes. Many of the proteins produced during this phase are thought to be involved in replicative EBV DNA synthesis. Some of the early genes which have been identified either functionally or through homology with other herpesviruses are: DNA polymerase (BALF5) (Baer et al., 1984), major DNA binding protein (BALF2) (Quinn and McGeoch, 1985), large and small sub-units of ribonucleotide reductase (BORF2 and BARF1 respectively) (Gibson et al., 1984), thymidine kinase (BXLF1) (Littler et al., 1986), DNase (BGLF5) (Zhang et al., 1987), dUTPase (BLLF2) (Davison and Taylor, 1987). Several other EBV genes have homologues in HSV and VZV, but their function has yet to be identified (Davison and Taylor, 1987; McGeoch et al., 1988).

b) The Replication of DNA in the Lytic Cycle

Since viral DNA in latently infected cells is in a closed circular form prior to induction, replication must proceed from this as an initial substrate. For some time it was not known whether the DNA could replicate as a circular molecule or go through a linear intermediate. The products of replication from the EBV origin of plasmid replication, oriP (discussed later), are circular plasmid

molecules identical to the template DNA. However, it was shown that oriP does not mediate the amplification of viral DNA during the lytic phase of the EBV life cycle (Yates and Camiolo, 1988), but rather maintains a stable number of plasmid molecules per cell in a dividing cell population. In contrast, Herpes simplex viruses, type 1 and 2, had been shown to amplify their template DNA 100-1000 times within one cell cycle via a mechanism of rolling circle replication. The product of this replication is a head-to-tail concatemer which is synthesised by a viral DNA polymerase (Roizman and Batterson, 1985).

Two origins of lytic replication (oriLyt) have been identified. One is between bases 52642 and 53697 in the B95-8 genome about 40 Kbp away from oriP (Hammerschmidt and Sugden, 1988). The other is deleted from the right hand end of B95-8, but is present in other EBV strains such as Raji and M-ABA (between bases 3554 and 4609 of the 12kbp deletion in B95-8) (Hammerschmidt and Sugden, 1988; Parker et al., 1990). Unlike the lytic origins of HSV, oriLyt is complex and has several regions that are required for its complete function. OriLyt overlaps an efficient promoter/enhancer that functions during the lytic phase of the EBV life cycle and which had previously been shown to be transactivated by the BZLF1 gene product (Chevallier-Greco et al., 1986). Also, plasmid constructs containing oriLyt could be amplified 100- to 1000-fold when a second vector expressing the BZLF1 gene was introduced. This suggests that BZLF1 may not only transactivate viral genes, but also play a role in activation of replication directly. The amplification of this plasmid containing oriLyt yielded head-to-tail concatemers, and this appeared to be dependent on the EBV DNA polymerase (Hammerschmidt and Sugden, 1988). Analysis of the terminal repeats has also revealed that there is a concatenated replicative intermediate form of the viral DNA in the productive cycle (Sato et al., 1990). These findings suggest that,

although the structure of oriLyf of EBV differs from that of the lytic origins of HSV-1 and -2, the mode of replication used by these different viruses in their productive life cycles appears to be similar.

c) Late Gene Expression and Virus Maturation

Expression of the late lytic cycle genes is entirely dependent upon lytic viral DNA synthesis, as it is sensitive to phosphonoacetic acid and acyclovir, which selectively inhibit the virally encoded DNA polymerase (Nyormoi et al., 1976; Summers and Klein, 1976; Colby et al., 1980). Members of this class of genes make up components of VCA and MA, discussed earlier. Recently, extensive sequence homology was reported between an EBV late gene, BCRFI, and a mouse cDNA clone for interleukin 10 (Moore et al., 1990). The virus is assembled in the nucleus, and, eventually, immature virus particles with a diameter of 80nm can be detected both in the nucleus and cytoplasm of productively infected cells (Epstein et al., 1965). Finally, the virus buds through the nuclear or plasma membranes, being engulfed in the process to give a mature virus particle with a diameter of about 120nm.

1.4.3 Latent Gene Expression

Of the eighty or so genes encoded by EBV, to date only eleven of these have been shown to be expressed during latency in in vitro immortalised B cells (Sugden, 1989). These genes must therefore all be candidates for being required both to initiate and to maintain the immortalised state of the infected cell. The products of these genes are two small RNAs (the EBERs), three membrane proteins (LMP, TP1 and TP2) and six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and LP). The approximate location of these genes in the EBV genome is represented in Figure 1.2. Latent gene expression differs between the three main

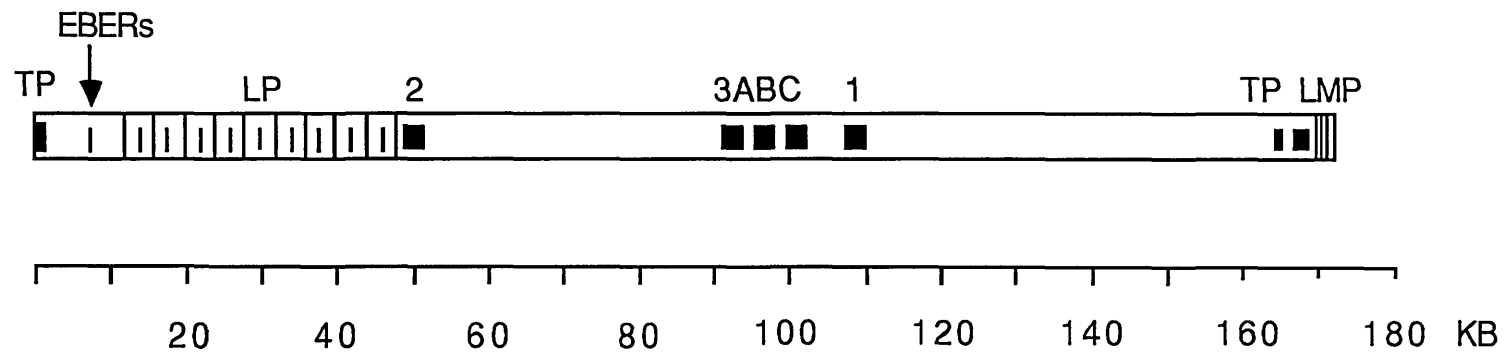


Figure 1.2 Linear representation of the B95-8 Epstein-Barr virus genome showing the approximate location of the known latent cycle genes. The six Epstein-Barr virus nuclear antigens (EBNAs) are 1, 2, 3A, 3B, 3C and Leader Protein (LP); LP is encoded within the major internal repeat region (IR-1). The Epstein-Barr virus encoded RNAs (EBERs 1 and 2) and the latent membrane protein (LMP) are expressed from the left and right hand end of the genome respectively. The genes for the terminal proteins (TP) span the terminal repeats of EBV, so that they are interrupted in the linear virion DNA and reconstructed by circularisation.

EBV-carrying cell prototypes: in vitro immortalised lymphoblastoid cell lines (LCLs), EBV-carrying Burkitt lymphoma (BL) cells and nasopharyngeal carcinoma (NPC) cells (Rowe et al., 1986; reviewed by Klein, 1989). During prolonged in vitro culturing, EBV-carrying but not negative BLs tend to "drift" towards a more LCL-like phenotype, but it is the representative (type 1) BL cell (as described by Rowe et al., 1985) that is referred to here. EBNA 1 is expressed in all three phenotypes, while the other EBNAs are only expressed in LCLs. LMP is also on in LCLs and off in type 1 BLs, and, as discussed earlier, its expression varies markedly in different NPC tumour biopsies. The different latent cycle genes will now be discussed separately.

a) Epstein-Barr virus encoded RNAs EBER-1 and EBER-2

The EBER RNAs, which are by far the most abundant viral transcripts in lymphocytes infected with EBV, were the first genes to be mapped (Rymo, 1979). The EBER genes are adjacent to each other and are transcribed constitutively in lymphocytes (Weigel et al., 1985) by the cellular RNA polymerase III (Rosa et al., 1981; Jat and Arrand, 1982). Although they are pol III transcripts, they have some pol II regulatory elements near them and these affect the gene transcription (Howe and Shu, 1989). All other transcription of EBV is thought to be by the cellular RNA polymerase II. The EBER RNAs are about 160 (EBER-1) and 180 (EBER-2) nucleotides in length (Arrand and Rymo, 1982) and have sequence homology with each other (98 identical residues). The RNAs are not polyadenylated and stop codons occur in all three reading frames, indicating that they do not code for proteins. In situ hybridization with probes specific for EBER-1 or -2 has suggested that the EBERS are predominantly nuclear (Howe and Steitz, 1986). As mentioned before, they display minor type-specific

sequence variation with the A and B types of EBV (Arrand et al., 1989).

Functional analysis of the EBERs has focused on their striking similarity to the virus associated (VA) RNAs of adenoviruses, and that they can partly complement VA RNA functions in a VA defective adenovirus mutant (Rosa et al., 1981; Bhat and Thimmappaya, 1983, 1985). Transcription of the adenovirus genome late in infection is thought to generate dsRNA from opposing transcription units. This activates a latent protein kinase, induced in interferon treated cells (Lebleu et al., 1976; Roberts et al., 1976; Zilberstein et al., 1976), which in turn phosphorylates a protein synthesis initiation factor. This results in the inhibition of total protein synthesis (Farrell et al., 1977, 1978). VA RNA binds to and sequesters the inactive protein kinase and prevents its activation by dsRNA (Reichel et al., 1985; O'Malley et al., 1986; Kitajewski et al., 1986), and consequently a VA RNA defective adenovirus is restricted in its ability to grow. Although the EBERs differ from the VA RNAs in that the latter are cytoplasmic and are only found in high accumulation late in the infectious cycle, it appears that EBER 1 does in fact have an analogous function; high concentrations of EBER 1 regulate protein synthesis by blocking the activation of the double-stranded RNA-dependent protein kinase DAI (Clarke et al., 1990).

b) The Latent Membrane Protein (LMP)

The most abundant of the latent cycle pol II transcripts is transcribed from the BamHI Nhet fragment at the right-hand end of the genome near the TR, and encodes a latent membrane protein from the BNLF1 open reading frame (Fennewald et al., 1984; Hennessy et al., 1984; Hudson et al., 1985a; Mann et al., 1985). The LMP gene has three closely spaced exons and gives rise to a 2.5kb mRNA. Analysis

of the predicted amino acid sequence for the LMP protein revealed that it would have six hydrophobic stretches near the N terminus, that are likely to be membrane spanning domains, followed by a globular C terminal portion containing many acidic amino acids. LMP can be phosphorylated, mainly on serines, and has a short half-life of between 2-5 hours (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987). This protein has been shown to be located in the plasma membrane of EBV-infected cells, and an even number of trans-membrane domains means that both the N and C termini are thought to be located inside the cell (Liebowitz et al., 1986). The phosphorylated form of LMP is associated with the cellular cytoskeletal protein vimentin (Liebowitz et al., 1987; Birkenbach, 1989), the network of which is abnormal in EBV-infected cells. More recently it has been shown that transfection of an EBV-negative BL line, Louckes, with a construct expressing LMP can result in an increase in vimentin expression (Birkenbach et al., 1989).

LMP is considered to have an important role in the immortalisation potential of EBV. First, LMP is a broad inducer of B cell activation markers (Wang et al., 1988b, 1990). Transfection of the LMP gene, followed by its expression at levels comparable to those found in EBV infected lymphocytes, can lead to changes in cell morphology and reduced serum requirement for Rat 1 fibroblasts (Wang et al., 1985) and Balb/3T3 cells (Baichwal and Sugden, 1988). Higher levels of LMP expression can be toxic to fibroblasts (Hammerschmidt et al., 1989). Rat 1 cells expressing LMP were tumourigenic in nude mice, indicating that LMP can act as a transforming gene, and more recently it has been shown that this function is localised to the N terminal part of the protein that contains the membrane spanning regions (Wang et al., 1988a,b; Baichwal and Sugden, 1989). LMP has no homology to membrane bound proto-oncogenes, such as c-src, c-yes, c-ras, c-fps, c-sis and

c-abl but LMP has loose similarity in structure to known ion channel proteins, and it has been speculated that it may alter Ca^{2+} regulation in cells. As already discussed, LMP may play a direct role in the development of NPC as it can prevent the differentiation of human epithelial cells in culture (Dawson et al., 1990) and can transform a human keratinocyte cell line (Fahraeus et al., 1990b).

c) The Terminal proteins, TP1 and TP2

The open reading frames for TP1 and TP2 (Laux et al., 1988,1989), which are also known as LMP2A and LMP2B respectively (Sample et al., 1989) span the terminal repeats of EBV. Therefore, the genes are always interrupted in the linear virion DNA and are reconstructed by circularisation. Rightward RNAs had been partly mapped to the left terminus of the genome map (Hudson et al., 1985b) and cDNA cloning and Northern analysis finally elucidated the structures of the mRNAs for TP1 and TP2 (Laux et al., 1988, 1989; Sample et al., 1989). The first exons of both genes are located close to the right end of the linear genome, and the TP1 and TP2 mRNAs are spliced across the joined terminal repeats to the same exons near the left end of the genome.

TP1 protein has been detected in lymphocytes latently infected with EBV (Rowe et al., 1990). From the protein structure, TP1 and TP2 were predicted to be membrane proteins. In fact, TP1 can be detected in both the nuclear and plasma membranes of the infected lymphocyte; in the latter it appears to be colocalised with LMP (Rowe et al., 1990; Sample et al., 1989; Longnecker and Kieff, 1990). This may imply a close association of TP1 and LMP and a functional relationship between the two, but further research is required to clarify this.

d) Expression of EBNA_s

The genes which encode the various EBNA_s are spread out over approximately 100 kb of the EBV genome. However, EBNA mRNAs constitute a family of transcripts sharing common 5' exons that are joined to 3' coding exons, which are unique to each EBNA (Bodescot et al., 1986; Bodescot and Perricaudet, 1986; Sample et al., 1986; Speck and Strominger, 1985; Speck et al., 1986). This results from the EBNA_s sharing the same two promoters at the left-hand-end of the genome (Figure 1.3). One is in the U_S region (BamHI C fragment) and is called the C promoter (Cp) (Bodescot et al., 1987), and the other is in each of the major internal repeats (BamHI W fragments), and is called the W promoter (Wp) (Sample et al., 1986). Transcripts initiating at Cp contain at their 5' ends two small exons (C1 and C2) encoded near the right-hand-end of the short unique region (U_S), whereas transcripts initiating from Wp contain at their 5' ends a short exon (W0) encoded in the major internal repeat (IR-1), located about 150 bp upstream of the region encoding the W1 exon (Sample et al., 1986; Speck et al., 1986). Transcription from either Cp or Wp includes a variable number of two small exons (W1 and W2) from the IR-1 and these make up the 5' exons present in all the EBNA mRNAs that have been analysed (for a summary of known EBNA cDNAs, see Figure 4.4 in Chapter 4). These "leader" exons are coding in the EBNA-Leader Protein gene, and this will be discussed later.

S1 nuclease analysis has revealed that utilisation of Cp or Wp is mutually exclusive in clonal cell lines established from either BL tumours or LCLs (Woisetschlaeger et al., 1989), and there is no clear correlation between promoter usage and cell type. Further analysis of two Wp-utilising LCLs revealed that, in both cases, the region encompassing Cp was deleted from the endogenous viral genome (Woisetschlaeger et al., 1990). In addition, when both promoters were

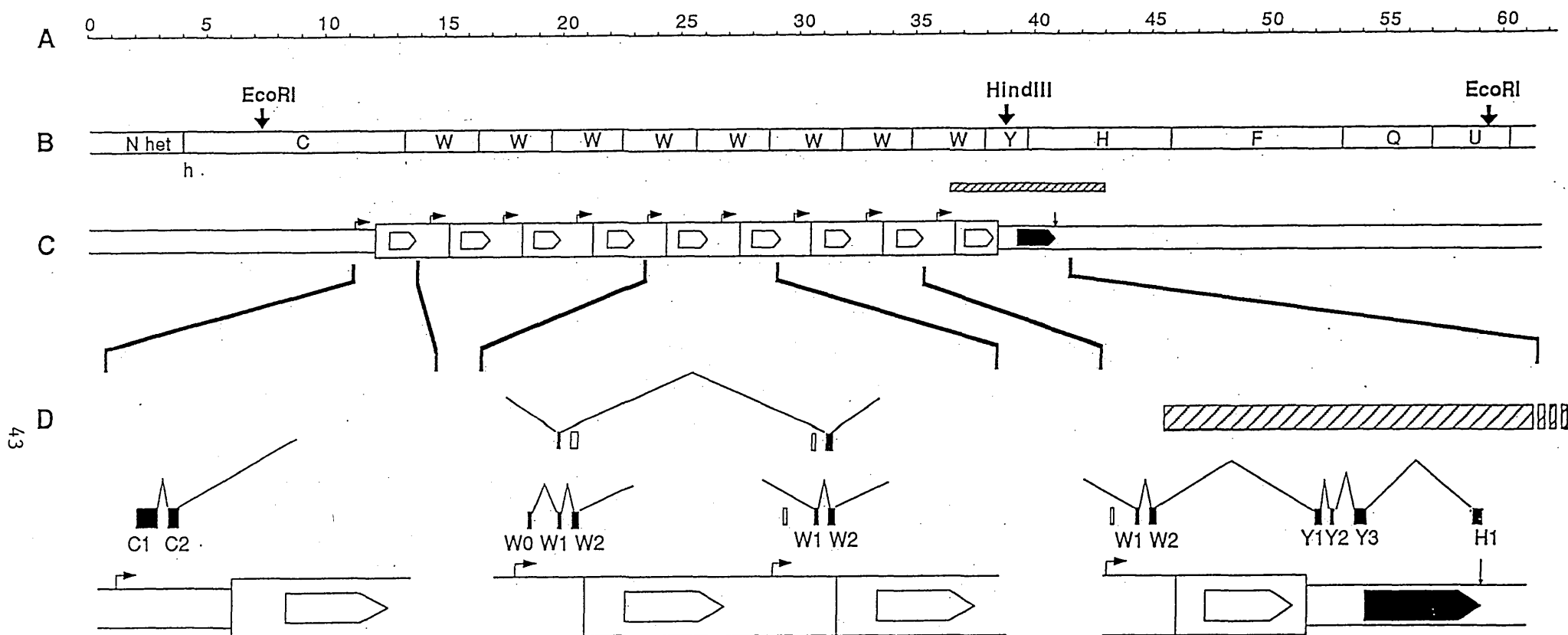


Figure 1.3 The Major Internal Repeat region (IR-1). A) shows a scale in kilo base pairs. B) represents the BamHI restriction map of this region of EBV. Eight BamHI W repeats are shown in this map since this is a typical number (determined by the FICE analyses in Chapter 3). C) represents the double-stranded EBV genome with the enlarged region being the IR-1 repeats. Hooked arrows indicate known latent promoters. The white boxes represent the BWRFl open reading frame. The black box is the BYRFl EBNA 2 open reading frame. The hatched bar above the diagram shows the extent of the P3HR-1 deletion. D) shows the EBNA-LP exons (black bars) incorporated into known cDNA clones. Two alternative splicing mechanisms of generating the reiterated W1W2 exons are indicated.

present on a reporter construct, Cp was always preferentially utilised by cell lines, irrespective of whether the endogenous viral genomes use Cp or Wp. Thus, it seems that Cp is the preferred promoter for driving transcription in established latently infected cell lines. There is now evidence that the C promoter has an upstream enhancer element, which is closer to the transcription start than that in oriP (Lupton and Levine, 1985; Reisman et al., 1985; Reisman and Sugden, 1986), and whose activity is increased in the presence of EBNA 2 (pers. comm. N. Sung). However, S1 analysis of RNA taken at different time points following infection of peripheral B cells with Akata EB virus, showed that Wp activity could be detected after 18 hours, whereas Cp activity only appeared after 3 days (Woisetschlaeger et al., 1990). Taken together, this data suggests a model where Wp activity precedes that of Cp. It may be that Wp has evolved to function in resting or pre-B cells, and that, after the viral antigens induce partial differentiation of the infected cells, the appropriate transcription factors for driving Cp become available and a switch from Wp to Cp occurs. This would represent a programmed progression from the initial stages of infection to latency.

S1 analysis of total RNA from the 5-Azacytidine treated Burkitt line, Rael, could detect Wp and Cp activity (pers. comm S Speck). No activity from either of these promoters could be detected in untreated cells, but there was still clear EBNA 1 expression. This suggests that EBNA 1 may be able to be transcribed from a separate EBV promoter.

e) EBNA 1 and oriP

EBNA 1 protein is encoded by the BKRF1 open reading frame in the BamHI K fragment of EBV (Summers et al., 1982; Hearing et al., 1984; Fisher et al., 1984). To date, four cDNAs corresponding to EBNA 1

mRNAs have been sequenced (Speck and Strominger, 1985; Bodescot and Perricaudet, 1986; Sample et al., 1986). Three of these use different combinations of exons in the 5' region of the message (Fig 4.4) and two have been shown to initiate from the C promoter. The BKRF1 open reading frame includes a 708bp repeat sequence that consists of only three in-frame triplets; GGG, GGA and GCA (Heller et al., 1982). These repeats encode only alanine and glycine, which make up about 40% of the protein (Hennessy and Keiff, 1983). The size of the protein varies in different EBV-infected cell lines between about 70 and 85kDa (Hennessy et al., 1983; Allday and MacGillivray, 1985; Kallin et al., 1986), and this is due to heterogeneity in the number of repeat elements in the protein coding sequence (Hennessy et al., 1983). It appears likely that the gly-ala repeats separate two functional domains in the EBNA 1 protein (Polvino-Bodnar et al., 1988) and, in fact, the repeats can be deleted without losing the activity of the protein (Yates et al., 1985; Milman et al., 1985).

EBNA 1 is found in the nuclei of all cell lines latently infected with EBV, and binds to cellular metaphase chromosomes (Grogan et al., 1983) as well as to three sites on the EBV genome (Rawlins et al., 1985). The known function of EBNA 1 is to maintain the episomal EBV DNA in latently infected cells, and this is mediated by EBNA 1 binding to the maintenance origin of viral DNA replication, oriP. This function for EBNA 1 was first identified when plasmids containing both oriP and EBNA 1 could be shown to replicate in a variety of cell types as stable replicons (Yates et al., 1984, 1985; Reisman et al., 1985).

OriP (origin of plasmid replication) comprises a 1.8kb region in the BamH1 C fragment. Using this origin, EBV DNA is replicated in the early S phase of latently infected cells, using cellular enzymes

(Hamper et al., 1974; Summers and Klein, 1976). The minimal origin has been shown to be composed of two cis acting elements, both of which are required for activity (Reisman et al., 1985). One consists of a region of twenty 30 bp tandem repeats and the other is a region of dyad symmetry, which could form a stem-loop structure. Intermediate replicative forms of the EBV genome were analysed on 2D gels and this revealed that the site of initiation of DNA replication is at or very near the dyad symmetry element, while the tandem repeats contain a replication fork barrier and the termination site (Gahn and Schildkraut, 1989). Thus, replication from oriP proceeds in a predominantly unidirectional manner. Deletions within this dyad element destroy the function of oriP, which is also dependent on a minimum number (6-8) of the 30bp repeats in plasmid replication (Chittenden et al., 1989). EBNA 1 binds to both of these elements in oriP (Jones et al., 1989; Ambinder et al., 1990; Gahn and Schildkraut, 1989), and it is the carboxy terminus of the protein which contains the DNA binding specificity (Milman et al., 1985). Gel retardation assays have demonstrated that the active DNA binding form of EBNA 1 is a dimer (pers comm R.F. Ambinder), and it may be that interaction between two EBNA 1 proteins bound to the two elements in oriP is required to initiate replication. EBNA 1 also binds to a region in the BamHI Q fragment, but the significance of this is unknown (Jones et al., 1989). The essential nature of the binding of EBNA 1 to oriP for maintenance of EBV episomes in a dividing cell population is reflected in the invariant presence of the EBNA 1 protein in latently-infected cells; it is the only EBNA expressed in type 1 Burkitt's lymphoma cells (Rowe et al., 1986, 1987, 1988).

The 30bp repeats can also act as a transcriptional enhancer when activated by EBNA 1 binding (Lupton and Levine, 1985; Reisman et al., 1985; Reisman and Sugden, 1986). This leads to transactivation of the

Cp promoter, and thus it seems that EBNA 1 can regulate its own expression and possibly that of other EBNAs (Sugden and Warren, 1989; Wysokenski and Yates, 1990).

f) EBNA 2

The EBNA 2 gene was mapped by cDNA cloning to the rightward BYRF1 open reading frame, which begins in the BamH1 Y fragment (van Santen et al., 1983). EBNA 2 protein could be detected in extracts from cells transfected with a plasmid that contained BYRF1 downstream from a promoter (Dambaugh et al., 1984; Rymo et al., 1985; Hennessy and Kieff, 1985) and antibodies raised against EBNA 2 oligopeptides, could detect this nuclear antigen in cells infected with EBV (Dillner et al., 1985). Analysis of a 3kb cDNA encoding EBNA 2 revealed that it initiated from the Wp promoter (Sample et al., 1986) and that it was bicistronic, since the 5' exons were coding for EBNA-LP (Wang et al., 1987b). Recent work has revealed that the C promoter has an upstream enhancer element, which has greater activity in the presence of EBNA 2 (pers. comm N. Sung).

The EBNA 2 open reading frame contains variations which are characteristic of two distinct viral types, A or B (Dambaugh et al., 1984; Young et al., 1987; Zimmer et al., 1986). As a result, the size of the EBNA 2 protein varies between A (82-87 kDa) and B (75 kDa) type virus and the two forms are antigenically distinct (Rickinson et al., 1987), with the type-specific epitopes clustered at the carboxy terminus (Rowe and Clarke, 1989). It should be noted that the growth characteristics differ in cells infected with A or B-type virus, and that A-type viruses are more efficient at establishing a transformed phenotype within a cell (Rickinson et al., 1987; Rowe et al., 1986, 1987). This may be attributed to the variation in EBNA 2, but, as

discussed earlier, differences between A- and B-type viruses extend to other latent cycle genes. The EBNA 2 gene in B95-8 virus has 14 repeats of CCCCCACCA resulting in a long stretch of prolines within the protein (Dambaugh et al., 1984).

The importance of EBNA 2 in EBV-mediated immortalisation was deduced from the inability of the P3HR-1 viral strain to transform infected cells (Miller et al., 1974; Delius and Bornkamm, 1978). This strain contains an approximately 7 Kbp deletion (Allan and Rowe, 1989), which removes the entire EBNA 2 gene (Fig 1.3 C.D) (Miller et al., 1974; Menezes et al., 1975, Raab-Traub et al., 1978; Delius and Bornkamm, 1978; Rabson et al., 1982). A second viral strain, Daudi, has an almost identical deletion to that found in P3HR-1 virus and this strain has also been reported to be non-immortalising (Jones et al., 1984). These deletions also remove the 3' unique sequences of EBNA-LP (discussed later) so it was not clear whether it was the loss of EBNA 2, the truncation of EBNA-LP, or both that led to the loss of immortalising potential. Superinfection of the non-producer Raji cells with P3HR-1 virus has resulted in the rescue of immortalising viruses, which have arisen from recombination between the two EBV genomes to restore the deleted sequences from P3HR-1 (Skare et al., 1985). More recently, it was demonstrated that recombination events which restored EBNA 2, but not a full length EBNA-LP, also restore immortalising ability to P3HR-1 virus, while the converse is not true (Hammerschmidt and Sugden, 1989; Cohen et al., 1989). Therefore, EBNA 2 is required for EBV-induced B-cell immortalisation, although it is not sufficient alone.

Transfection of EBNA 2 into Rat-1 cells resulted in a lower serum requirement for growth, and the cells grew in clumps rather than as a uniform layer (Dambaugh et al., 1986). EBNA 2 alone can induce the

expression of the B-cell activation markers CD23 and CD21 (Wang et al., 1987a; Cordier et al., 1990). It is now clear that it can also induce the expression of LMP (Abbot et al., 1990) by relieving the repression of a negative transcription element at the LMP promoter (Fahraeus et al., 1990a). EBNA 2 and LMP together can induce higher levels of CD23, than either of them separately (Wang et al., 1990). More detailed mutational analysis of the EBNA 2 gene has revealed that at least 30% of EBNA 2 is dispensable for immortalisation, but that all transformation competent mutants still transactivate LMP (pers. comm. J. Cohen). This suggests that transactivation of LMP is a prerequisite for immortalisation. Other effects have been attributed to EBNA 2 which may be relevant to its role in immortalisation. These include its suggested involvement in the neutralisation of the antiproliferative activity of α -interferon (von Gabain and Aman, 1990) and the possible EBNA 2 stimulation of the c-fgr oncogene (Knutson, 1990).

g) EBNAs 3A, 3B and 3C

The EBNA 3 series of latent cycle proteins was identified as heterogeneous high molecular weight bands on Western blots using EBV specific antisera (Hennessy et al., 1985; Kallin et al., 1986; Hennessy et al., 1986; Rowe, D. et al., 1986, 1987). The three genes are homologous in both their organisation and part of their primary structure, and it is likely that they have arisen from the triplication of a single viral gene. EBNA 3A, 3B and 3C are also known as EBNA 3 (Hennessy et al., 1985), EBNA 4 and EBNA 6 (Ricksten et al., 1988; Allday et al., 1988) respectively. The coding region of EBNA 3A is made from splicing together BLRF3 and BERF1, EBNA 3B comes from BERF2a and BERF2b, and EBNA 3C from BERF3 and BERF4. To date, all the EBNA 3 cDNAs identified have initiated from the C region (Bodescot and Perricaudet, 1986; Kerdiles et al., 1990), but there is

no reason to believe that the EBNA 3 family cannot be transcribed from the W promoter as well. These proteins are expressed early following infection and shortly after EBNA 2 expression (Allday et al., 1989; Rooney et al., 1989a).

Using specific antisera, the EBNA 3 proteins were identified in extracts from cells which had been transfected with the appropriate DNA (Hennessy et al., 1986; Ricksten et al., 1988; Joab et al., 1987; Shimizu et al., 1988; Petti and Kieff, 1988, Petti et al., 1988). To date, the only function which has been ascribed to these proteins, is that EBNA 3C can induce the expression of the EBV receptor antigen CD21 on the surface of B-lymphocytes (Wang et al., 1990). There is considerable homology within the N-terminal region of the three proteins, but it is not known whether this reflects a common function.

h) EBNA-LP

The Epstein-Barr virus nuclear antigen, Leader Protein (EBNA-LP) (Sample et al., 1986), is also known as EBNA 4 (Rowe et al., 1987), EBNA 5 (Dillner et al., 1986) and EBNA W/Y (Sauter et al., 1988). It derives the name Leader Protein from the fact that most of the coding exons in the gene comprise the 5' "leading sequences" in nearly all of the known EBNA mRNA molecules (Figure 4.4 in Chapter 4). EBNA-LP is the major topic of study in this thesis.

i) Structure of EBNA-LP

The structure of the LP gene (Figure 1.3 D) was deduced from analysis of highly spliced cDNAs which spanned the major internal repeat (IR-I) region of the virus. The first cDNA identified (T1 cDNA) was isolated from Raji cells and was incomplete at both the 5' and 3' ends (Fig 4.4 in Chapter 4) (Bodescot et al., 1984). Two full-length

cDNA clones were eventually found expressed from the B95-8 EBV in IB4 cells, IB4-WY1 (Speck et al., 1986) and IB4-T65 (Fig 4.4) (Sample et al., 1986). These were shown to have both been transcribed from a W promoter and contained part of the non-coding W0 exon at the 5' end, followed by a repeated sequence of W1 (66bp) and W2 (132bp) exons from the IR-1 repeats. At the 3' end were two coding exons Y1 (33bp) and Y2 (123bp, 102bp coding) and a non-coding exon Y3 (Fig 1.3 D). IB4-WY1 ended in the Y3 exon, whereas IB4-T65 had the EBNA 2 coding sequence downstream. The IB4-WY1 cDNA could be transcribed and translated in vitro to produce LP (Speck et al., 1986), whereas the bicistronic IB4-T65 could produce both LP and EBNA 2 protein (Wang et al., 1987b). LP can be expressed from the C promoter (Woisetschlaeger et al., 1989; Rogers et al., 1990) and cDNAs originating from here contain the non-coding exons C1 (144bp) and C2 (31 bp) at their 5' end (Bodescot et al., 1986), as opposed to W0 from a W promoter start (Fig 1.3 D). It is envisaged that LP mRNA can be monocistronic, when the polyadenylation signal is contained in the HI exon downstream of the EBNA 2 gene (the T1 cDNA contains a Y3 to HI splice, that spans the EBNA 2 open-reading frame; Fig 1.3 D). It is also possible that, like IB4-T65, LP mRNAs exist which are bicistronic in their 3' region for one of the other EBNA genes.

As the LP gene is primarily composed of multiple copies of repeated exons within the major internal repeat, the protein product must be composed of a number of 66 amino acid repeats, followed by 45 amino acids of unique carboxy terminal sequence. LP was first identified in Western blot analysis using polyclonal antibodies raised against oligopeptides with BamHI W and Y sequences (Dillner et al., 1986) and human sera from EBV positive individuals (Dillner et al., 1986; Rowe et al., 1987). Subcellular fractionation of IB4 cells localised LP to the nucleus. It was found in these experiments that LP was expressed

in all LCL lines studied, whereas Namalwa was the only BL line with detectable protein. In addition, a distinctive ladder of proteins was observed between 22 to 70 kDa with a regular spacing of 6 to 8 kDa, consistent with the repetitive 66 amino acid structure of the protein. A mouse monoclonal antibody, JF186, was also raised against an LP oligopeptide (Finke et al., 1987), and this verified multiple LP protein expression in different cell lines carrying the B95-8 virus. It was suggested here and in earlier work (Rowe et al., 1987) that the 6 kDa size differences could arise from different-sized messages produced by alternative splicing in the LP coding regions, or use of different internal W promoters (Finke et al., 1987). It was also pointed out that B95-8 LP proteins existed which were considerably larger than the then predicted coding capacity of the gene, and one possible explanation was that the protein had an anomalous size on SDS-gels due to its unusual primary structure, with high proline and arginine content (Finke et al., 1987).

ii) Expression of EBNA-LP

Transcriptional control

Transcriptional regulation of EBNA-LP expression is not yet well understood. Experiments using nuclear run-on assays (the nuclei were isolated from IB-4 cells) suggest that there is considerably higher levels of EBNA-LP and EBNA 2 mRNA during latency in immortalised lymphocytes (Sample and Kieff, 1990). However, these experiments involved hybridisation of labelled RNA to genomic fragments which represented the individual latent-infection genes; this may be artefactually high for EBNA-LP because of the multiple repeats in its sequence.

Splicing control

EBNA-LP expression appears to be dependent upon specific splicing events taking place. The LP cDNAs which could be in vitro translated contained near the 5' end an alternative splice which used the acceptor site 4bp further downstream than in the "conventional" W0-W1 splice (Fig 1.4) (Speck et al., 1986; Sample et al., 1986). This alternative splice is termed a W0-W1' splice, and it brings together the AT at the end of the W0 exon and the G at the beginning of the W1' exon, to form an initiator methionine codon, ATG. Clearly, EBNA-LP can also be expressed from the C promoter (Woisetschlaeger et al., 1989). The non-coding C2 exon also ends with AT, but, to date, the only cDNAs analysed which originate from the C promoter, have a C2-W1 splice and consequently no initiator codon. It should be noted that in different systems, translation initiation can sometimes occur from certain CUG condons (Clements et al., 1988; Hann et al., 1988; Prats et al., 1989), and that two of these codons lie within the C2 exon of EBV (presented in chapter 4). However, S1 protection assays, that used antisense splice junction oligonucleotides, demonstrated that a C2-W1' splice oligo was protected in Cp-utilising cell lines, while a W0-W1' oligo was protected in a Wp cell line (Rogers et al., 1990). It was also shown that the W0-W1 and C2-W1 splices were favoured over the alternative W1' splices, in the appropriate cell lines. This, and data presented in chapter 4, is the first time a C2-W1' splice has been recorded (Fig 1.4). These splicing events are important, not only for the expression of EBNA-LP, but also for the other EBNA's. One can imagine that where EBNA-LP is encoded at the 5' end of a multicistronic message, the translation of LP will reduce that of other EBNA's downstream. This is in fact what was observed; EBNA 2 was translated only about 5-10% as efficiently from the in vitro transcribed bicistronic IB4-T65 cDNA, compared with when the

DONOR

ACCEPTOR

C2 GCACGTGAGCATGTATCTGAAA...

...CCGCCATCCAAGCCTAGCCTAGGGGAGACCG **W1**

...CCGCCATCCAAGCCTAGCCTAGGGGAGACCG **W1'**

W0 GTCCACAATGTAAGAGGGGG...

Figure 1.4 Splicing control of EBNA-LP. Transcription from either the C or W promoter includes a non-coding exon, C2 and W0 respectively, which ends in the nucleotides AT. There are no initiator methionine codons present in EBNA-LP mRNAs when these exons are spliced to the coding exon W1. The alternative splice from either C2 or W0 to W1' creates the initiator ATG.

EBNA-LP sequences were removed from the 5' region (Wang et al., 1987).

Post-translational modification

Within 24 hours after in vitro infection of B cells by EBV, the first Epstein-Barr virus nuclear antigens, EBNA 2 and multiple species of EBNA-LP, can be detected (Kallin et al., 1986; Moss et al., 1986; Dillner et al., 1986; Finke et al., 1987). Post-translational modification of LP was detected after running EBV-infected cell lysates on 2-D gels, and immunoblotting these with LP-specific antibody (Sauter et al., 1988; Petti et al., 1990). Several isoforms of the protein were seen in this way, and further 2-D gel analysis with ³²P metabolically labelled cell extracts was able to show that these isoforms had arisen from phosphorylation.

iii) Function of EBNA-LP

The precise function of the EBV Leader Protein is as yet unknown. Several observations about the protein's nuclear localisation have led to speculation about its potential role in EBV infected cells. The 44-amino acid repeat encoded by the W2 exon has two runs of basic amino acids, Arg-Arg-His-Arg and Arg-Arg-Val-Arg-Arg-Arg, which could be nuclear localisation signals. Indirect immunofluorescence with the LP specific monoclonal antibody, JF186, and LP specific affinity purified antibodies, gives a characteristic picture of coarse nuclear granules, which may suggest the concentration of LP in a few subnuclear particles (Finke et al., 1987; Petti et al., 1990). In addition, subcellular fractionation experiments have shown that LP is localised to the nuclear matrix fraction (Dillner et al., 1986; Petti et al., 1990). Others have shown that EBNA-LP can be trapped on a DNA-cellulose column, after passing cell extracts through, and it was concluded from this that LP exhibits specific DNA binding activity

(Sauter et al., 1988). However, it is not at all clear from these experiments whether LP is involved in sequence specific DNA binding. Also, it may not have bound to the column directly but via interaction with other viral or cellular proteins.

A homology has been reported between sequences within the unique carboxy terminal region of EBNA-LP and conserved domain 1 of the adenovirus Ela protein (Huen et al., 1988). This comprises six identical amino acids and one conservative replacement. (Figure 5.1 in Chapter 5) This is interesting when one considers that these seven residues lie over the core sequences of conserved domain 1, which are absolutely required for Ela mediated transformation (Zerler et al., 1987; Subramanian et al., 1988; Whyte et al., 1988a). It is too early to say whether this homology has any functional significance. However, as already mentioned, the non-transforming EB viral strains, P3HR-1 and Daudi, contain deletions which not only remove the EBNA 2 gene, but also the 3' unique sequences of EBNA-LP (Miller et al., 1974; Menezes et al., 1975; Raab-Traub et al., 1978; Delius and Bornkamm, 1978; Rabson et al., 1982; Jones et al., 1984). Therefore, these viral strains have also lost the homology between EBNA-LP and Ela. It should also be noted that the homology to Ela is less close with EBNA-LP from B-type EB virus (Huen et al., 1988), which is considered to be less efficient at establishing a transformed phenotype than A-type virus (Rickinson et al., 1987; Rowe et al., 1986, 1987).

More recently, it was demonstrated that recombination events which restored EBNA 2 to P3HR-1 virus, without restoring full-length EBNA-LP, also restored immortalisation potential (Hammerschmidt and Sugden, 1989; Cohen et al., 1989). This shows clearly that the carboxy sequences of LP are not required for the initiation of

immortalisation of B-cells by EBV. However, EBNA-LP may still be considered as a possible accessory to transformation, because cells which have been immortalised with the EBV strain containing a truncated LP gene (called 633 EB virus) are slow to proliferate initially and then grow poorly (Hammerschmidt and Sugden, 1989).

Various aspects of the molecular biology of EBNA-LP are presented in this thesis. The objective was to characterise more fully this important Epstein-Barr viral antigen and devise an assay for its function.

MATERIALS AND METHODS

2.1 Solutions and Buffers

NET

100mM Sodium chloride

1mM Ethylenediamine tetracetic acid (EDTA)

10mM Tris (hydroxymethyl) aminoethane hydrochloride (Tris-HCl)

pH7.5

TE

10mM Tris-HCl pH7.5

1mM EDTA

10 X TBE per litre

108g Tris base

27.5g Boric acid

20ml 0.5M EDTA pH 8.0.

20X SSC per litre

175.3g Sodium Chloride

88.2g Sodium Citrate adjusted to pH 7.0

10% SDS

10% W/V Sodium dodecyl sulphate

10 x Phosphate buffered saline

Per litre 7.33g Sodium Chloride

2.36g di-Sodium hydrogen orthophosphate

1.32g Sodium dihydrogen orthophosphate

2xHepes buffered saline pH 6.9 (used for Calcium phosphate precipitation)

0.265 M Na_2HPO_4

12 mM Dextrose

0.274 M NaCl

10 mM KCl

37 mM Hepes

Phenol/Chloroform

Ultra pure phenol saturated with water (Rathburn Chemicals Ltd., Walkerburn, Scotland) was mixed with an equal volume of chloroform.

50 X Denhardt's solution

5g Ficoll

5g polyvinylpyrrolidone

5g bovine albumin fraction V

per 100ml water. After dissolving the ingredients the solution was filtered through a $45\mu\text{m}$ filter, aliquoted and stored at -20°C until used.

10xMSH

60mM 2-Mercaptoethanol

10xDTT

10mM Dithiothreitol

10x Ligase buffer

500mM Tris-HCl pH7.5

100mM Magnesium chloride

100mM DTT

10mM Adenosine triphosphate

10xTMC

100mM Tris-HCl pH7.5

100mM Magnesium chloride

100mM Calcium chloride

Restriction enzyme buffers (10X)

	0	N1	N2	N3	N4	K1	K2	K3
Tris-HCl								
pH 7.5	100mM	100mM	100mM	100mM	1M	100mM	100mM	100mM
Magnesium								
Chloride	100mM	100mM	100mM	100mM	50mM	100mM	100mM	100mM
Sodium								
Chloride	-	60mM	600mM	1.5mM	500mM	-	-	-
Potassium								
Chloride	-	-	-	-	-	60mM	200mM	660mM

(10x) In vitro transcription buffer

400mM Tris-HCl pH7.5

60mM MgCl₂

20mM spermidine - HCl

DNA mini-prep solutions

Solution I:- 25mM Tris-HCl pH8.0

10mM EDTA

50mM glucose

2mg/ml lysozyme

Solution II:- 1% SDS

0.2M NaOH

Solution III:- 3M Sodium acetate pH 4.8

Made by diluting glacial acetic acid with H₂O and
bringing to pH with solid NaOH and 4N NaOH.

Hanks Staining Solution for Immunofluorescence in Fibroblasts

5% Donor calf serum

Hanks balanced salt without sodium bicarbonate (Gibco Biochemicals)

20mM Hepes pH 6.9

5ml of 10% Sodium azide

2.2 Gels

Agarose gels

0.8-1.5% w/v high gelling temperature agarose (Bethesda Research Laboratories, Gaithersburg, MD, USA) in 1xTBE containing 10µg/ml ethidium bromide

Loading buffers

50% w/v sucrose

0.1M EDTA pH 8.0

0.1% Bromophenol blue

0.1% Xylene cyanol FF

(with 1µg/ml RNase when analysing miniprep DNA)

Polyacrylamide gels

Denaturing gels (6%)

24g urea

10ml 30% w/v Acrylamide

10ml 1% w/v NN' Methylenebisacrylamide

5ml 10 x TBE

10ml water

70 μ l Tetramethylethylenediamine (TEMED)

70 μ l 25% w/v Ammonium persulphate

Non-denaturing gels (6%)

10ml 30% w/v acrylamide

10ml 1% w/v NN' Methylenebisacrylamide

5ml 10xTBE

10ml water

70 μ l TEMED

70 μ l 25% w/v Ammonium persulphate

Gradient sequencing gels

0.5 x TBE gel mix

150ml 40% solution (38% w/v acrylamide, 2% w/v NN' methylenebisacrylamide)

50ml 10xTBE

460g urea

water to 1 litre

5.0 x TBE gel mix

150ml 40% solution (38% w/v acrylamide, 2% w/v NN'

methylenebisacrylamide)

500ml 10 x TBE

460g Urea

50mg Bromophenol blue

water to 1 litre

For each gel, two premixes were prepared:-

7ml 5.0 x TBE gel mix + 14 μ l TEMED + 14 μ l 25% w/v Ammonium persulphate.

45ml 0.5xTBE gel mix + 90 μ l TEMED + 90 μ l 25% w/v Ammonium persulphate

A gradient gel was poured by taking up 6ml of 0.5 x TBE premix followed by 7ml 5.0 x TBE premix into a 25ml pipette. Mixing was performed by passing a few air bubbles through the liquid in the pipette. The gel mix was then poured down the edge of the gel plates. The remaining 0.5X TBE gel mix was then poured in a similar manner.

Bacteriology products

2xTYE

16g tryptone

10g Yeast extract

5g sodium chloride

water to 1 litre

pH 7.4

TYE plates

10g tryptone

5g yeast extract

8g sodium chloride

15g agar

water to 1 litre

Ampicillin plates:- as for TYE plates, but including 100 μ g/ml ampicillin, added after autoclaving and cooling to 50°C.

Top Agar

10g tryptone

8g sodium chloride

8g agar

water to 1 litre

2.3 Cell Lines

2.3.1 Bacterial Cell lines

The *Escherichia coli* strains TGl and HB101^f (Boyer and Roulland-Dussoix, 1969) were used for preparing DNA preps of M13 and plasmid clones respectively.

Mammalian Cell Lines

2.3.2 B Cell Lines and EBV strains

IARC/BL18, IARC/BL29, IARC/BL74, Wewak 1, Wewack 2, Raji and Jijoye are Burkitt's lymphoma cell lines, (gifts of G. M. Lenoir, IARC France, and A. B. Rickinson, CRC, Birmingham, England). IARC/BL41 is an EBV negative Burkitt's lymphoma line used here in transfection studies. Human LCLs had been made by infecting peripheral blood lymphocytes from a seronegative donor with EBV from BL74, BL18, BL29, WW1 and WW2 lines (Rickinson et al., 1987). P3HR-1 (a line derived from Jijoye; Hinuma et al., 1967), which contains highly rearranged heterogeneous (het) EBV (Miller et al., 1985; Countryman et al., 1986). C-16 is a subclone of P3HR-1 which has been shown not to contain this particular "het" DNA (Rabson et al., 1982). P3HR-1/633 is a recombinant virus which has the P3HR-1 deletion restored with the exception of the 3' EBNA-LP sequences (Hammerschmidt and Sugden,

1989). JC-5 is a tightly latent human cord LCL containing the Jijoye strain of EBV (Miller et al., 1974). An EBV negative cell line, BJAB, was used as the negative control in all the structural DNA analysis experiments.

2.3.3 Fibroblast lines

Primary rat embryo fibroblasts which had been immortalised with a temperature sensitive SV40 large T, tsa 14 cells, (Jat and Sharp, 1989) were used to test immortalising potential of EBNA-LP. Cells were grown in Dulbecco's Modified Eagle Medium (Gibco, Paisley, Scotland) containing 10% fetal calf serum (Flow Laboratories, Irvine, Scotland), 2mM glutamine, 200µg/ml streptomycin and 60µg/ml penicillin at 37°C, 10% CO₂. Also, in collaboration with Lawrence Banks, ras cooperation assays were carried out in baby rat kidney (BRK) cells (Land et al., 1983).

2.4 DNA extraction from B-cells and restriction digestion

All B-cell lines were grown in RPMI 1640 (Gibco, Paisley, Scotland) containing 10% heat inactivated fetal calf serum (Flow Laboratories, Irvine, Scotland), 2mM glutamine, 200µg/ml streptomycin and 60µg/ml penicillin at 37°C, 10% CO₂. Cells (10⁸) were spun down at 1,300 r.p.m (500g) for 5 mins at 4°C. The cell pellet was washed twice in ice cold PBS before resuspending it in 2ml of NET. 200µl of 10% sodium dodecyl sulfate was added, followed by Proteinase K (3µg/ml). This was then left on a roller overnight at room temperature. After two phenol extractions (1:1 vol), the samples were incubated at 37°C with 10µg/ml RNase for 30 min, extracted with phenol/chloroform, ethanol precipitated and redissolved in approximately 200µl of TE. Pipette tips which had been widened at the bottom were used and

vortexing was avoided, so that high-molecular weight DNA molecules could be preserved. Virion DNA was kindly provided by Dr P. Farrell.

The DNA was digested with a two to five fold excess of the appropriate restriction enzyme (all enzymes from New England Biolabs, Massachusetts, U.S.A.).

2.5 Field Inversion Gel Electrophoresis

Field inversion gels (Carle et al., 1986) were run using the DNA star module, (Madison, WI, USA). Upto 3 μ g of DNA, which had been digested with the restriction enzymes EcoRI and HindIII, was loaded onto a 0.7% agarose slab gel. The gels were run at 150V for 30 min, and then pulsed for 20hr across a ramp of 3 sec in the forward direction, 1.5 sec backward at the start of the run, down to 0.3sec forward, 0.1 sec backward at the finish. Molecular weight DNA size markers in the range of 8 to 48kb were obtained from Bethesda Research Laboratories. The markers were end-labelled with [α -³²P] (as described in Maniatis et al., 1989, page 5.36).

Fragments were transferred to nitrocellulose (Schleicher and Schull, Dassel, West Germany) by the method of Southern (Southern, 1975). The Epstein-Barr virus BamHI W fragment was excised and 0.1 μ g was labelled by nick-translation (Rigby et al, 1977) (kit from Amersham International, Aylesbury, U.K.) with 50 μ Ci [α -³²P] CTP (800 Ci/mM) (Amersham). Nonincorporated triphosphates were removed on a sephadex G-50 column in TE. Hybridisation was carried out in polythene bags in a buffer containing 6 X SSC, 0.5% SDS, 5 x Denhardt's solution, 100 μ g/ml denatured salmon sperm DNA, and up to 5 x 10⁷ cpm of labelled probe which had been heat denatured for 5 mins in a boiling water bath. Hybridisation was performed at 65°C for at least 15hr.

Filters were washed at room temperature for 5 min in 2xSSC , 0.5% SDS, followed by a 15-min wash, in 2 X SSC, 0.1% SDS. Finally there was a 2-hr wash followed by a 30-min wash, both in 0.1x SSC, 0.5% SDS at 65°C.

2.6 DNA Manipulation

The cDNA clones from M. Perricaudet's laboratory were subcloned as EcoRI fragments into M13mpl8 (Yanisch-Perron et al., 1985) in both orientations so that they could be sequenced.

S. Speck's seven repeat LP cDNA clone (Speck et al., 1986), and deletion derivatives thereof, were at the EcoRI site of SP64 (Melton et al., 1984). These were subcloned into different plasmids during the course of this work.

1) pUC-MoMlv-LP was used for LP expression in the tsA 14 fibroblasts. It contained the LP gene from SP64 as a BamHI fragment in the BamHI site of pUC-MoMlv. The LP BamHI fragment was obtained by cutting the SP64 clone with EcoRI, filling in the EcoRI site with Klenow DNA polymerase and addition of BamHI linkers (CGCGGATCCGCG). pUC-Molv is pUC19 containing the Mouse Moloney virus LTR promoter upstream of the polylinker (Edmonds and Vousden, 1989).

2) p3.1W-LP was used for LP expression in CAT assays and for assessing CUG initiation of translation in BL41 cells. It contained the LP gene from SP64 as an XbaI-EcoRI fragment between the XbaI and EcoRI sites of p3.1W. p3.1W is pUC19 containing a 3.1 kbp partial PstI fragment from BamHI W of EBV (EBV map positions 42017 to 45089 in the W repeats 10 and 11 of B95-8) in the PstI site of the

polylinker region with the promoter 5' to the EcoRI site (kindly provided by G. Packham).

3) pSNOC was derived from pCMV19 (Grieves and O'Hare, 1989), which is a pBR322 derivative containing the CMV immediate early promoter upstream of the pUC19 polylinker region. pSNOC contains the SV40 early promoter poly A signal, oriP from EBV and the neomycin resistance gene. The poly A signal was cut out of pSV2-CAT (Slater et al., 1985) as a 135bp BamHI-HpaI fragment. The BamHI site was filled in with Klenow DNA polymerase and the fragment was cloned into the SspI site of pCMV19, 191bp downstream of the polylinker.

The oriP element was originally cloned between the EcoRI-SmaI sites in pUC19 as an EcoRI-StuI fragment (EBV map positions 7316 to 9659) (pers. comm. D. Rowe). It was cut back out as an EcoRI-BamHI fragment, the sites were filled in with Klenow DNA polymerase and the blunt fragment was cloned into the NdeI site within a part of the CMV sequences of pCMV19 not required for promoter function (Boshart et al., 1985).

An NdeI-BamHI fragment containing the bacterial neomycin resistance gene downstream of the SV40 early region promoter was cut out of pSV2-Neo (Southern and Berg, 1982). The BamHI site was filled in and the blunt fragment was subcloned into the StuI site of pCMV19, resulting in pSNOC. This plasmid has the SV40 and CMV promoters in the same orientation. pSNOC was used as a negative control in the 633 virus analysis described in Chapter 5.

4) pSNOC-LP was used to express a full length LP in B cells immortalised with 633 virus. It contained the LP gene from SP64 as a XbaI-EcoRI fragment between the XbaI and EcoRI sites of pSNOC,

described above. The structure of pSNOC-LP is represented in Figure 5.7.

Standard procedures for subcloning were employed (Maniatis et al, 1989). All the restriction enzymes and the ligase used in these experiments were obtained from New England Biolabs and the calf intestine alkaline phosphatase from Boehringer Mannheim. DNA fragments were extracted from agarose gels by electroelution using a biotrap (Schleicher and Schuell, Dassel, W. Germany). Finally, plasmid constructs were purified on caesium chloride gradients (Maniatis et al., 1989, page 1.42).

Restriction Endonuclease Digestions

A typical reaction containing 1 μ g of DNA would be;

18 μ l DNA + H₂O

2 μ l appropriate 10x restriction buffer (listed earlier)

$\approx$ 10 units of restriction enzyme

Reactions were incubated at the recommended temperature for approximately 1-2 hours. The reaction could then be stopped by adding 0.5M EDTA (pH 8.0) to a final concentration of 10mM.

If the digested DNA was to be loaded directly onto a gel, 6 μ l of loading buffer was added to the restriction digestion reaction. If the digested DNA was to be purified, it was extracted once with phenol/chloroform (1:1 vol) and once with chloroform alone. It was then precipitated with ethanol.

Dephosphorylation

Following the digestion, 1 μ l of calf intestinal alkaline phosphatase (CIP; 24 units/ μ l) was usually added to a 20 μ l restriction reaction

and left for a further 30 min at 37°C to remove the 5' phosphate groups. The DNA was then purified by phenol/chloroform extraction and ethanol precipitated.

Ligation

A typical ligation reaction would be;

200ng of dephosphorylated plasmid DNA

200ng of insert DNA carrying compatible phosphorylated termini

1μl 10x ligation buffer

1μl 10mM ATP

H₂O to 9.5μl

0.5μl T4 DNA Ligase (400 units/μl)

10μl Total

The reaction was incubated overnight at 15°C.

Linkers

If linkers were to be ligated, they were kinased prior to use.

2μl linker DNA (≈2μg)

1μl buffer 0

1μl 10x MSH

1μl 10mM ATP

4.5μl H₂O

0.5μl T4 polynucleotide kinase (10 units/μl)

10μl Total

The reaction was incubated for 2 hours at 37°C and then for 10 min at 65°C to heat inactivate the kinase. The kinased linkers could then be stored at -20°C.

A typical linker reaction would be;

6.5 μ l	digested DNA ($\approx$ 0.5 μ g)
1 μ l	kinased linkers
1 μ l	10x ligation buffer
1 μ l	10mM ATP
0.5 μ l	T4 DNA ligase (400 units/ μ l)
10 μ l	Total

The reaction was incubated overnight at 15°C. After ligation the excess linkers were cut off by digestion with a 10-fold excess of the appropriate restriction enzyme in a large volume ($\approx$ 80 μ l).

Klenow Repair

Following digestion of DNA with restriction enzymes it was often necessary to fill the recessed 3' termini using the large fragment of DNA polymerase I (Klenow fragment) from E. coli. A typical reaction containing 1-5 μ g of digested DNA would be;

20 μ l	DNA + H ₂ O
3 μ l	buffer 0
1 μ l	10x MSH
1 μ l	Klenow (5 units/ μ l)
4 μ l	dNTP's (2mM dATP, 2mM dTTP, 2mM dCTP, 2mM dGTP)
30 μ l	Total

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

Electroelution of DNA from agarose gels

DNA fragments were extracted from agarose gels by electroelution using the Schleicher and Schuell Biotrap (Dassel, West Germany). The

Biotrap is placed in a horizontal electrophoresis chamber filled with buffer (1xTBE). The DNA is cut out of the agarose gel and the block placed inside the central elution chamber of the Biotrap. An electric field was applied (150 Volts for 2 hours). The DNA molecules migrate from the elution chamber into a trap (volume 200-800 μ l) formed by two different types of membranes, that allow the passage of current through the device. The first membrane in the trap (nearer to the elution chamber) acts as a prefilter which excludes large particles, while the second membrane retains the DNA molecules. Fragments from 14 bp to 150 kbp can be trapped in this way. The contents of the trap can be removed using a yellow tip, and the DNA can be digested or ligated without further treatment.

Plasmid Transformation into *E. coli*

Competent *E. coli* were prepared as follows; 1 ml of 2x TYE was inoculated with a bacterial colony and this was left shaking overnight at 37°C. On the next day the cells were diluted 100-fold in 10 ml of fresh 2x TYE and this was left shaking for approximately 2 hours until the cells had reached an OD₆₀₀ of 0.3-0.5. The cells were spun out by centrifugation at 3000 rpm for 5 min at 4°C in a Sorvall GS3 rotor and resuspended in 5 ml of cold 50mM calcium chloride. This was left on ice for 15 min and then the cell were spun out as before. Finally the cells were resuspended in 1 ml of cold 50mM calcium chloride. They were then ready for transformation. Optimally this should yield 1×10^6 transformed colonies per microgram of supercoiled plasmid DNA.

A typical transformation would consist of;

5 μ l	DNA ($\approx$ 50ng)
5 μ l	10x TMC
40 μ l	H ₂ O
100 μ l	competent cells
150 μ l	Total

Controls would include no DNA and vector DNA ligated in the absence of insert DNA. The tubes were then left on ice for 30 min, before heat shocking them for 2 min at 37°C. The tubes were left for 10 min at room temperature before adding 100 μ l of 2x TYE to each of them. The cells were incubated for a further 30 min at 37°C, before being plated onto Ampicillin plates. The plates were incubated overnight at 37°C.

2.7 Sequencing of M13 clone inserts

The dideoxy chain termination method was employed (Sanger et al, 1977). 2 μ l of a M13 clone was placed in four wells (labelled T, C, G, A) of a 96 well microtitre plate. This was repeated for each clone to be sequenced. To each well 2 μ l of a primer mix was added. The mix comprised of:

1 μ l (0.2pmol) M13 sequencing primer
1 μ l of 100mM magnesium chloride
6 μ l water

The plate was centrifuged briefly, covered in Saran wrap and incubated at 55°C for 60 minutes. The condensation produced by the

incubation was collected by brief centrifugation. Four different nucleotide mixes (NTPs) were added to the wells.

	NTP mixes			
	T	C	G	A
0.5mM dTTP (μ l)	25	500	500	500
0.5mM dCTP	500	25	500	500
0.5mM dGTP	500	500	25	500
10mM ddTTP	50			
10mM ddCTP		8		
10mM ddGTP			16	
10mM ddATP				1
TE	1000	1000	1000	500

The T mix was added to the wells labelled T, the C mix added to the wells labelled C and so on. On completion, 2 μ l of NTP mixes had been added to each well. Each mix was a combination of deoxynucleotide triphosphates (dNTP) and dideoxynucleotide triphosphates (ddNTP). To each well, 2 μ l enzyme mix, comprising of:

1 μ l 100mM DTT
 0.5 μ l ³⁵S dATP 5 μ Ci/ μ l
 0.2 μ l 5U/ μ l Klenow DNA polymerase
 6.3 μ l water

was added and then incubated at room temperature for 15 minutes following which 2 μ l chase dNTPs (0.25mM dCTP, 0.25mM dGTP, 0.25mM dATP) were added to each well and incubated again at room temperature for 15 minutes. The sequencing reaction was terminated by the addition of 2 μ l formamide dye to each well and incubation at 80°C for 15 minutes. The contents of each well were loaded on the gel in the

order T, C, G, A. The gel was run for 140 minutes at 37 watts. On completion of the electrophoresis the glass plates were separated and the glass plate with the gel adherent to it was immersed in 10% acetic acid, 10% methanol for 15 minutes to fix the gel. The gel, after being removed from the fixer and drained, was transferred to 3MM Whatman filter paper and covered with Saran wrap. The gel was dried using a heated vacuum gel drier (30 minutes at 80°C). The Saran wrap was then removed and X-ray film exposed to the gel overnight at -70°C.

2.8 Transfections

2.8.1 Transfection of DNA into B cells by Electroporation

On the day prior to electroporation, the B cells were diluted 2-fold in fresh growth medium and left to incubate overnight. They were centrifuged at 1,300 r.p.m (500g) for 5 mins at 4°C and resuspended in cold growth medium at 3.2×10^7 cells/ml. The supernatant was saved as conditioned medium to be used later. 250 μ l of cell suspension (8×10^6 cells) was transferred into electroporation cuvettes (Bio-Rad) and 20 μ g of the appropriate DNA was added and mixed using a yellow tip. The cuvettes were then left on ice for 10 mins. The Bio-Rad "Gene Pulser" was used to electroporate the cells (250 volts, 960 capacitance) and the cuvettes were placed back on ice for a further 10 mins. 1 ml of conditioned medium was added to each cuvette and the cells were agitated carefully to break up clumps before they were transferred to tissue culture flasks. The cuvettes were then rinsed out with a further 2mls of conditioned medium and the cells were resuspended in a final volume of approximately 7 mls. The cells were left for at least 24 hours before testing for gene expression or applying drug selection.

2.8.2 Transfection of DNA into tsa-14 fibroblasts by calcium phosphate treatment

These experiments involved co-transfection of a second plasmid called pHYG (puc19 containing the gene for hygromycin resistance behind the HSV thymidine kinase promoter; Sugden et al., 1985), at a 10-fold lower concentration than pUC-MoM1V-LP. Drug selection was therefore likely to give clones which had also integrated the LP construct into the cellular DNA. On the first day, the fibroblasts were seeded into 60mm wide petri dishes (Nunc, Kamstrup, Denmark) at 5×10^5 cells/dish, and left to incubate overnight at 33°C, 10% CO₂. The next day the medium was sucked off and replaced with 4mls of fresh medium containing 15µg/ml of chloroquine. 1ml aliquots of precipitate were made up in sterile bijou bottles, in the following order :-

Sterile H₂O to 1ml

10µg of appropriate plasmid DNA

1µg of pHYG (Sugden et al., 1985)

0.5ml 2 x Hepes buffered saline

60µl 2M Calcium Chloride (added slowly at end)

The bijou bottles were allowed to stand at 37°C for 30 mins before adding the precipitates to the plates. The precipitate was left on the cells for approximately 6 hours at 33°C, before sucking off the liquid and washing the cells with 4ml of serum free Dulbecco's modified Eagle medium. 5ml of complete DMEM containing 10% FCS medium was added to each plate and the cells were again left overnight to incubate at 33°C. On the third day the cells were trypsinised, 2-fold into duplicate plates and then incubated at 33°C. Early on day four, the medium was replaced with medium containing 100µg/ml of hygromycin and all the plates were returned to the 33°C incubator. Later in the

day, one set of the duplicate plates was transferred to the 39°C incubator. From this point on, the cells were fed with medium containing 100µg/ml hygromycin.

2.9 SDS - PAGE and Western Immunoblotting

Whole-cell extracts were prepared by resuspending 10^7 cells per gel track in approx 20µl H₂O and 20µl 2 x sample buffer (0.0625M Tris, pH 6.8, 2% SDS, 0.1% 2-mercaptoethanol, 10% glycerol, and 0.02mM bromophenol blue). The extracts were sonicated for about 30 secs, if viscous, and boiled for 5 mins immediately before loading onto the gel. Proteins were electrophoretically separated on 1.5mm thick and 180mm long gels, consisting of 10% resolving gel and 5% stacking gel (acrylamide: bisacrylamide, 30: 0.8). Generally, 30µl of sample representing 2.3×10^6 cells was electrophoresed at 60V for 12hr. Proteins were electrophoretically blotted onto nitrocellulose (Towbin et al., 1979; Burnette, 1981; Fischer et al., 1984) and the nonspecific antibody binding sites on the filter were blocked with 5% skimmed milk in 150mM NaCl, 10mM Tris-Cl pH 8.0 (Johnson et al., 1984). The filters were then incubated at 4°C for at least 12hr with the appropriate antibody at the recommended dilution; for example the anti-EBNA polyclonal human serum, RM, was diluted 1/200 in Tris-saline, whereas the mouse monoclonal JF186 (Finke et al., 1987) was diluted 1/10. After washing in Tris-saline (10mM Tris-HCl pH 8.0, 150 mM NaCl), filters being analysed with polyclonal serum were probed for 4hr at room temperature with 50nCi/ml [α -¹²⁵I] protein A in Tris-saline, whereas those with mouse monoclonal antibodies were probed with 50nCi/ml sheep anti-mouse IgG conjugated with ¹²⁵I (both supplied by Amersham, UK). Prestained protein size markers were obtained from Bio-Rad.

2.10 Immunofluorescence

2.10.1 In Suspension Cells

Following electroporation, the B-cells were returned to the incubator for at least 24 hours before being tested for EBNA expression by immunofluorescence. The cells were then counted using a haemocytometer (Weber, England), washed once in PBS and resuspended at 6×10^7 cells per ml. $5\mu\text{l}$ (3×10^5 cells) were then spotted onto a slide and allowed to air dry, before fixing the cells in Acetone: Methanol (2:1) for 5 mins. The spots were then covered with $20\mu\text{l}$ of RM serum diluted 1/10 in PBS and incubated at 37°C for 30 mins. At no point following this were the slides allowed to dry out. After the excess serum was blotted off, it was replaced by $20\mu\text{l}$ of human complement diluted 1/8 in PBS, and the slides returned to 37°C for a further 30 mins. Three 10 min washes in PBS were then carried out before spotting on $20\mu\text{l}$ of fluorescein isothiocyanate (FITC) conjugated goat anti-human C3 complement (Atlantic Antibodies, Scarborough, ME, USA) diluted 1/30 in PBS. Following a further 30 mins incubation at 37°C and then washing as before, the slides were rinsed in distilled water. The spots were finally mounted in PBS-buffered glycerol before placing cover-slips on top. The slides could then be stored at -20°C before looking for immunofluorescence.

2. In fibroblasts

Circular cover slips were placed in 24 well plates before 0.5ml of cells (2×10^4 cells/ml) was pipetted on top. Plates were incubated overnight to allow the fibroblasts to adhere to the cover slips. The slips were subsequently handled using forceps and at no time were they allowed to dry out. First, the slips were washed by dipping them

in Hanks Staining Solution followed by sterile water. The cells were then fixed by placing 100 μ l of Methanol on top and storing them at -20°C for 20 mins. The slips were blotted before putting on 50 μ l of RM serum diluted 1/10 in PBS. The same procedure was then followed as with the B cells, except that the intervening washes were all done in Hanks Staining Solution and water. Finally the cover slips were placed onto slides which had DABCO antifade on them, and finally secured in place using nail varnish. Slides were then stored at -20°C.

Chloramphenicol Acetyl Transferase (CAT) Assay

Following transfection (normally 24-48 hours) the cells were counted, washed once in 1xPBS, and then resuspended in 0.25M Tris-HCl pH 7.5, at 10⁶ cells/ 10 μ l. The cells were then freeze-thawed three times(5 mins at 37°C, then 5 mins in dry ice/ethanol bath) and subsequently then placed at 65°C for 10 mins. Cell debris was centrifuged out at 2,000 rpm for 10 mins at 4°C in the microfuge, and the supernatant transferred to a new tube. The reaction mix was as follows :

25 μ l cell extract
2 μ l ¹⁴C Chloramphenicol (25 μ Ci/ml)
5 μ l 5mM Acetyl Coenzyme A (Sigma)
18 μ l Water

50 μ l

The reaction was incubated for 1 hour at 37°C. The chloramphenicol was extracted by adding 1 ml of ethyl acetate and transferring the upper phase to a new tube. This was then lyophilised and redissolved in 20 μ l of ethyl acetate. The ethyl acetate was finally spotted onto

cellulose Thin Layer Chromatography plates (Merck, Darmstadt, W. Germany) and the plates placed in a chromatography tank containing 200ml of chloroform:methanol (95 vol: 5 vol). After the solvent front had reached the top of the plates, they were taken out, air-dried and exposed to X-ray film.

In vitro transcriptions and translations

For the in vitro transcription experiments, the Promega SP64/65 plasmids were employed, in which transcription is directed from the SP6 promoter (SP6 RNA polymerase supplied by New England Biolabs). Prior to transcription, 10 μ g of the plasmid DNA was linearised at a restriction site downstream of the cloned sequences, and the digestion checked on a mini-gel. Following phenol/chloroform extraction and ethanol precipitation, 5 μ l of DNA (approx 0.5 μ g/ μ l) was added to 45 μ l of transcription mix and incubated at 40°C for one hour. In this reaction the RNA yield was approx 5-10 μ g of RNA per μ g of plasmid.

Transcription Mix (45 μ l)

5 μ l 10 x Transcription Buffer

5 μ l 5mM ribonucleotide triphosphates (ATP, UTP, CTP, GTP)

5 μ l 0.1M DTT

2.5 μ l CAP (7-methyl-guanosine triphosphate) from Boehringer Mannheim

1.25 μ l (35 units) RNasin Ribonuclease inhibitor (Promega)

2.5 μ l (>100 units) SP6 RNA polymerase

23.75 μ l H₂O

After a further 15 mins incubation at 37°C with 10 units of DNase I (Pharmacia), the RNA was phenol/chloroform extracted and ethanol precipitated prior to *in vitro* translation.

As EBNA-LP contains no cysteine residues and has only the initiator methionine which may be cleaved off following translation, it was not possible to label with ^{35}S -Cys or ^{35}S -Met, which are the commonly used radiolabelled amino acids in *in vitro* translation experiments. Instead, L-[2,3- ^3H] arginine from Amersham was used, as Leader protein is rich in this particular amino acid. Rabbit reticulocyte lysate, depleted in amino acids, was used for the translations (Amersham N.150 lysate) and a 20mM stock solution of amino acids, minus Cys, Asn, Lys and Arg, was prepared. 20 μl of ^3H -Arg (1 $\mu\text{Ci}/\mu\text{l}$; 54Ci/mM) was lyophilised and resuspended in 5 μl of 30 μM Arg, 2M potassium chloride. 5 μl of the amino acid stock solution and 80 μl of lysate was added and this mix was sufficient for 10 translation reactions, each consisting of 9 μl mix and 1 μl RNA (1.25ng). The reactions were incubated at 30°C for 30-60 mins, then terminated by adding 5 volumes of SDS-PAGE sample buffer. If the proteins were not to be analysed on a polyacrylamide gel straight away, they were stored at -70°C. Sample buffer was not added if they were going to be used in functional studies, such as Rb binding.

Immunoprecipitation

Rabbit reticulocyte lysate containing *in vitro* translated Epstein-Barr virus Leader Protein (^3H -Arg labelled) and Retinoblastoma (Rb) protein (^{35}S -Met labelled) were mixed together (35 μl of each) and kept on ice for 1-2 hours. To this was added 8 μl of the C36 monoclonal antibody (Whyte et al., 1988), which recognises the Rb protein. This was left for a further 1 hour on ice. 65 μl of

Protein A sepharose beads (from Pharmacia) was now added and these were mixed by rotating the tube for 1 hour at 4°C. The beads were then washed three times in buffer at room temperature.

Buffer for washing Protein A sepharose beads

250mM	Sodium chloride
50mM	Hepes pH 7.0
1mM	Phenylmethylsulphonyl fluoride
5mM	EDTA
0.5mM	DTT

Following transfer to a new tube, the beads were rotated for a further 10 mins at 4°C and then washed twice as before. Finally, 65µl of SDS sample buffer was added, the proteins were boiled for 10 mins and the samples were loaded onto a 10% polyacrylamide gel. The gel was run overnight at 40 volts and then fixed in 10% Acetic acid-45% Ethanol (3x30 mins) and enhanced with "Enlightning" fluid (Biotechnology Systems, Boston, MA.). The gel was then dried down and exposed to X-ray film.

Measurement of ³H-Thymidine Incorporation

In the doubling dilution experiments, B cells immortalised with 633 virus were plated out in 96-well plates at a volume of 200µl of RPMI 1640, containing ³H-thymidine, to each well. The final concentration of label (supplied at 25 Ci/mM) was 5µCi/ml and this was left on the cells for 4 hours. In the second set of experiments, 2ml of growth saturated cells was diluted two fold and then 200µl aliquots were dispensed into 96-well plates. Cells were then pulse labelled with ³H-thymidine at 2 hour intervals, ie 0-2 hrs, 2-4 hrs, 4-6 hrs, 6-8 hrs, 8-10 hrs after dilution. The cells were harvested on to glass

fibre filters using a cell harvester (Skatron Ltd, Suffolk, England). The wells were flushed briefly with water, which simultaneously lyses the cells and sucks the contents of the well onto the filter. This is followed by a five second wash in water, which removes the unincorporated label, while the labelled DNA remains on the filter. A second five second wash in methanol was tested (pers. comm. S. Pickup) to fix the DNA to the filter, but it was found that this made no difference to the eventual counts. An alternative method in which the DNA was T.C.A. precipitated onto the filters following harvesting with either 1 x P.B.S. or water was also tested, but again this did not change the counts. The filters were air-dried and added to scintillation vials before determining the counts per minute with a scintillation counter (Beckman, Irvine, CA).

Size and Stability of the Epstein-Barr Virus Internal Repeat (IR-1)

3.1 Structure of the B95-8 major internal repeat (IR-1)

Variable protein sizes for EBNA-LP have been detected by several researchers (Dillner et al, 1986; Finke et al, 1987; Rowe et al, 1987; Wang et al, 1987), and a variety of mechanisms utilising alternative splicing and promoter usage or different numbers of IR-1 repeat units have been suggested to account for these observations. The relationship between the number of 66 amino acid repeats in Leader Protein and the repeat copy number in the viral genome has not been defined previously.

As a basis for investigating this phenomenon, experiments were undertaken to determine the number of IR-1 repeat units within the B95-8 genome. Total cell DNA was digested with the restriction enzyme EcoRI or double digested with EcoRI and HindIII. These enzymes cleave EB viral DNA at sites flanking IR-1 (shown in Fig 1.3 B) generating a high-molecular-weight restriction fragment containing IR-1. Fragments that are 20 kb or more cannot be resolved by conventional agarose gel electrophoresis, so these digests were analysed by field inversion gel electrophoresis (FIGE) (Carle et al, 1986) tuned to separate molecules in the range of 10 to 60 kbp (Figure 3.1). The gels were Southern blotted and probed with the IR-1 repeat sequence (BamHI W fragment). A fragment containing IR-1 that was produced by an EcoRI single digest was extrapolated to be 52,600 bp in length, whereas an EcoRI - HindIII double digest produced a fragment of 31,500 bp, (Figure 3.2 A). Since there are 4685 bp of unique short region DNA between the 5' EcoRI site and the start of the repeats and 2247 bp of

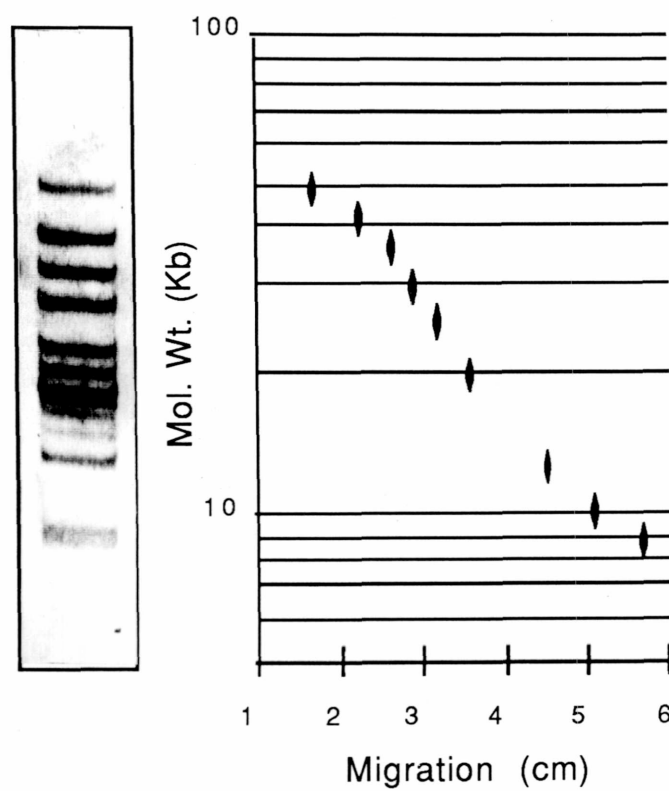


Figure 3.1 The left-hand panel is a field-inversion gel showing the resolution of end-labelled high-molecular-weight DNA size markers in the range of 48.5 kbp down to 8.6 kbp (Bethesda Research Laboratories). The right-hand panel is the plot of fragment size against distance migrated on the gel.

unique long region DNA between the end of the last full repeat and the 3' HindIII site (including the 1851 bp partial repeat found at the 3' end of IR-1), this leaves approximately 24,300 bp made up entirely of repeats within the EcoRI-Hind III IR-1 fragment. As each repeat has a size of 3072 bp, it was possible to estimate that the B95-8 viral strain has 8 full repeats in IR-1. Thus, including the partial repeat, the large internal repeat region of the viral DNA in these B95-8 cells contains 8.6 repeats.

In these experiments the EcoRI single digests provided a useful double check, but were particularly relevant in analysing the size of the IR-1 in mutant virus isolates (discussed later), where there is a deletion at the 3' end of the IR-1 that removes the HindIII site. The broad, smeared band of hybridisation seen at about 20 kb is thought to result from shearing of the EBV DNA during isolation and varied from preparation to preparation.

These results differed from previous estimates of 12 repeat units in the B95-8 genome (Hayward et al, 1980). This estimate was based on low percentage agarose gel migrations of genomic fragments obtained from virion DNA. Because of this, the experiments described here were repeated on DNA obtained from virions (Fig 3.2 B) and on B95-8 cell lines obtained from three other laboratories (Fig 4.2 in Chapter 4). Again, a 31.5kbp fragment containing the repeats was detected, which corresponded to a repeat copy number of 8.6. Thus, in this analysis, repeat copy number was not observed to change between cell-associated and virion-derived genomes.

3.2 Repeat copy number variations as a consequence of deletion

The non-immortalising mutant P3HR-1 strain of EBV arose as a clonal

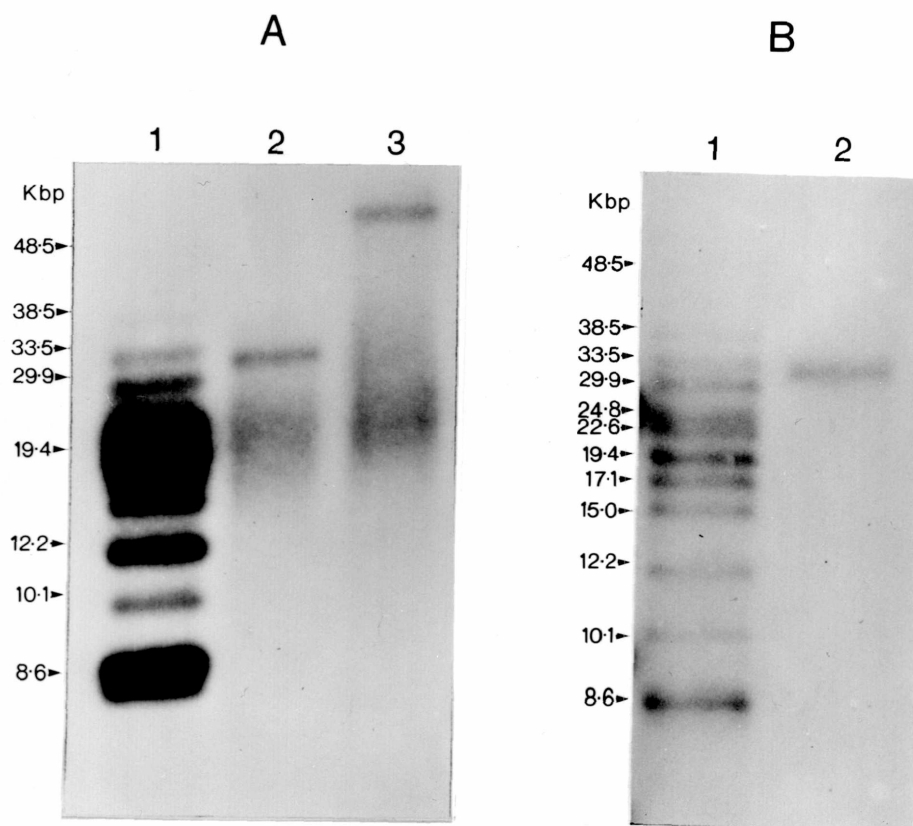


Figure 3.2 Southern blots of Field Inversion Gels of B95-8 cells. Lane 1 of both panels contains $[\alpha\text{-}^{32}\text{P}]$ -end labelled high molecular weight markers ranging from 48.5 to 8.6 kbp. A) lanes 2 and 3 are EcoRI-HindIII and EcoRI digests respectively of B95-8 cellular DNA. B) the virion DNA (lane 2) has been digested with EcoRI-HindIII. Both blots were probed with nick-translated BamHI W.

variant of the Jijoye cell line (Hinuma et al., 1967). The viral genome in P3HR-1 cells has undergone a deletion of sequences in the U_L region at the right side of IR-1 which removes the entire EBNA 2 gene and the 3' end of EBNA-LP (Fig 1.3 C,D) (Miller et al., 1974; Menezes et al., 1973; Raab-Traub et al., 1978; Rabson et al., 1982). Although both ends of the deletion have been sequenced (the deletion removes the (1851 bp) partial copy and 149 bp from a complete repeat unit) (Jeang and Hayward, 1983) the true size of the deletion is unknown because the number of repeats that have been lost has not been established.

To determine the true extent of this deletion, the repeat copy number of IR-1 in the parental Jijoye BL cell line and the P3HR-1 line were examined by the FIGE method (Fig 3.3). The measured sizes of the IR-1 containing fragments from these lines imply a repeat copy number of 6.6 in Jijoye, but only 5.9 in P3HR-1. These results show that the deletion only extends in to the most 3' full repeat of the parental Jijoye virus indicating that none of the W1 or W2 exons have been lost (see Fig 1.3 C,D). The size of the deletion is therefore 6806 bp.

Examination of a human umbilical cord LCL, JC-5 (Miller et al., 1974), established with Jijoye virus revealed multiple bands, which presumably represent subpopulations of genomes with five, six, or eight repeats in IR-1 (Fig 3.3). The cloning of P3HR-1 has produced a strain, C-16, which retains the same number of repeats as P3HR-1, but also contains a smaller fragment containing IR-1 sequences. The C-16 DNA was probed with part of the EBNA 2 sequence and this demonstrated that the lower fragment also lacks the sequences deleted from P3HR-1 (Fig 3.4). Analysis of BamHI digests of C-16 do not reveal any genomic rearrangement similar to "het" DNA (Rabson et al.,

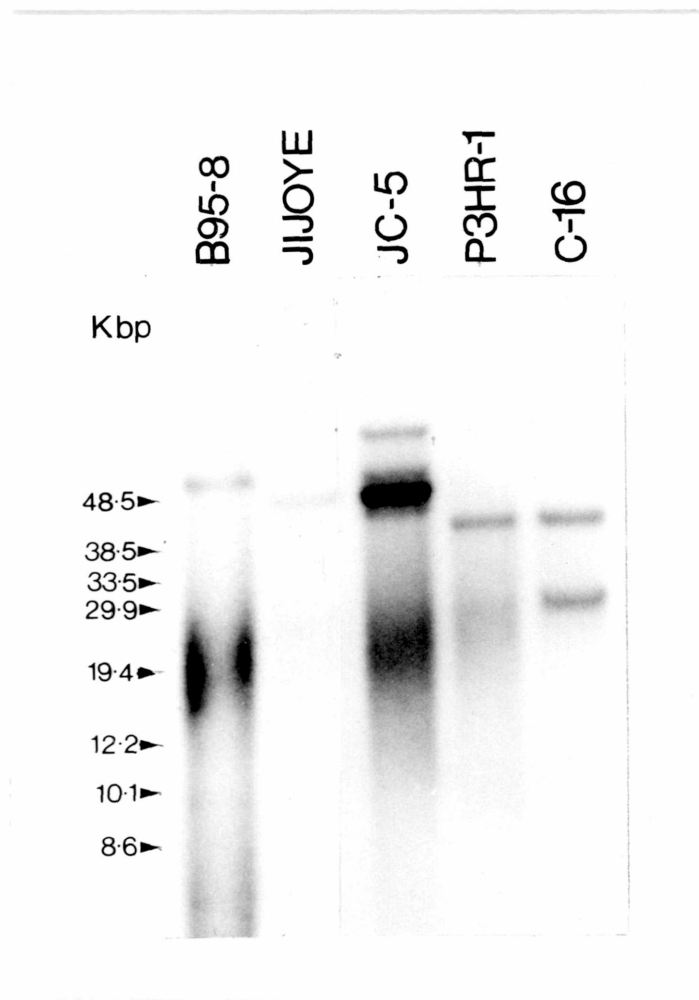


Figure 3.3 Sizing of the P3HR-1 deletion. Southern blot of field inversion gel comparing EcoRI digests of Jijoye BL and cord cell line JC-5, made with Jijoye virus, and the deletion clone P3HR-1 along with its subclone C-16. The blot was probed with BamHI W. The size of the IR-1 containing EcoRI fragment in Jijoye is 48,500 bp. There is 4658 bp of unique short region DNA between the 5' EcoRI site and the start of the repeats and 23,327 bp of unique long region DNA between the end of the last full repeat and the 3' EcoRI site. Therefore, 20,488 bp is made up entirely of repeats. This estimates 6-7 full repeats in Jijoye. An EcoRI-HindIII digest produced a 25,500 bp IR-1 fragment in Jijoye which gives a more accurate estimate of 6 full repeats (data not shown). The EcoRI fragment in P3HR-1 is 41,000 bp. This is a drop in size of 7,500 bp compared to the parental Jijoye virus fragment (close to the predicted 6,806 bp deletion size). This means that the P3HR-1 deletion only extends 149 bp into the most 3' full repeat of Jijoye. Therefore, P3HR-1 contains 5.9 repeats.

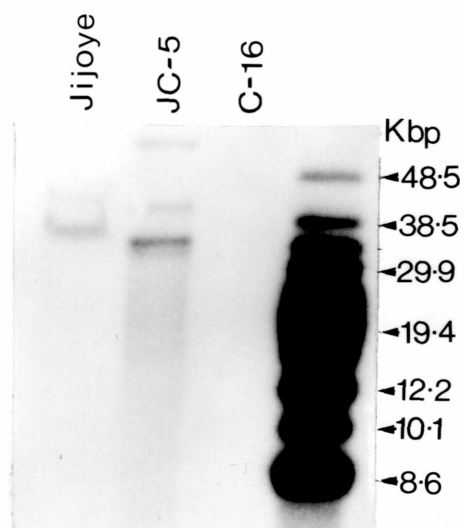


Figure 3.4 Southern blot of a field inversion gel comparing EcoRI digests of Jijoye, JC-5 and C-16. On the right are [α - ^{32} P]-end labelled high molecular weight markers. The blot was probed with a nick-translated M13 clone, 142.JOY, which contains EBNA 2 sequences from Jijoye EBV.

1982). If the shorter fragment has only lost IR-1 DNA, the C-16 EBV molecules from which this was derived would appear to contain between 1 and 2 internal repeat units. This might yet represent a form of rearranged viral DNA in the C-16 cells.

3.3 Stability of repeat copy number in different viral isolates

To establish whether there is much interstrain variation and perhaps derive an average repeat copy number for EBV, several other cell lines containing EBV genomes were analysed as before. These lines contained viral genomes which had a range of repeat copy numbers from 3 to 12. In addition, multiple bands appeared in some lines (WW-1, JC-5, BL18, BL74, and BL29). The repeat copy number of several viral isolates and the predicted maximum size of EBNA-LP proteins possible from this coding capacity are summarised in Table 3.1. The data supporting this is shown in the various field inversion gels presented in this Chapter (the data is not shown for EB3). The observed protein sizes appear to be within the range of the IR-1 coding capacity. Some cell lines carry only one detectable size of IR-1 (for example, B95-8 and Raji) and yet express multiple species of EBNA-LP protein. This suggests that differential W promoter usage and/or alternative splicing, rather than multiple genomes, is largely responsible for the size variations in EBNA-LP. The distribution of repeat copy number in all the cell lines that were investigated is shown in Fig 3.5. This represents 31 different genomes from 17 different cell lines. There appears to be a wide variation in repeat number in naturally occurring isolates with a normal distribution around a mean of 6 repeats.

Some indication that changes in the average IR repeat copy number might occur when EB virus was induced from latently infected cells

Table 3.1

CELL LINE	REPEAT NUMBER	MAXIMUM PREDICTED PROTEIN SIZE	OBSERVED PROTEIN SIZE
B95-8	8.6	60kDa	66,60,54,48,42 kDa (Finke et al., 1987) 41,36 kDa (Rowe et al., 1987)
Raji	7.6	54kDa	51,47,44,42,37 kDa (Fig 3.7, C; Allan and Rowe, 1989)
Jijoye	6.6	47kDa	35 kDa (Finke et al., 1987)
JC-5	12.6	88kDa	62,53 (major), 38 kDa
JC-5	7.6	54kDa	(Rowe et al., 1987)
JC-5	6.6	47kDa	
JC-5	5.6	40kDa	
P3HR-1	5.9	35kDa	27 kDa (Wang et al., 1987) 20 kDa (Finke et al., 1987)
C-16	5.6	35kDa	48,28 kDa (major) (Rowe et al., 1987)
C-16	1.6-2.6	12-18kDa	
EB-3	3.6*	26kDa	N.D.

N.D., not detected with the JF186 monoclonal antibody (Finke et al., 1987)

* Unique region deleted unknown.

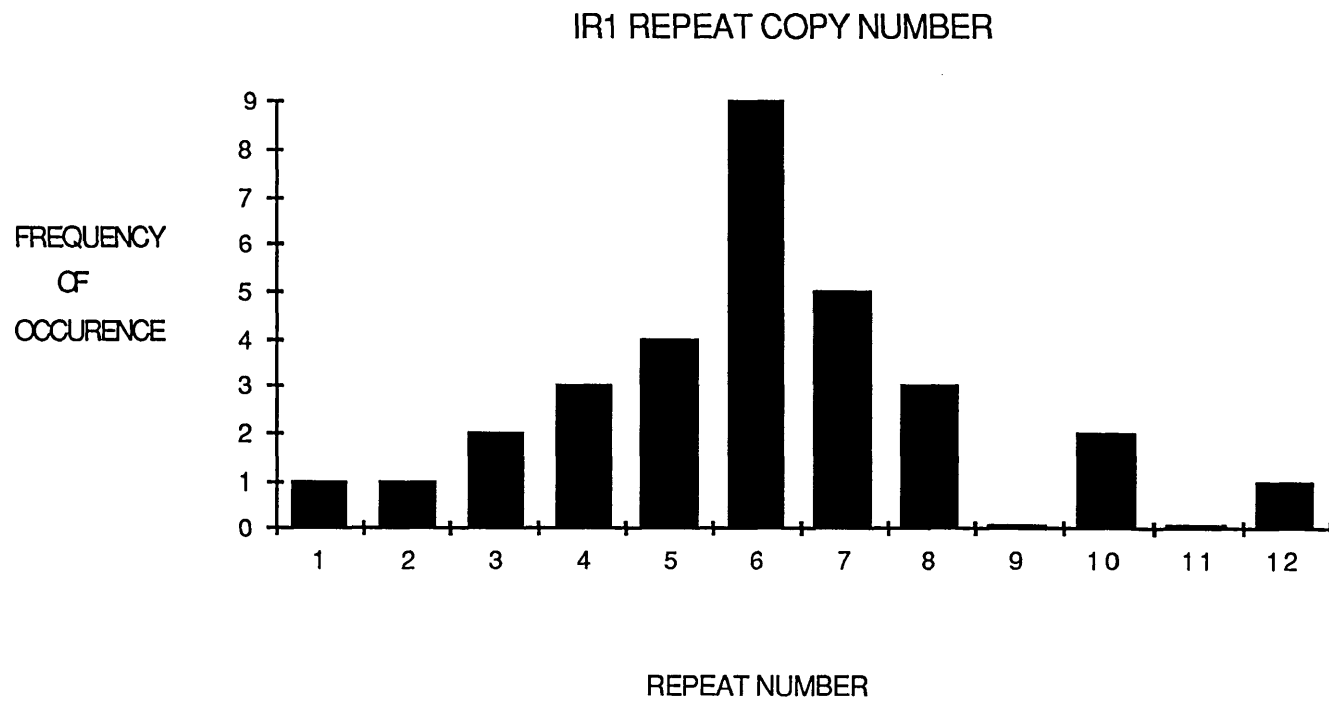


Figure 3.5 Cumulative distribution of repeat copy number in all EBV genomes characterised.

and then used to infect and immortalise fresh lymphocytes had already come from a comparison of the lymphoblastoid cell line (LCL) JC-5 and the parental BL line Jijoye (Fig 3.3). Establishing new LCLs with virus from the BL lines requires the genome to undergo lytic replication, and analysis of the established lines indicates the effect of this on IR-1 repeat number. It was necessary therefore, to compare repeat copy numbers in other established BL/LCL cell line pairs using the same strategy as before (Fig 3.6). With Wewak-2 virus, there was no change in repeat number between the BL and the corresponding LCL. Similarly for Wewak-1 and BL74, where there were two and three separate genomes respectively in the BL lines, there was no alteration in the sizes of the bands in the LCLs; a marked change in the relative abundance of each genome type has occurred with BL74. In the LCLs derived from both BL18 and BL29, multiple genome copies have arisen with different repeat numbers compared to the single genome in the parental BL line.

Although I did not pursue this point further, the clonality of cell lines containing multiple EBV episomes with respect to the IR-1 region could also be addressed by analysis of the fused EBV termini (Raab-Traub and Flynn, 1986; Brown *et al.*, 1988). A single terminus type would confirm that the variation in IR-1 in these lines developed after circularisation and amplification of the EBV genomes.

3.4 Stability of repeat copy number during latency

Another source of variation in IR-1 might involve the maintenance replication of the genome that occurs during latency. To test whether the size of IR-1 was stable during latency, samples of the latently infected cell line, Raji, were collected from different laboratories, where the cells have been cultured in isolation for long period (>15

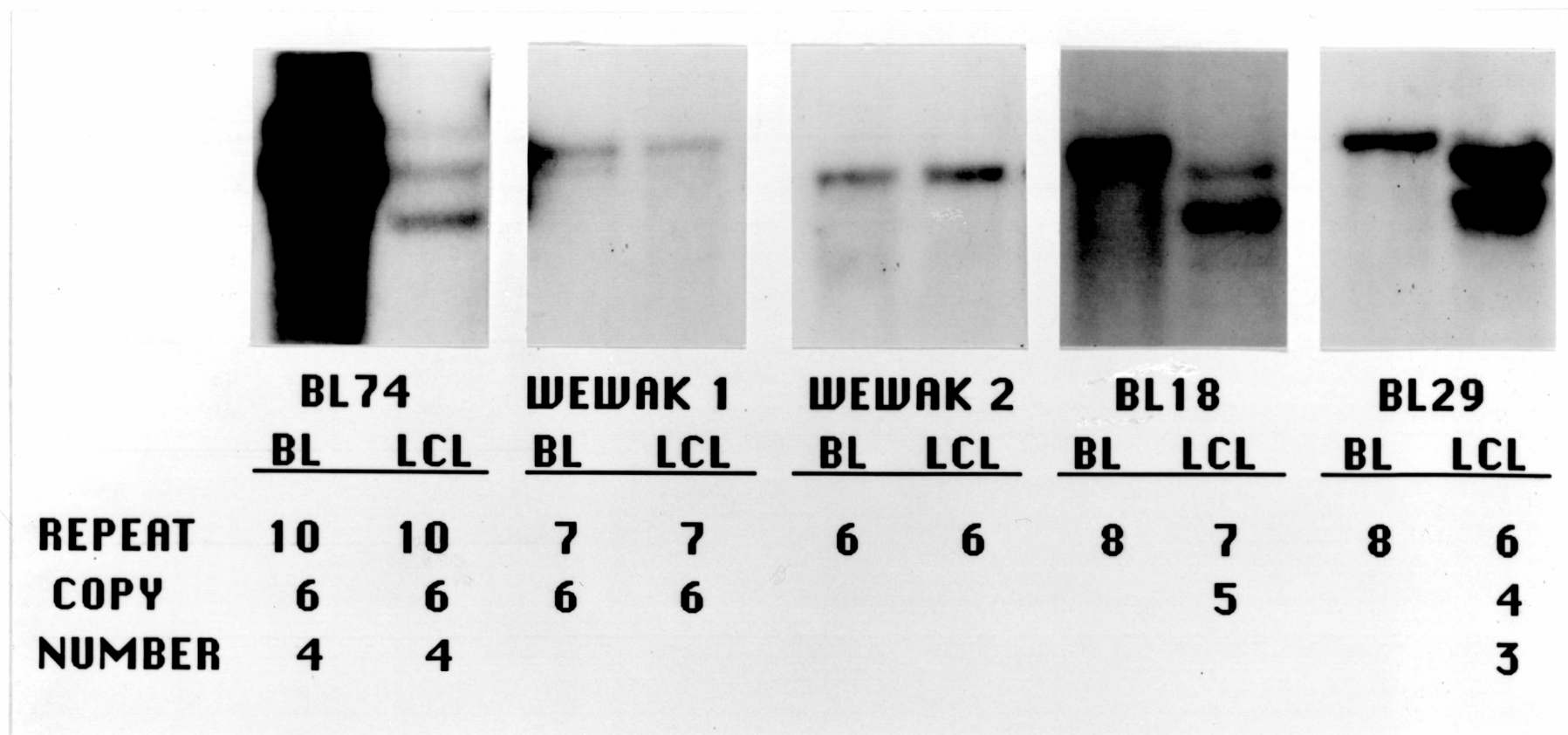


Figure 3.6 Change in repeat copy number in lymphoblastoid cell lines (LCL) produced by infection with virus from Burkitt's lymphoma lines (BL). Southern blots of field inversion gels comparing EcoRI-HindIII digests of BL and LCL cell lines, probed with BamHI W.

years in some cases). Raji is a strain of EBV which has a defect in lytic replication; induced Raji cells produce early antigens, but no viral capsid antigens and have no productive viral DNA replication (Polack et al, 1984). Thus, the horizontal spread of Raji virus within this cell line is extremely unlikely, and maintenance of the genome must depend entirely upon latent replication of EBV.

In order to verify that all these cell lines were indeed Raji, their latent proteins were analysed on Western blots probed with a human anti-EBNA serum (RM) (Fig 3.7 B). The sizes of EBNA 1 and EBNA 2 proteins vary among EBV strains and may be considered as characteristic of a virus strain. Since the size of these proteins was the same in all the collected samples, there was a strong indication that all the lines were Raji. In addition, the EBV genomes in all the lines contained the deletion in the BamHI E fragment (Polack et al, 1984; Hatfull et al, 1988), which is characteristic of Raji (Fig 3.8).

The FIGE analysis of the DNA of eight isolates (seven of which are shown in Fig 3.7 A) demonstrated that (with one exception; kindly provided by G. Miller) the IR-1 repeat copy number had remained constant at seven. The one isolate which appeared to have more repeats (9-10 repeats) was checked for variation in size of the unique sequences flanking IR-1 and for the loss of the EcoRI or HindIII restriction sites used in the analysis. Both restriction sites were present and there was no indication of insertions into the flanking regions that would account for the increased size of the band (data not shown). It seems from these results that during latent episomal replication there is little opportunity for change in the repeat number within IR-1. The one Raji isolate containing more

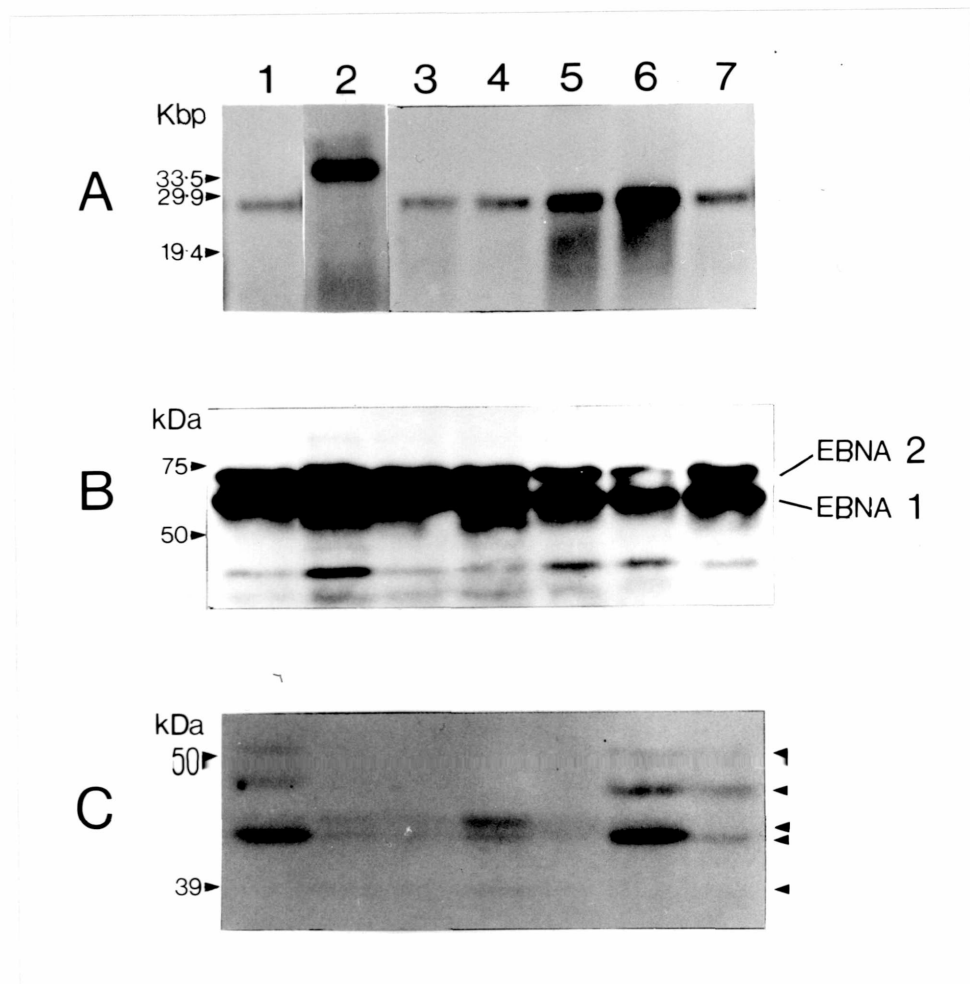


Figure 3.7 Stability of repeat number in latently infected Raji cells. A) southern blot of field inversion gel. Lanes 1-7 contain EcoRI-HindIII digested total cellular DNA from Raji's obtained from different laboratories. B) and C) show Western blots of whole cell extracts from the same Raji strains as above, probed with human anti-EBNA serum, RM, and the murine monoclonal, JF186, respectively. Positions of molecular weight standards are indicated.

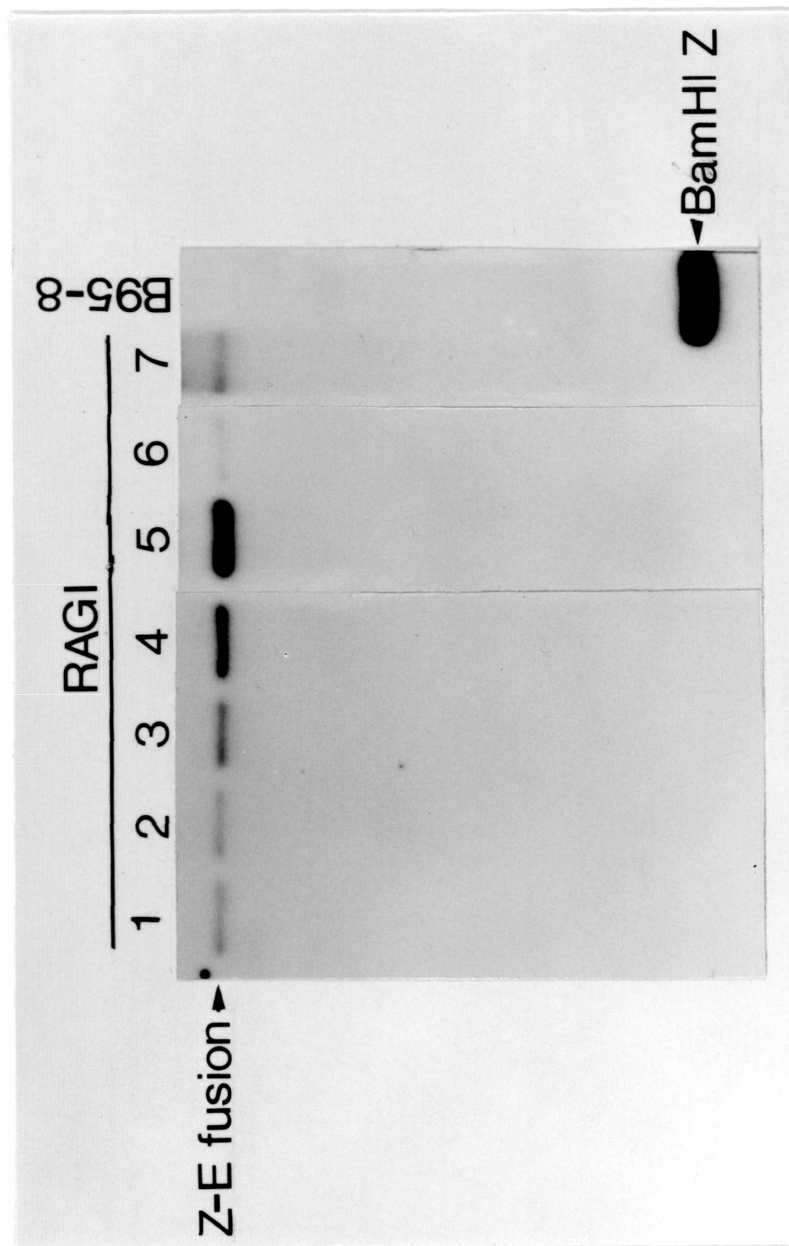


Figure 3.8 Identification of the Raji E-Z deletion. Southern blot of a gel containing BamHI digests of total cellular DNA. Lanes 1-7 are the various Raji cell lines obtained from different laboratories, while the right-hand lane is B95-8. The blot was probed with nick-translated BamHI Z. A band of 1.8 kbp representing the BamHI Z fragment can be seen in the B95-8 lane. The deletion in Raji removes the BamHI site between Z and E fragments, creating a fusion fragment of 8 kbp.

repeats may have arisen from a recombination event in the latently infected cells.

Western blots of the Raji lines were also probed with a monoclonal antibody JF186 (Finke et al, 1987), which recognises an epitope encoded by the W1 exon in EBNA-LP (Fig 3.7 C). Curiously, this antibody detected two distinctive banding patterns of EBNA-LP among the Raji strains (compare lanes 1, 6 and 7 with lanes 2, 3, 4 and 5). One pattern consisted of specific protein bands of molecular weight 51, 47, 44 and 42 kDa. The other group appeared to express proteins of 44, 42 and 37kDa. It should be noted that the one isolate which contains more repeats, number 2, shared this second pattern of EBNA-LP banding.

3.5 EBNA-LP clones with different numbers of repeats

Following the IR-1 repeat copy number analysis, it was interesting to speculate whether the highly repetitive structure of Leader Protein is important for its function. Therefore, the IB4-WY1 cDNA clone (Speck et al., 1986) within SP64 was digested partially with the restriction endonuclease StyI, which cuts once within each repeat. These digests were religated to generate a set of SP64 constructs containing LP cDNAs with 2 to 7 repeats. These could be in vitro transcribed and translated to produce a series of Leader Proteins which increase in size in a stepwise manner (Fig 3.9). The StyI site is lost in the first repeat due to the W0W1' splice, but a 1 repeat clone was also obtained by removing a SmaI fragment from the 2 repeat construct. It is hoped that these clones can eventually be used to determine the relevance of the repetitive structure of LP when its function/s are finally known. In addition, this demonstrated that in

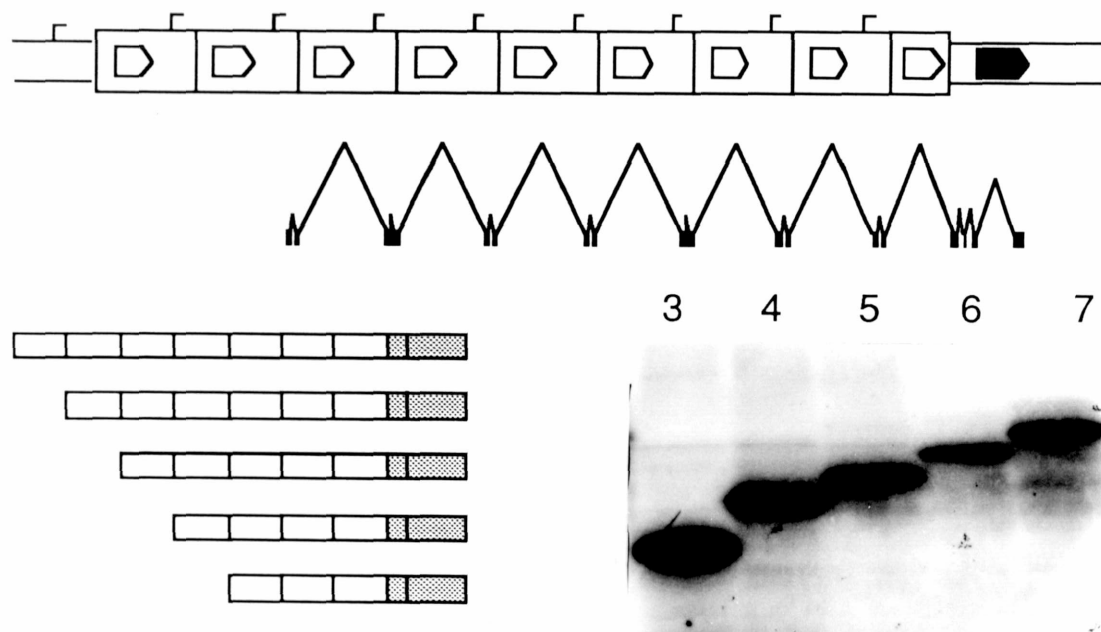


Figure 3.9 Leader Protein Ladder. In vitro translated Leader Proteins with varying repeat copy numbers (3-7) are shown in the lower right panel. The proteins were translated in rabbit reticulocyte lysate, labelling with ^3H -Arg, electrophoresed and autoradiographed. The proteins are represented schematically on the lower left, where white boxes are the repeats and the shaded boxes are the unique C termini. The upper figure represents the major internal repeat region of B95-8 EBV; below is one possible splicing pattern for producing a 7 repeat LP mRNA.

vitro translated Leader Proteins migrate on denaturing protein gels at their predicted sizes.

3.6 Discussion

This work has characterised the repeat copy number in the major internal repeat region (IR-1) of Epstein-Barr virus in cell lines representing a variety of viral isolates. There was a range of repeat copy numbers between the different strains that varied between less than 1 full repeat up to 12 repeats, with a mean copy number of 6. In B95-8 genomes there were 8.6 repeats, which is less than a previously reported estimate of 12.6 (Hayward et al., 1980). Although this study also analyzed the size of EcoRI-HindIII fragments from virion DNA which contain the IR-1 region, 0.3% agarose cylindrical gel electrophoresis was used, which is not as accurate as field inversion gel electrophoresis for resolving high-molecular-weight fragments. Analysis of purified virions from B95-8 cells revealed only the presence of an 8.6 repeat IR-1.

Leader Protein is almost entirely encoded within the repeat region. Although there was a general trend for smaller proteins to be made from genomes with fewer repeats, there was not a strong correlation between repeat copy number and the size of Leader Protein observed (Table 3.1). It is easy to speculate how smaller proteins could arise from a potentially larger coding capacity in the repeats. Expression of a shorter mRNA could be achieved either from transcription initiation at any of the internal repeat W promoters or by splicing out of excess repeats (see Fig 1.3). Alternative splicing or promoter usage has been offered as an explanation for the ladder pattern of Leader Proteins seen in some cell lines run on Western blots (Finke et al., 1987).

It is more difficult to explain the presence of Leader Proteins which are much larger than the theoretical size range determined from the

repeat copy number (Finke et al., 1987, reported 130kDa Leader Proteins from B95-8 virus, although the FIGE analysis presented here would predict a maximum size of 60kDa). To account for this, it has been hypothesised that the unusual primary structure of Leader Protein may result in anomalous gel migration (Finke et al., 1987). In the work here it is clear that in vitro translated LPs migrate on denaturing gels at their predicted sizes (Fig 3.9). In addition, one of these clones was expressed in vivo by transfection (Fig 5.5; Fig 5.8 in Chapter 5) and the migration of the protein was the same as before. Therefore, post-translational modification of LP in vivo does not appear to alter the mobility of the protein in SDS-gels. This would imply that the observed size of LP in different EBV strains is directly related to the number of repeats in the protein. However, there may be a mechanism for producing these larger proteins which involves as yet undetected splicing events between sequences originating in the repeat region and large open reading frames downstream (Walls and Gannon, 1988). The heterogeneity observed in repeat number raises important questions about the nature of LP. For example, if the highly repetitive structure of the protein is important for its function, then do viruses with more or less repeats have different interactions with host cells?

Experiments presented later in Chapter 5 show that the naturally occurring deletion in the P3HR-1 virus affects LP function. The FIGE analysis has determined that this deletion only extends into the most 3' IR repeat. Therefore, this 6806bp deletion removes the partial repeat and all the 3' unique sequences for EBNA-LP (as well as the entire EBNA 2 gene), but does not remove any of the W1 or W2 exons of the parental immortalising virus, Jijoye (Fig 1.3 C,D). The assumption that the inability of P3HR-1 to immortalize lymphocytes in tissue culture is due to the loss of EBNA 2 only (Dillner et al.,

1985; Hennessy et al., 1985; Hammerschmidt and Sugden, 1989) does not take into account there being a truncated LP which may or may not retain its function (discussed in more detail later). In this analysis, C-16 virus generates a second lower EcoRI-HindIII fragment, which contained IR-1 sequences (1-2 repeats), but none from within the P3HR-1 deletion (Fig 3.4). EBV DNA from the parental Jijoye cell line may be particularly susceptible to rearrangements. Polymerase chain reaction analysis has shown that in vivo rearrangements of EBV genomes in the region of the P3HR-1 deletion may be a more common event than previously realised (Patton et al., 1990).

In certain circumstances the number of IR repeats can increase or decrease. This is most evident in the comparison between the genomes in Burkitt's lymphoma cell lines and lymphoblastoid cell lines transformed with the BL viruses (Fig 3.6). While there was no change in some cell lines, for example Wewak-2, other LCLs derived from BL18, BL29 and Jijoye had generated multiple genome copies with different repeat numbers compared to the single genome in the parental BL line. A multiple infection may have occurred in the generation of the LCL from BL74, because the same three genomes are present in both the BL and LCL lines (although there is a change in the relative abundance of each type). Alternatively, if the LCL is clonal, then the same three genomic forms detected in the BL line were apparently spontaneously generated again in the LCLs. From these results, it appears quite common for the dominant number of repeats to change through the process of induction and reinfection. This may imply a fluidity in repeat copy number in the lytic cycle of the virus.

In contrast, it was found that the repeat copy number is relatively stable in different cultures of the Raji cell line, where there is no virus production and genomes must be transmitted vertically as the

cells grow in culture. Use of the JF186 monoclonal antibody (Finke et al., 1987) demonstrated that there were two distinct LP banding patterns in the Raji strains. Therefore, it appears that heterogeneity in EBNA-LP expression is possible even when the IR-1 repeat copy number is constant.

Expression of EBNA-LP4.1 Structure of Large EBNA-LP cDNAs

The work described so far had shown that the repeat copy number of IR-1 in a given EB viral strain does not always correlate with the reported size of Leader Protein expressed from this region. Most of the published EBV cDNA sequences which contain W1 and W2 exons from IR-1 are either partial (in the sense that the 5' end is missing) or contain very few W1 W2 exons, accounting for less than half the potential number of exons identified in IR-1. This has led to the intriguing suggestion that not all of the potential exons within any one transcript can be used (Sample et al., 1986). As a means of analysing the splicing mechanism of IR-1 and the potential for EBNA-LP protein expression, large cDNA clones (13.13 and 13.19; kindly provided by M. Perricaudet), that contained many W1, W2 repeats were characterised .

13.13 and 13.19 had been isolated from a B95-8 cDNA library using a probe prepared by randomly primed labeling of the pDK10 plasmid which contains part of the BamHI C fragment of EBV DNA around the C1 exon (Bodescot et al., 1986). In order to determine how many W1 W2 repeats were present in each cDNA, the clones were linearised by complete HindIII digestion, and then partially digested with low concentrations of the restriction enzyme SmaI, which cleaves once in every W1 exon. The digests were run on agarose gels containing ethidium bromide (0.5µg/ml) and were then photographed on an ultra-violet light box. The restriction digest pattern was identical for both 13.13 and 13.19, and the results of the 13.13 partial

digestions are shown in Fig 4.1. In the right hand lane there has been little cutting with SmaI, so the upper band of 4,800 bp corresponds to the pUC9-EBNA-LP recombinant. When increasing concentrations of SmaI were used, this band was progressively broken down and a ladder of bands appeared, representing different numbers of repeats still attached to the pUC vector. The liberated SmaI repeat fragments were detected on the original as a very faint ladder further down the gel, but are not illustrated in Fig 4.1. With the highest concentration of restriction enzyme, a complete digest produced a single lower band representing pUC and the 5' unique sequences of the 13.13 cDNA. By counting the number of bands in the ladder, it was deduced that both 13.13 and 13.19 contain 9 repeats.

This contradicts the previous FIGE analysis, which demonstrated that there were 8 repeats in the B95-8 IR-1. The B95-8 cells which were used to generate the cDNAs had been cultured in isolation from those used in the FIGE analysis for approximately 4 years. It was therefore decided to repeat these experiments with the B95-8 cells from which the cDNAs were derived. The results of this are shown in Fig 4.2. The second lane contains the B95-8 strain from our laboratory, which again shows a single fragment corresponding to an 8 repeat IR-1 region. This is also the case for B95-8 cells obtained from two other laboratories; lanes 4 and 5. However, a faint higher molecular weight restriction fragment was detected in the DNA from B95-8 cells from M. Perricaudet's laboratory, as well as the major one representing 8 repeats; lane 3. The upper fragment (indicated with an arrow in lane 3 of Fig 4.2) was not a result of partial digestion and its size did not correlate with the loss of either of the restriction enzyme sites used in the analysis; it would therefore appear to represent a genome containing 12 full repeats in IR-1.

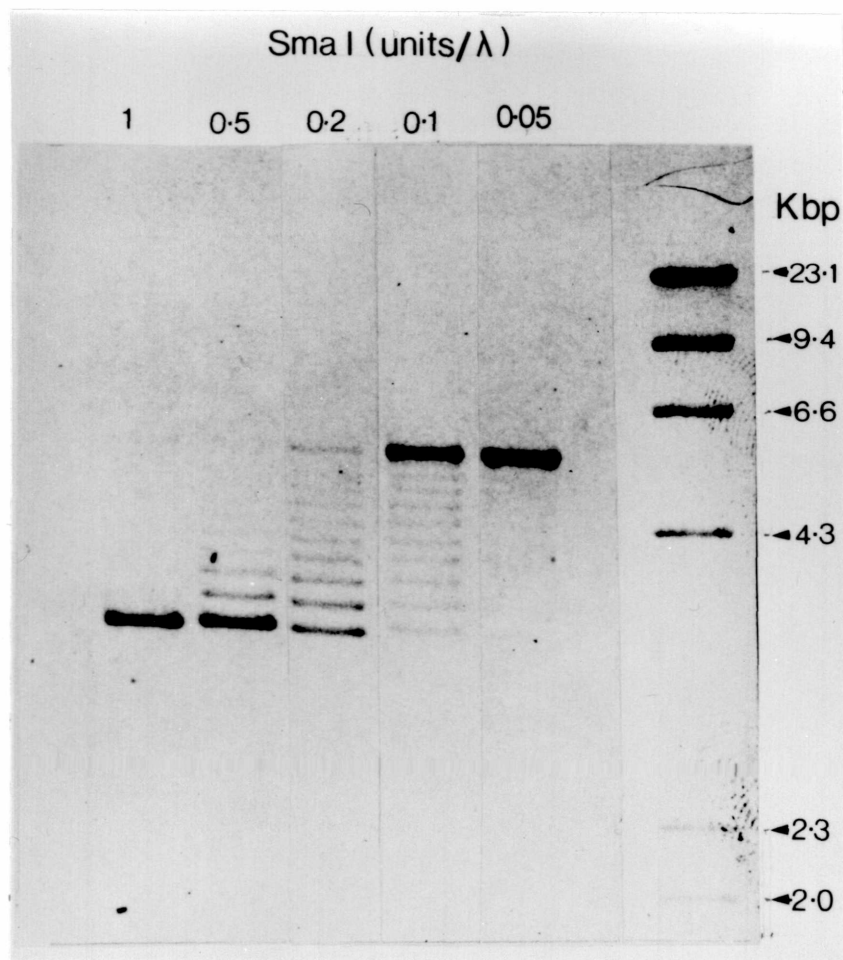


Figure 4.1 Partial SmaI digests of the 13.13 cDNA. The right-hand lane contains λ -HindIII DNA size markers and the sizes are indicated. From left to right in the other lanes are SmaI digests of the 13.13 cDNA using decreasing concentrations of restriction endonuclease. The gel contained 0.5 $\mu\text{g/ml}$ of ethidium bromide so that the DNA could be photographed on an ultra-violet light box.

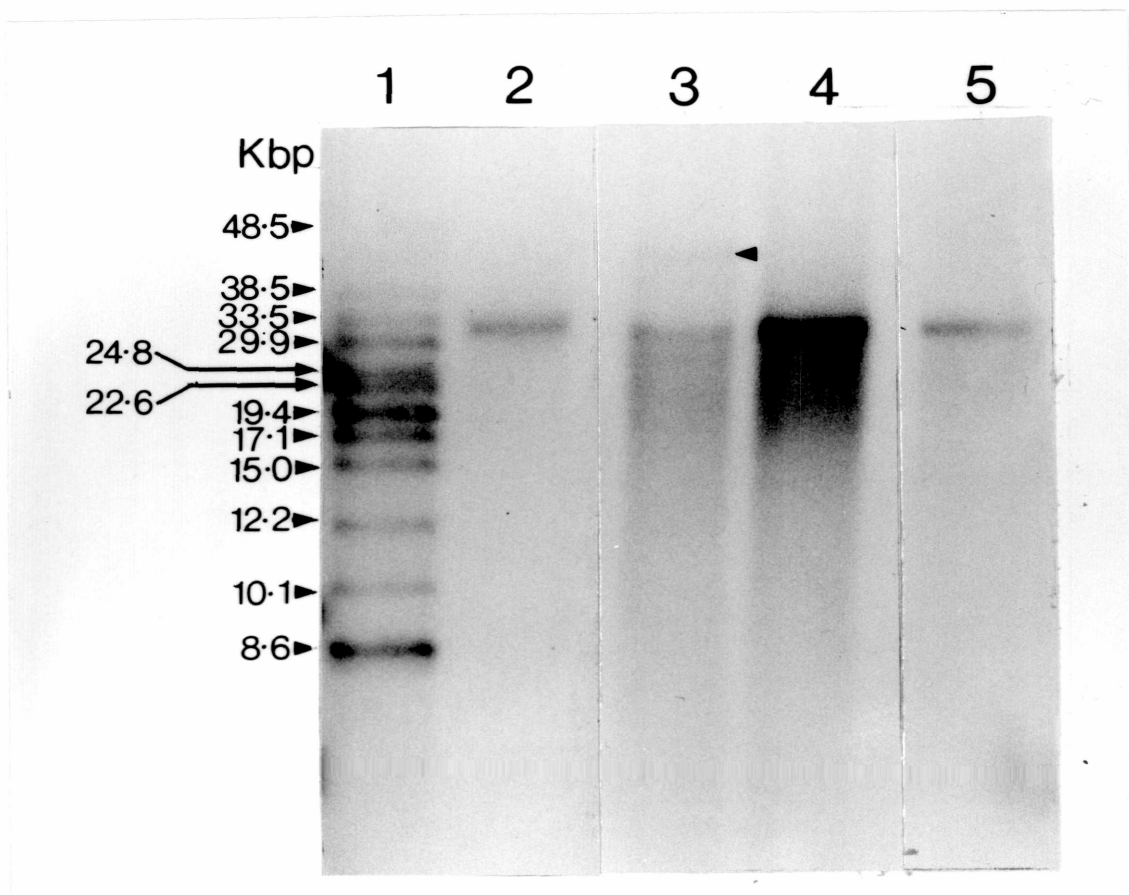
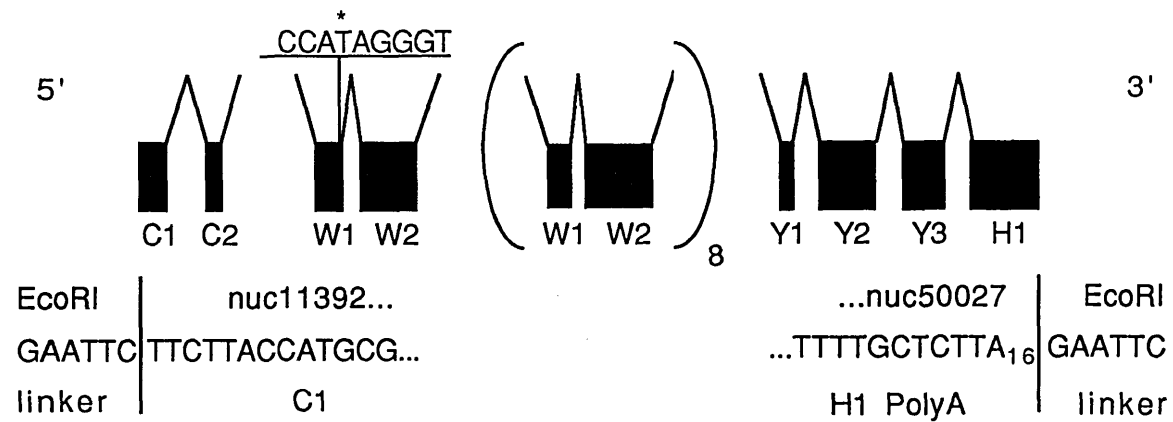


Figure 4.2 Southern blot of a field inversion gel of DNA from B95-8 cells. Lane 1 contains [α - ^{32} P]-end labelled high molecular weight markers ranging from 48.5 to 8.6 kbp. Lanes 2-5 contain EcoRI-HindIII digested total cellular DNA from B95-8 cells obtained from different laboratories. Lane 2 contains the B95-8 strain from our laboratory, while lane 3 contains B95-8 cells from M. Perricaudet's laboratory.

After subcloning the cDNAs into M13mpl8 in both orientations as EcoRI fragments, their 5' and 3' regions were sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977). The nucleotide sequence of the leader exons used to make the 13.13 and 13.19 cDNAs was identical to other C start cDNAs that have been reported previously (Bodescot et al., 1987). The structures of 13.13 and 13.19 are represented in Figure 4.3.

13.13 has a 5' end corresponding to nucleotide 11,392 of the B95-8 sequence (Baer et al., 1984), that is 87 base pairs downstream of the TACAAA putative TATA-like sequence of the C promoter (Fig 4.3). The C1 donor (11,479) and C2 acceptor (11,626) join the two exons derived from the left side of IR-1 in a splice which is typical of C promoter initiated mRNAs (Fig 4.4). The sequence was read for 196 bp through C2 and the first W1 (66 bp) into the first W2 (132 bp). The C2W1 splice (nucleotide 11,657 to 14,554) used the acceptor which is normally used within the reiterating W1W2 exons and not the alternate acceptor at 14559 which generates an AUG codon across the junction. The 3' end of the cDNA contains the Y1 (47,761 to 47,793), Y2 (47,878 to 47,999), Y3 (48,386 to 48,444) and H1 (49,853 to 50,027) exons and 16 A residues presumably derived from the polyA tail 21bp downstream of an AAUAAA polyA consensus signal. 13.13 therefore has a 3' structure that is typical of an EBNA-LP monocistronic mRNA. Since the C2W1 splice does not generate the initiator AUG codon, this mRNA would appear to be non-coding.

1313



1319

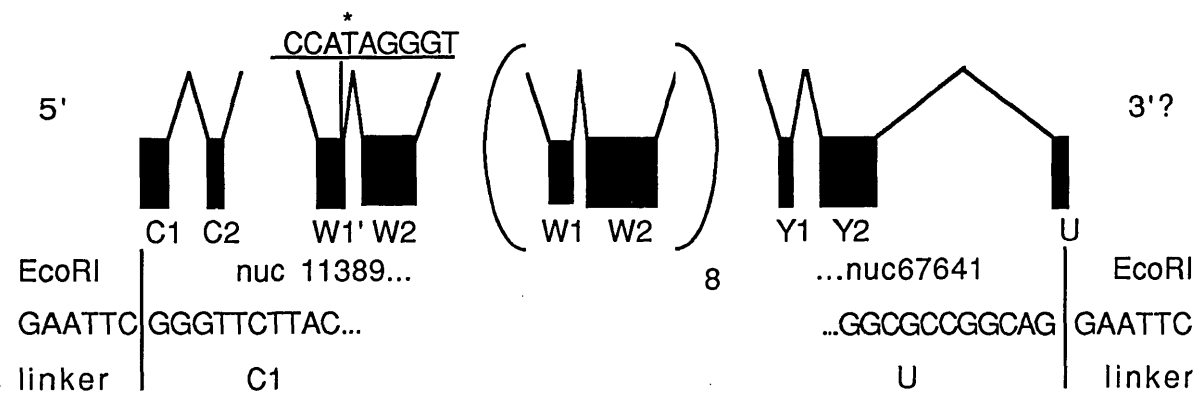
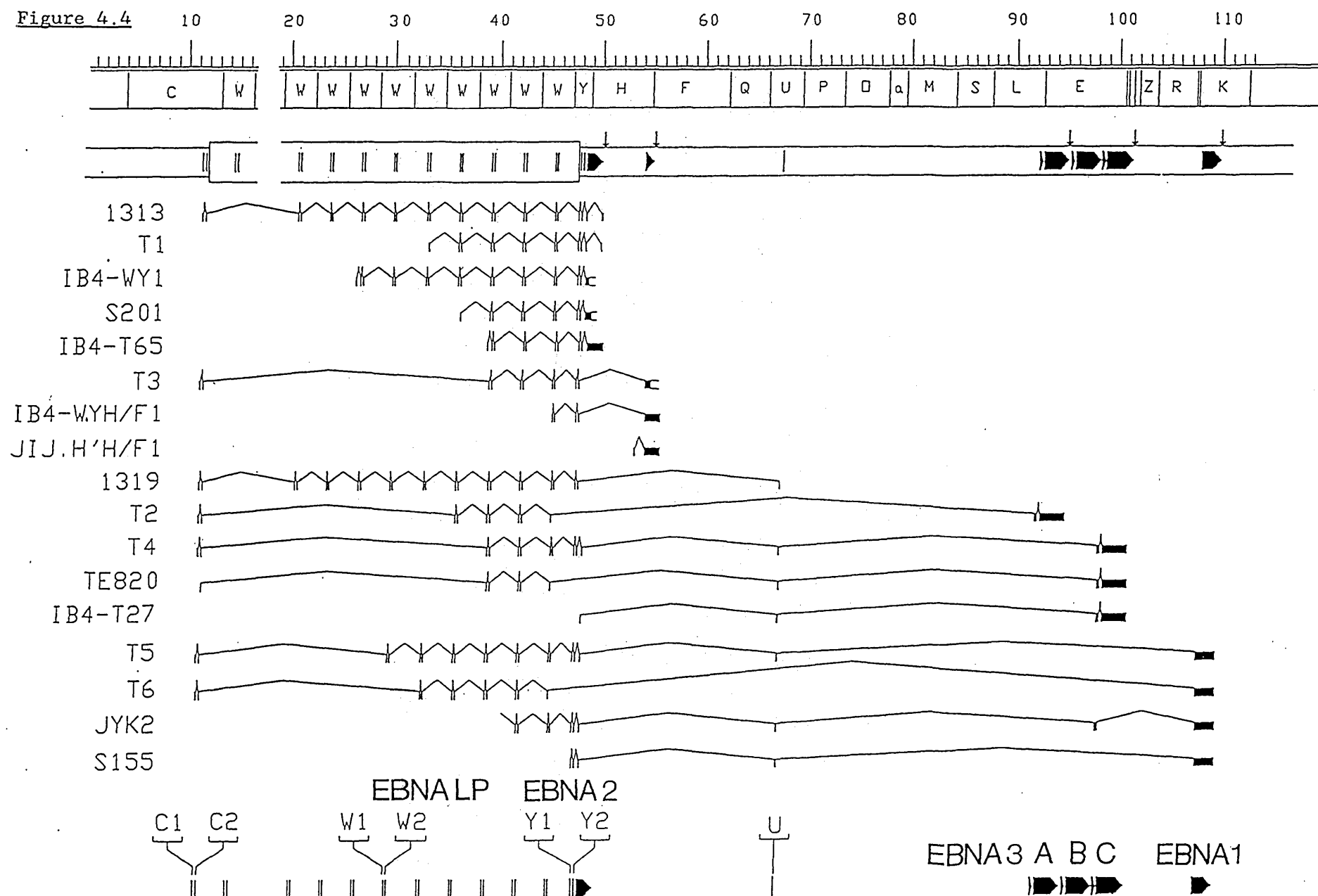


Figure 4.3 Structure of 13.13 and 13.19 cDNAs. The diagrammatic representation of the exon structure of 13.13 and 13.19 shows the exact location of the first and last nucleotides of the cDNAs. The sequence of the region containing the point change relative to the standard B95-8 sequence is shown.

Figure 4.4 A map of published cDNA structures for EBNA-LP messages. The scale is in kilobases and the BamHI fragment map of the left 110 kbp of the EBV genome are shown at the top. The bar represents the physical genome and exons incorporated into the mRNAs are shown as horizontal arrows and vertical stripes. The structures of 15 published cDNAs and 13.13 and 13.19 are shown below the map. Complete cDNAs start within W (IB4-T65 and IB4-WYI) or C (13.13, 13.19 and T2-T6). Only 13.19, IB4-T65 and IB4-WYI contain the splice which creates an EBNA-LP start codon. 13.19 is the only C start cDNA with an EBNA-LP initiator codon. 13.13 and 13.19 have the longest leader structures. Below the cDNA structures the named leader exons are shown and the exons which encode the EBNA proteins are identified. Two non-coding exons Y3 and H1 are present in some cDNAs (like 13.13), overlap the EBNA 2 open reading frame, and are not shown at the bottom.

Figure 4.4



The structure of 13.19 was determined in a similar manner. Both the 5' and 3' ends of the sequence showed differences compared to 13.13 (Fig 4.3). The 5' end contained a C2W1' splice which, at that time, had not been previously reported for C promoter initiated cDNAs. Recently, another laboratory has demonstrated the existence of this splice using C2W1' splice oligos in S1 protection assays (Rogers et al., 1990). The 3' end of the cDNA did not end in polyA. The sequence ended at nucleotide 67,641 (163 nucleotides into the U exon), eight bases upstream of the U donor site present in other cDNAs which extend further in the 3' direction. In the 5' direction the U exon acceptor is spliced to the Y2 donor forming a unique leader exon combination since all other cDNAs reported containing the U exon splice to the Y3 exon (Fig 4.4). Presumably, the 13.19 cDNA came from a message for one of the EBNA 3 family or EBNA 1. This would imply that the message would have been polycistronic since the C2W1' splice would have permitted the EBNA-LP sequences to be expressed.

4.2 A mutation in the first W1 exon of 13.13 and 13.19

Sequence polymorphisms within the W1 or W2 exons have not been observed (Baer et al., 1984). In the sequencing analysis of the 13.13 cDNA, a G to T transversion was detected in the first W1 exon converting the codon (GAG) for residue 21 of the protein into a stop codon (TAG) (Fig 4.3). The same mutation was detected in the first W1 exon of the 13.19 cDNA which renders unlikely the possibility that the mutation occurred during cloning and indicates that one of the IR-1 repeat units (possibly the first) of B95-8 contains a mutation which may have significant effects on the phenotype of the virus.

4.3 Expression of 13.13 initiator sequences in vitro and in vivo

Despite the presence of a mutation in one B95-8 W1 exon, there is another feature of the 13.13 cDNA structure which is relevant to EBV biology. Absence of an EBNA-LP initiator codon and lack of a downstream EBNA exon means no coding capacity employing a classical AUG start codon is envisaged for this message. In different systems, translation initiation can sometimes occur from CUG codons (Clements et al., 1988; Hann et al., 1988). Recent studies on human basic fibroblast growth factor (bFGF) have shown that this polypeptide can be synthesised by exploiting an alternative CUG initiation codon (Prats et al., 1989). The surrounding consensus for the bFGF CUG initiation sequences is similar to the sequence surrounding an in phase CUG codon in the C2 exon of 13.13. The ability of these C2 sequences to initiate EBNA-LP expression was examined by first digesting the IB4-WYI cDNA (within SP64; Speck et al., 1986) with HindIII/StyI and replacing the 5' W0-W1' region with a HindIII-StyI fragment containing the 13.13 C1C2 sequences. This effectively removes the stop codon mutation from the EBNA-LP open reading frame and positions the 13.13 C1C2 leaders in an in vitro transcription vector in one step.

In vitro transcribed RNA was purified and used to program translation in rabbit reticulocyte extracts. Since monovalent and divalent cations have significant effects on the fidelity of initiation of translation in vitro, these parameters were varied around optimal conditions; 110mM KCl, 1.5mM MgCl₂ (Fig 4.5). The positive control was a cDNA containing 3 repeats (3r) derived from IB4WY-1 by partial digestion, as described before. 13h is the hybrid 1 repeat clone described before, which has the 5' sequences of 13.13 joined to the 3' region of IB4WY-1, and 13c is the complete 9 repeat 13.13 clone

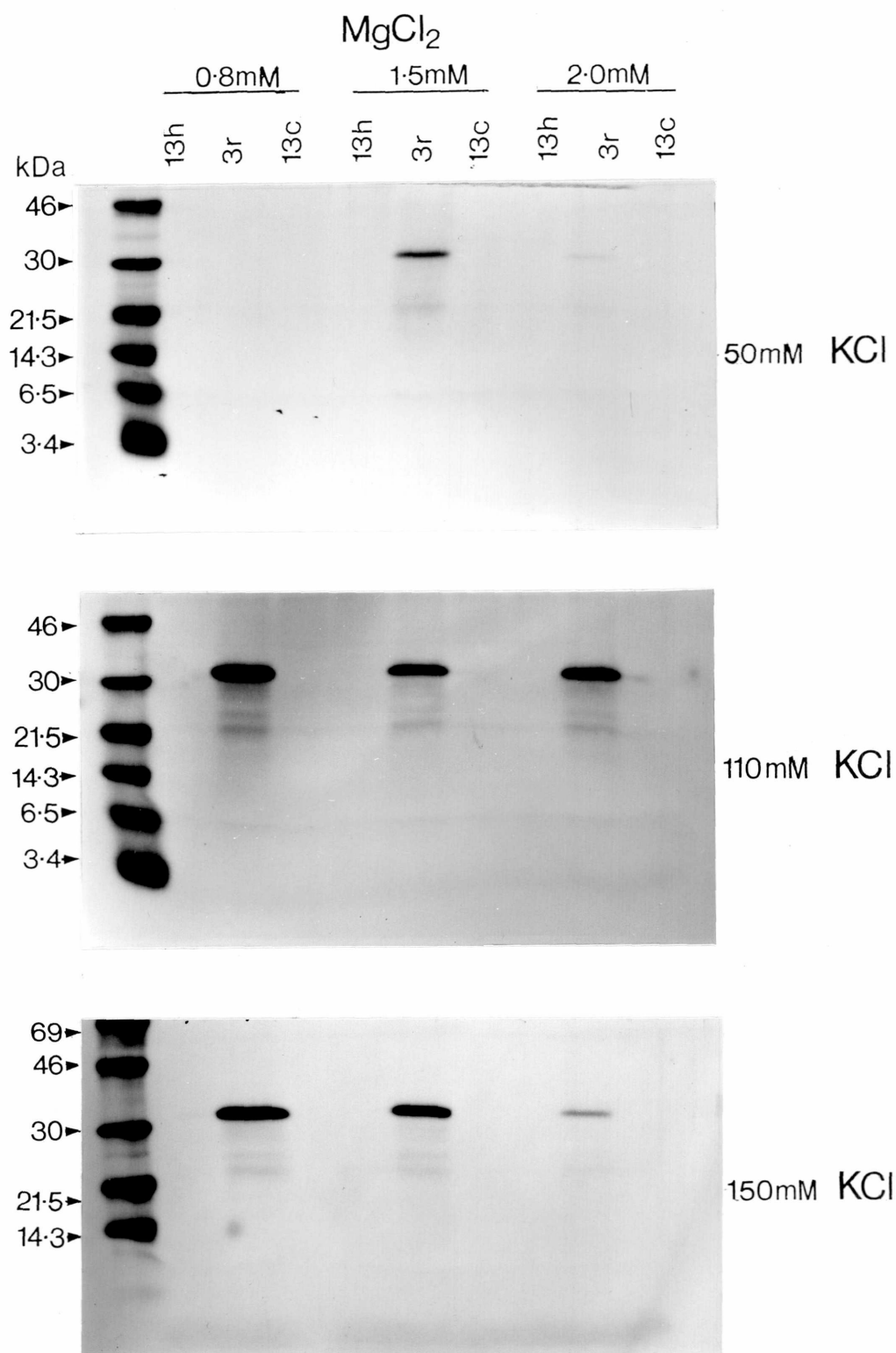


Figure 4.5 In vitro translation of the 13.13 cDNA. The C2W1 fragment of 13.13 was cloned into an SP64 vector containing the 3' sequences of the IB4-WYI cDNA. RNA synthesised from the plasmid (13h), from a plasmid containing 3 repeats with a translatable open reading frame (3r) and from the complete 13.13 cDNA (13c) was translated in a rabbit reticulocyte lysate under varying monovalent and divalent cation concentrations. The products were labelled with ^3H -arginine and visualised by autoradiography. On the left are standard molecular weight markers, sizes are shown in kilodaltons.

which has the premature stop codon mutation (subcloned into SP64 directly as an EcoRI fragment). The size of the predicted protein products would be 12 kDa and 65 kDa respectively. The yield from the 3r clone was maximal at 110mM KCl and 0.8 mM MgCl, and was severely affected by low KCl concentrations. However, neither the 13h or 13c clones produced a detectable translation product under any of these conditions.

These cDNAs were also subcloned into the eukaryotic expression vector ,p3.1W, described before in Chapter 2 (3r and 13h were cloned as XbaI-EcoRI fragments; 13c was cloned as an EcoRI fragment). They were then assayed for transient in vivo expression in the BL41 cell line. This was determined by indirect immunofluorescence using a human anti-EBNA anti-serum (Fig 4.6), and gave similar results to the in vitro translations, in that the 3r positive control was the only one seen to be expressed. In conclusion, there was no evidence from these experiments for use of the alternative CUG initiator codon in the 13.13 message.

4.4 Transactivation of the C promoter

Previous experiments in this laboratory (C. Rooney) had shown that the CAT expression from a Cp promoter-CAT plasmid (p1.4C-CAT) transfected into BL41 cells could be considerably increased if it was cotransfected with the plasmid pWOL, which contains the BamHI WWYH region of EBV and could thus potentially express both EBNA 2 and a short EBNA-LP. It had been shown by immunofluorescence that pWOL was expressing EBNA 2 and/or LP after transfection into BL41 cells, and this was presumed to be driven off the W promoter which lies within the repeats (pers. comm. C. Rooney). The antibody that was used in the immunofluorescence was the polyclonal human serum, RM, which

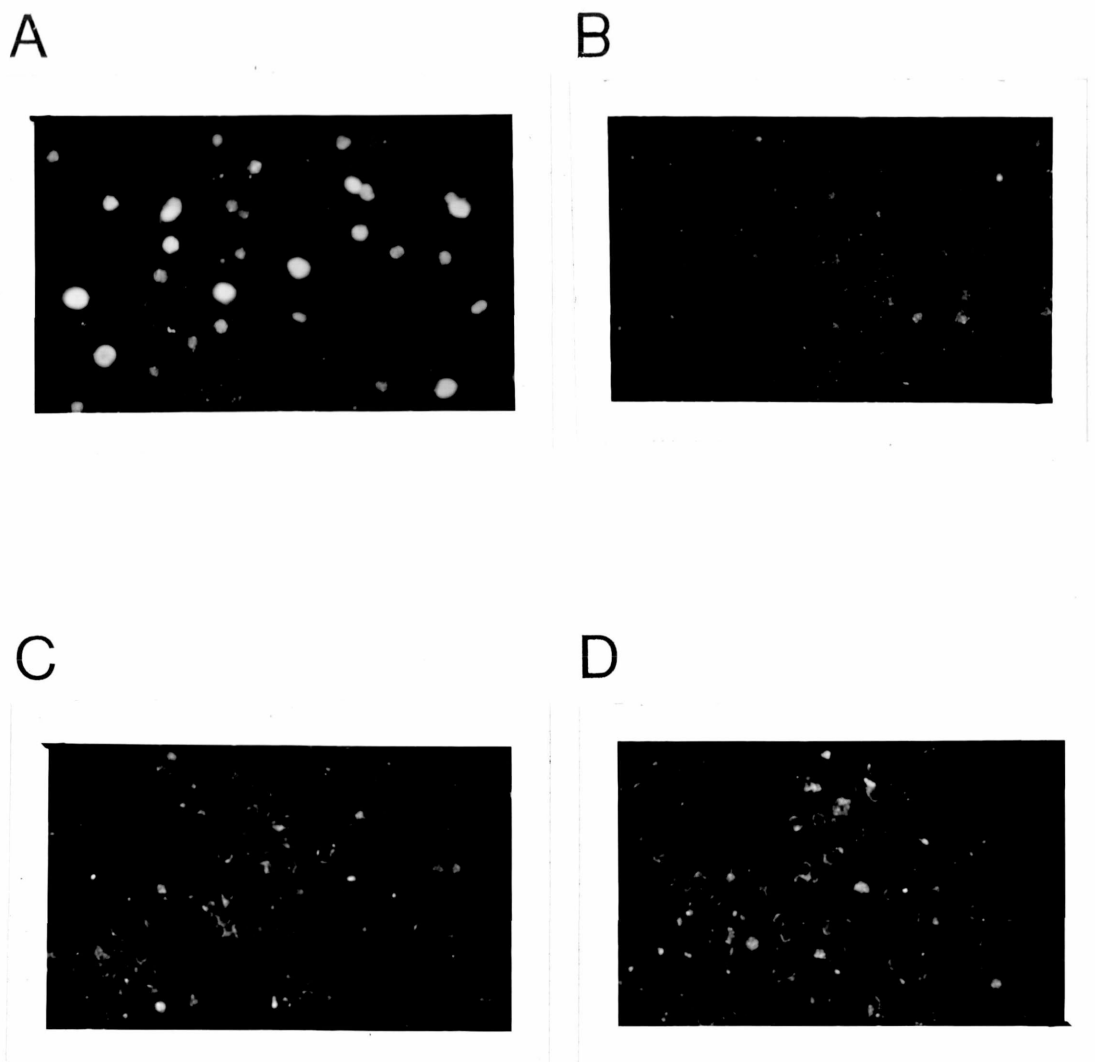


Figure 4.6 Immunofluorescence of transfected BL41 cells, using the human polyclonal antiserum, RM, to identify LP expression. The cells were transfected with: A) positive control p3.1W-3r B) p3.1W-13h C) p3.1W-13c D) negative control p3.1W with no insert.

recognises both EBNA 2 and EBNA-LP. I sought to determine whether this transactivation was a result of EBNA 2 and/or EBNA-LP expression. The strategy was to repeat the experiment with constructs that would express only EBNA 2 (pCMV19-EBNA 2) or EBNA-LP (p3.1W-LP). One C promoter-CAT construct (p1.4C-CAT) and one W promoter-CAT construct (p0.9W-CAT) were used.

The details of the constructs used in the CAT assay are as follows:

pWOL: derived from pKAN2 (Yates et al., 1984) by removing the sequences between the EBV KpnI site and the SalI site in the vector; this appears to improve the expression of EBNA 2 from the EBV sequences (pers. comm. C. Rooney). Therefore, pWOL contains a 12,081 bp BamHI partial-KpnI EBV fragment (EBV map positions 40864 to 52945), which includes two complete BamHI W fragments, a BamHI Y fragment, and 4,096 bp of BamHI H before the KpnI site.

p1.4C-CAT: derived from pSVO-CAT, which is pSV2-CAT (Slater et al., 1985) with the AccI-HindIII fragment removed and converted into BglII (pers. comm. C. Rooney). The MboII fragment of EBV BamHI C (EBV map positions 9911 to 11305) was cloned into the BglII site of pSVO-CAT.

p0.9W-CAT: The 0.9 kbp PstI fragment from BamHI W of EBV (EBV map positions 44188 to 45089 in the number 11 W repeat of B95-8) was cloned into the PstI site of pUC-CAT, upstream of the CAT gene. pUC-CAT is pUC18 containing the CAT gene between the HindIII and AatII sites; the CAT gene was subcloned as a HindIII-AatII fragment from pA10-CAT, which is pSV2-CAT (Slater et al., 1985) with the sequences between the BamHI and EcoRI sites removed (pers. comm. C. Rooney).

p3.1W-LP: The 5- and 3-repeat derivatives of the IB4WY-I cDNA (Speck et al., 1986) were cloned as XbaI-EcoRI fragments into p3.1W (a pUC19 plasmid which has a 3.1 kbp EBV partial PstI restriction fragment containing W promoter sequences upstream of the polylinker; described in detail in Chapter 2). Following transfection into BL41 cells, these constructs were shown to be expressing EBNA-LP by immunofluorescence (Fig 4.6 A; 4.7).

pCMV19-EBNA 2: pCMV19 is a pBR322 derivative containing the CMV immediate early promoter upstream of the pUC19 polylinker (Greives and O'Hare, 1989). EBNA 2 was cut out of pWOL above as a BstUI-NaeI blunt fragment (EBV map positions 48339 to 50382) and subcloned into the SmaI site of pCMV19.

The results of these experiments are shown in Figure 4.8, where each pair of lanes represents duplicate CAT assays. The positive control, p0.9W-CAT (lane 6), has W promoter sequences upstream of CAT and this promoter element appears to work well in transfected BL41 cells. The equivalent C promoter construct, p1.4C-CAT (lane 5), produces a much lower level of CAT activity in these cells. Cotransfection of pWOL with p1.4C-CAT results in a significantly increased level of CAT activity (lane 1). Cotransfection of the constructs expressing either a 5- or 3-repeat EBNA-LP has no significant effect on the level of CAT activity produced from p1.4C-CAT (lanes 3 and 4). In my hands, only a very modest activation was seen with the EBNA 2 plasmid (lane 2), although other workers (N. Sung; C. Rooney) have reported activation by EBNA 2 alone. From this data it seems that the transactivation of these C promoter sequences by pWOL is not simply a consequence of EBNA-LP expression.

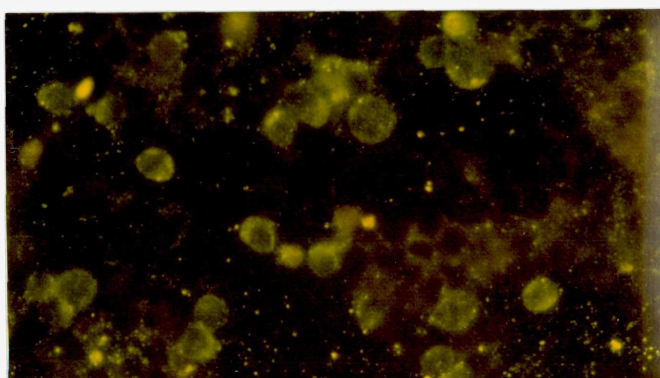
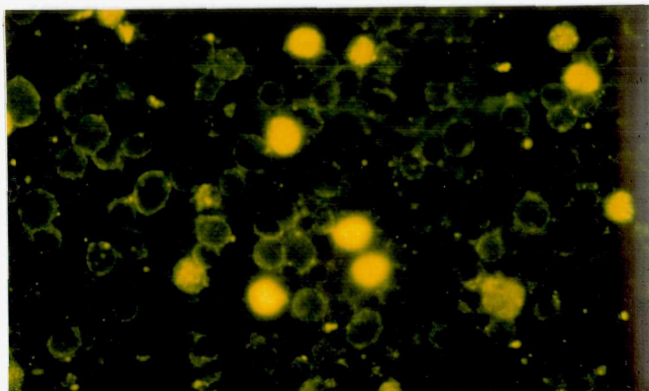


Figure 4.7 Immunofluorescence of BL41 cells. In the top panel the cells have been transfected with p3.1W-5rLP (the p3.1W plasmid containing the 5 repeat derivative of the IB4-WYI cDNA). The bottom panel shows cells transfected with p3.1W with no insert. The human polyclonal antiserum, RM, was used to identify LP expression.

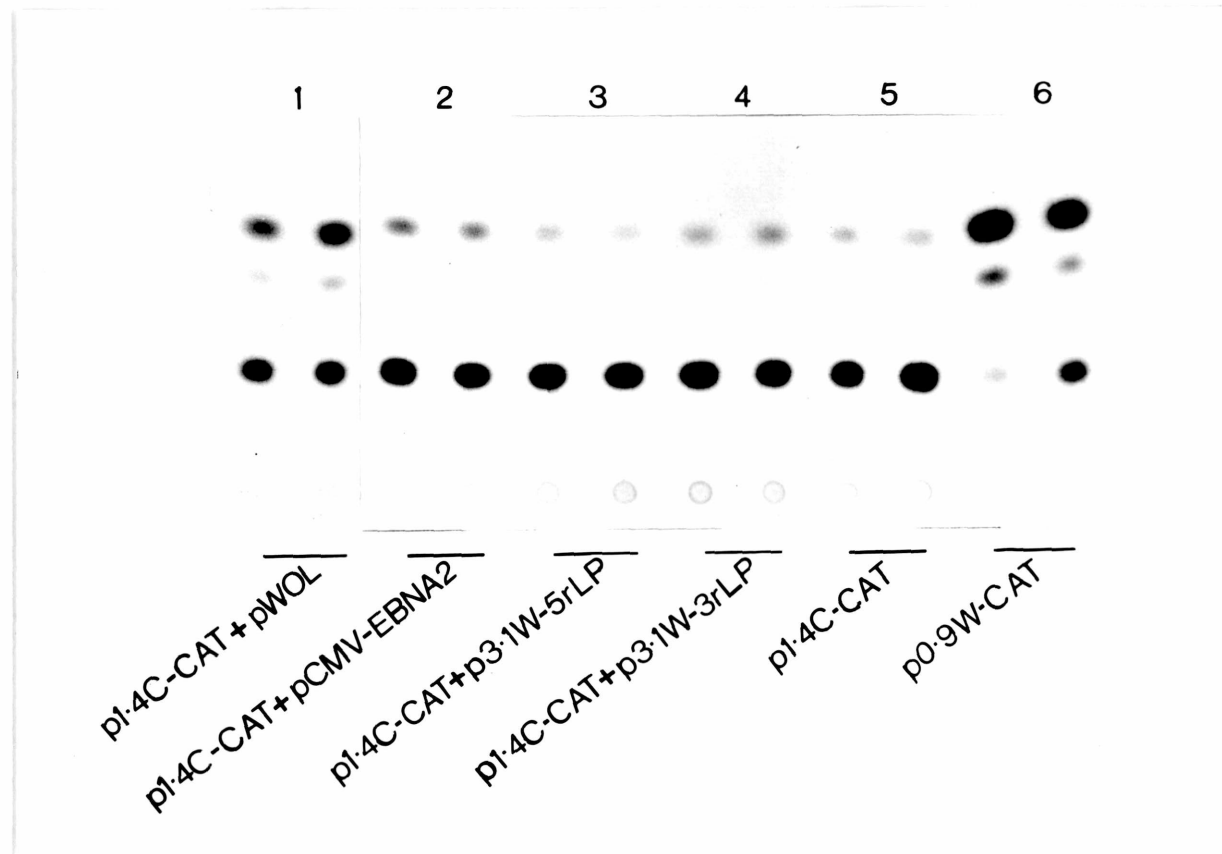


Figure 4.8 CAT assays in BL41 cells. The pairs of tracks 1-6 each represent duplicate transfections, and the transfected constructs are indicated below. CAT activity was determined 48 hours after transfection.

4.5 Discussion

Initially, it was surprising to find that there were 9 repeats in the EBNA-LP cDNAs, when it had already been determined that the EBV strain from which the original mRNAs had been produced, B95-8, contained 8 repeats in the genomic sequences. Several explanations may account for this. First, it is conceivable, given that there are splicing events spanning over 100kbp in the 170kbp EBV genome, that a type of "circum-genomic" transcription may exist, whereby the RNA polymerase travels around the circular viral episome at least twice. In this model the same W1 and W2 exons would appear more than once in the primary transcript, extending the size range of repetitive mRNA. Second, trans-splicing of leaders from one pre-mRNA to another would allow the building of extra long leader structures. Both of these models are attractive because they also explain the observation of Leader Proteins which exceed in size the coding capacity of the IR-1 exons (Finke et al., 1987). A third possibility is that there are low numbers of EBV genomes in the B95-8 cells from M. Perricaudet's laboratory, which contain more than 8 repeats in IR-1. The upper band in the FIGE analysis of these cells (Fig 4.2, lane 3), would certainly seem to support the hypothesis that there is a less abundant genome in these cells, which contains 12 repeats within IR-1. This may not be particularly surprising as B95-8 is a spontaneous producer of virus, and it has been shown here that the repeats in IR-1 are subject to change during the lytic phase of the viral life cycle. It may be coincidental that both the cDNAs analysed here originated from the minor species of EBV genome and contain the same number of repeats (we specifically sought to examine long cDNAs). Alternatively, this may underly a type of regulation, perhaps at the level of splicing, which promotes a certain number of repeat units in EBNA-LP mRNAs.

The sequencing has revealed that 13.13 has a C2W1 splice, which has been seen in all other C start cDNAs, but is unique in that it has the Y3H1 3' end of an EBNA-LP monocistronic message. 13.19, on the other hand, has the C2W1' splice not found previously in cDNA analysis, which creates an AUG codon for the EBNA-LP open reading frame. This supports the previous S1 protection analysis which identified this splice in cell lines utilising the C promoter (Rogers *et al.*, 1990). In addition, the 13.19 cDNA splice downstream into the U leader exon, which has thus far only been found in EBNA 3 and EBNA 1 messages (Bodescot *et al.*, 1986; Bodescot and Perricaudet, 1986; Shimizu *et al.*, 1988). It is reasonable to suppose that the 13.19 cDNA clone was derived from a mRNA which contained a downstream EBNA open reading frame and was therefore very probably polycistronic. The structures of 13.13 and 13.19 are presented diagrammatically in Fig 4.4, along with all the other EBNA cDNAs characterised to date.

The presence of a C2W1' splice conclusively shows that the formation of an initiator methionine codon, AUG, for the EBNA-LP ORF is dependent upon specific splicing events, irrespective of whether the C or W promoter is active. The possibility of the in phase leucine codon, CUG, in the C2 exon being used to initiate translation of EBNA-LP following transcription from the C promoter was studied using the 5' sequences of 13.13 in both *in vitro* and *in vivo* analysis. The failure to initiate from this alternative codon, shows clearly that the expression of EBNA-LP from both the W and C promoter is dependent on the AUG forming splice taking place.

This is important not only for the expression of EBNA-LP, but also for that of the downstream EBNA's. Where the EBNA-LP AUG is formed by splicing, translational polarity would lead to reduced expression of any other EBNA encoded in the 3' region of a bicistronic mRNA. This

is in fact what has been observed for the IB4-T65 cDNA which is bicistronic for EBNA-LP and EBNA 2 (Wang et al., 1987). Where the AUG is not formed, the 5' leader sequences of EBNA messages would be untranslatable. It may be that this splicing event is completely random, so that even messages which are monocistronic for EBNA-LP at their 3' end (such as 13.13), can still be untranslatable due to the absence of an initiator methionine codon. It is not possible to say whether the W1 acceptor site is closer to a common consensus, as there appears to be poor conservation between EBV splice acceptor sequences. However, the S1 protection analysis from a different laboratory has shown that the W0W1 and C2W1 splices are favoured over the alternative W' splices (Rogers et al., 1990), suggesting that there is some regulation over whether the W1 or W1' acceptor sequences are used in the splice. Such regulation may serve to maintain a balance of EBNA expression which is important for the immortalisation status of the cell.

Both cDNAs contained a G to T transversion which generated a stop codon in place of residue 21 in the first W1 exon. Although it is impossible to identify the repeat in which this mutation appears in the genomic sequence, it is not present in C start cDNAs with shorter leaders and therefore must sometimes be skipped during processing (Bodescot et al., 1986; Bodescot and Perricaudet, 1986; Shimizu et al., 1988). This makes it difficult to evaluate the impact of such a mutation on the phenotype of B95-8, since both truncated and full length EBNA-LP products would be present in the infected cells.

The cotransfection experiments with the p1.4C-CAT construct demonstrate that the transactivation of the EBV C promoter sequences by pWOL is not simply the result of EBNA-LP. Cp is now believed to be the preferred promoter for driving EBNA expression in established

latently infected cell lines, while Wp may function in resting or pre-B cells (Woisetschlaeger et al., 1990). Although other workers have obtained activation of Cp by EBNA 2 (pers. comm. N. Sung; C. Rooney) it is not clear that the level of activation of Cp with EBNA 2 plasmids is as good as that achieved with pWOL. Also, the EBNA 2 responsive element mapped by N. Sung and co-workers is upstream of the EBV content of p1.4C-CAT (positions 6878-6976), which is nevertheless enhanced considerably by pWOL. So it remains possible that there may be some cooperation between EBNA 2 and LP. Very recently, it has been shown that there are also important regulatory sequences downstream of the C promoter (between approximately 11,760 to 11,860 on the EBV map) (pers. comm. D. Walls), and all the previous work on Cp regulation will have to be reconsidered in the light of this. The work here has shown that, within certain sequence boundaries (map position 9911-11305), EBNA-LP expression alone has no effect on Cp activity, but may still contribute cooperatively.

Functional Analysis of EBNA-LP

The function of the Epstein-Barr virus Leader Protein is unknown, and at the beginning of this project it was not obvious how to address this problem. As described earlier, a role for EBNA-LP in the immortalisation process was implied by the loss of its unique 3' sequences in the non-transforming viral strain P3HR-1 (Miller *et al.*, 1974; Menezes *et al.*, 1975; Raab-Traub *et al.*, 1978; Delius and Bornkamm, 1978; Rabson *et al.*, 1982). This was supported by the report of a limited homology between the unique carboxy terminal sequences of LP and conserved domain 1 of the Adenovirus transforming protein, Ela (Huen *et al.*, 1988) (Fig 5.1). For these reasons most of the experiments described in this chapter were directed at the possible involvement of EBNA-LP in immortalisation by EBV. Various different ideas were tested.

5.1 Antisense EBNA-LP oligonucleotides

If it were possible to deplete the amount of LP in cells immortalised by EBV and consequently see changes in cell growth, that would provide a route to analyse LP function. An attempt was made to reduce the expression of EBNA-LP using antisense sulphur derivatised oligonucleotides (S-oligos). S-oligos have a sulphur atom substituted for the oxygen atom of the conventional phosphodiester bond of all naturally-occurring DNA internucleotide linkages. This substitution reduces the charge, thereby maintaining solubility in aqueous solutions and presumably enhancing compatibility with membranes. In addition, S-oligonucleotides have enhanced nuclease resistance and

HPV16	E7	HIS	GLY	ASP	THR	PRO	THR	LEU	HIS	GLU	TYR	MET	LEU	ASP	LEU	GLN	PRO	GLU	
AdV2	E1a	HIS	PHE	GLU	PRO	PRO	THR	LEU	HIS	GLU	LEU	TYR	ASP	LEU	ASP	LEU	VAL	PRO	GLU
EBV	EBNA-LP	HIS	PHE	GLU	PRO	PRO	THR	VAL	THR	THR									

Figure 5.1 Homology between adenovirus Ela and LP (after Huen *et al.*, 1988). The amino acid homology between the viral oncoproteins E7, from human papilloma virus type 16, and Ela, from adenovirus type 2, in their conserved domain 1 region is indicated in the top two sequences by closed boxes. The homology between the unique carboxy terminal region (encoded by the Y2 exon) of the EBV EBNA-LP and the core sequences of conserved domain 1 of Ela comprises six identical amino acids (boxed above) and one conservative replacement, Leu to Val.

biological stability. S-oligonucleotides, obtained from Synthecell (Rockville, MD, USA), were antisense to sequences in EBNA-LP, EBNA 1, EBNA 2, BZLF1 and human papilloma virus E7. All were 25 nucleotides in length and the latter two were used as negative controls in experiments aimed at reducing EBNA expression. The sequences of the antisense oligonucleotides are given below.

5' GGACTGGGCGACCGGTGCCTTCTTA 3' EBNA-LP

5' GAGCCTTCTGGTCCAGATGTGTCTC 3' EBNA 1

5' GGTTCCTCAAGACTATCCGTGTGAAC 3' EBNA 2

5' GGCACCTGGTATGGGTCAGGTGTAA 3' BZLF1

5' CATCCTCCTCCTCTGAGCTGTCATT 3' E7

The oligonucleotides were added to the growth medium of B95-8 cells (5×10^4 cells/ml) at a final concentration of 12, 25 or $50 \mu\text{M}$. The cells were incubated at 37°C , 10% CO_2 , for another 4 days before harvesting the cells and loading the extracts on to protein gels. The EBNA status of these cells was then checked in Western blots using the human polyclonal antiserum, RM (Fig 5.2). Unfortunately, the addition of the EBNA-LP S-oligo had no detectable effect on the level of LP expression. The fainter bands in the first lane in Fig 5.2 ($12 \mu\text{M}$ LP) are probably due to a loading error as all the EBNAs are effected equally and the reduction was not reproduced in a second experiment (not shown). However, there does appear to be slightly less EBNA 2 and EBNA 1 expression when either $25 \mu\text{M}$ or $50 \mu\text{M}$ of antisense EBNA-LP oligo is added to the cells, and this will be

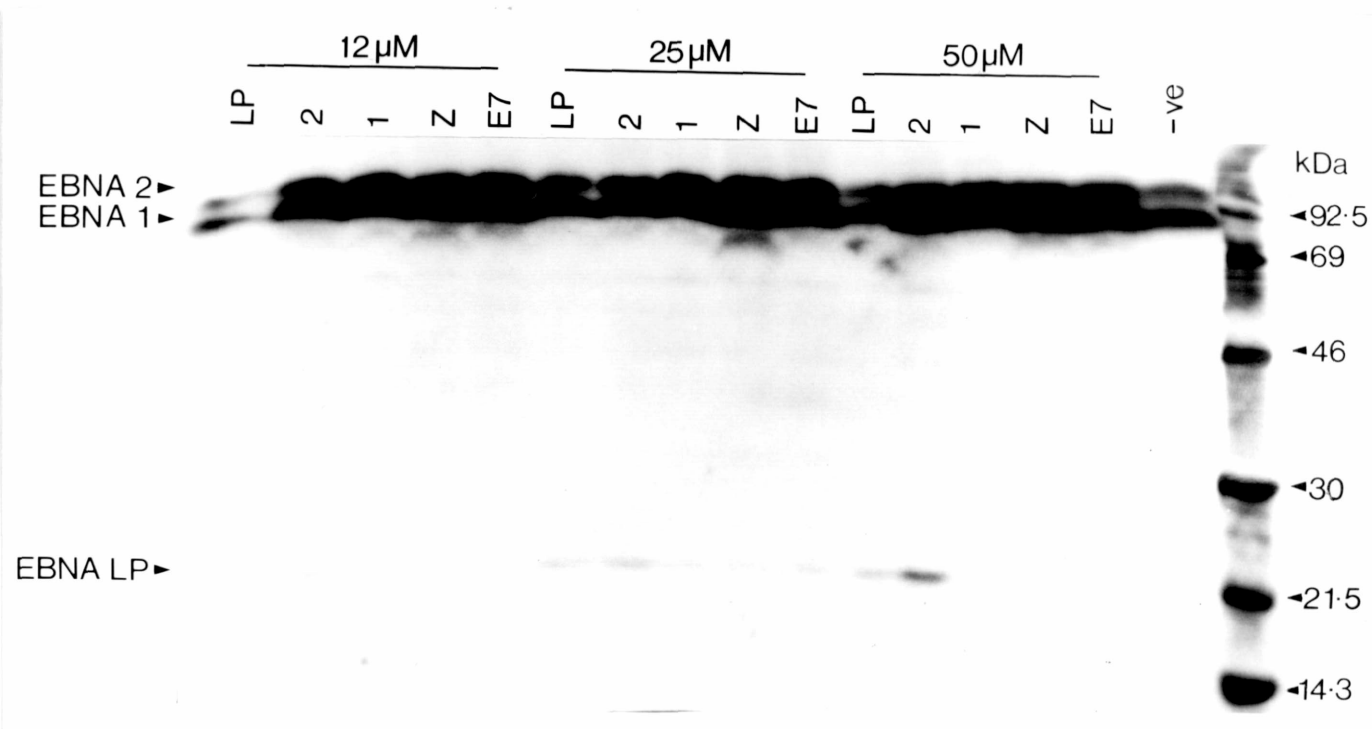


Figure 5.2 Western blot of B95-8 cell extracts. The blot was probed with the human polyclonal antiserum, RM, which recognizes a variety of EBNA proteins; the positions of EBNA 1, 2 and the major species of LP are indicated on the left. On the right are standard molecular weight markers, sizes are shown in kilodaltons. The s-oligonucleotides added to the cells are indicated at the top, along with their final concentration. From left to right, these oligonucleotides were antisense to sequences in the open reading frame for EBNA-LP (LP), EBNA 2 (2), EBNA 1 (1), BZLF1 (Z) and E7 from human papilloma virus type 16. The last two were negative controls for the effect of the oligonucleotides on EBNA expression; cells which had no oligonucleotides added (-ve) were also included.

discussed later. Other differences did not seem to be reproducible between the two experiments performed.

5.2 No evidence for LP binding to the Retinoblastoma gene product

The possibility of binding of EBNA-LP to Rb was investigated in collaboration with Dr K. Vousden. An assay had been developed earlier which demonstrates the binding of a human papilloma virus protein, E7, to the retinoblastoma susceptibility gene product, Rb. This entails mixing rabbit reticulocyte lysates which contain in vitro translated Rb and E7. The Rb-E7 protein complex can then be immunoprecipitated using antibodies that are specific to either of the two proteins, so that Rb comes down with an E7 antibody, and vice versa. EBNA-LP was introduced into this assay by mixing in vitro translated LP (labelled with ^3H -arginine) and Rb (labelled with ^{35}S -cysteine) together and immunoprecipitating with an anti-Rb monoclonal antibody, C36 (Whyte et al., 1988). The successful translation of LP was predicted on a separate gel (not shown) but in neither of the two attempts at this experiment was LP observed to co-precipitate with Rb (Fig 5.3). So no direct binding between LP of EBV and Rb could be detected under these conditions.

5.3 Transformation Rescue in Primary Rat Embryo Fibroblasts

The tsA 14 cell line is a rat embryo fibroblast line which expresses a temperature sensitive mutant of SV40 large T antigen (Jat and Sharp, 1989). At the permissive temperature (33°C) the protein is stable and the cells behave as an established line, whereas at the non-permissive temperature (39°C) the protein is degraded and the cells senesce rapidly within 2-3 days. In addition, it was shown that

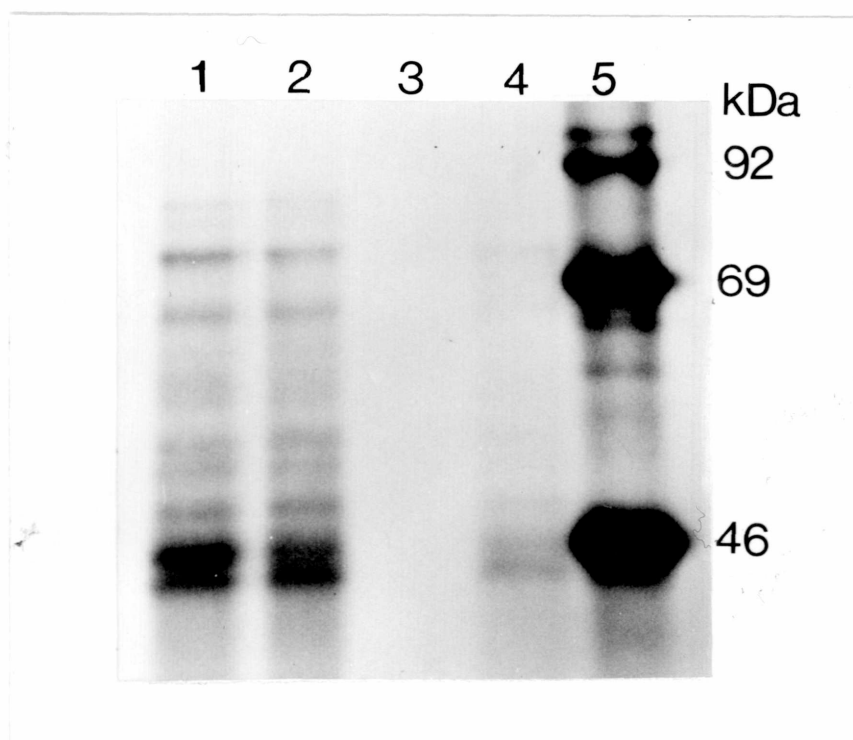


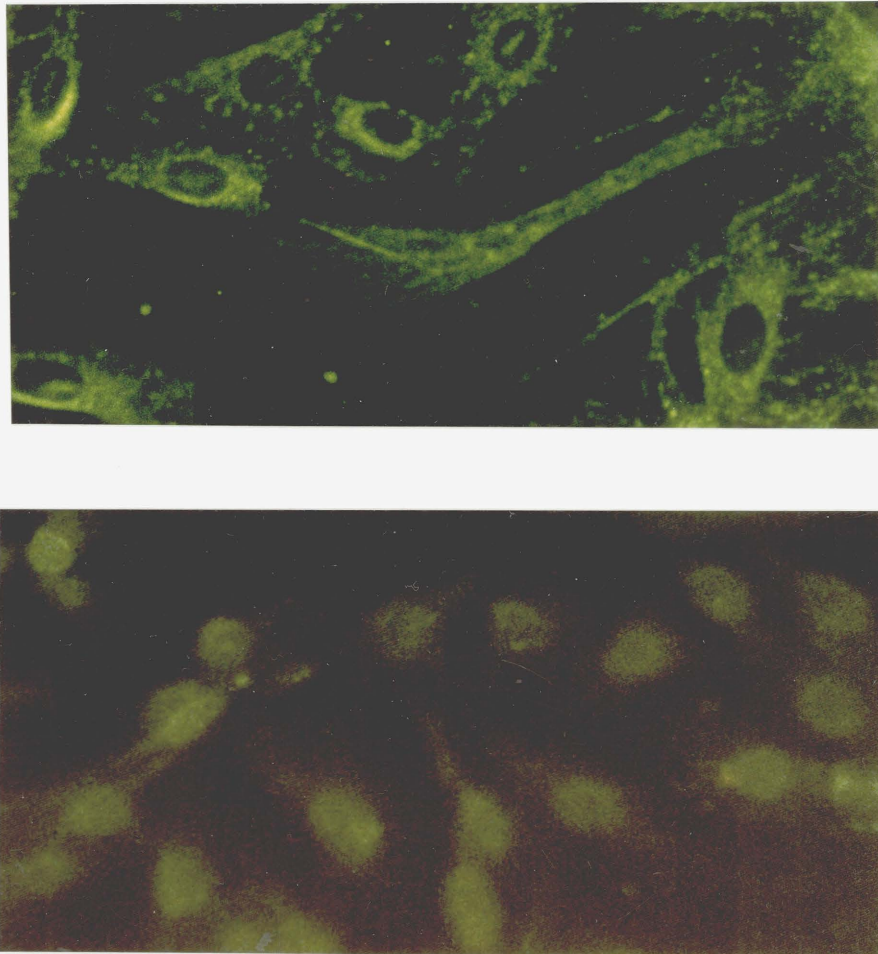
Figure 5.3 Rb Immunoprecipitations. Lane 5 contains standard molecular weight markers, sizes are shown in kilodaltons. Lanes 1, 3 and 4 are immunoprecipitates from rabbit reticulocyte lysates containing in vitro translated Rb alone (labelled with ^{35}S -cysteine); the immunoprecipitate in lane 2 is from a 1:1 mix of lysates containing in vitro translated Rb and 7-repeat LP (labelled with ^3H -arginine; molecular weight 51.4 kDa). The anti-Rb monoclonal antibody, C36 (Whyte et al., 1988b), was used in the immunoprecipitations in lanes 1 and 2, while a commercially available anti-Rb antibody (Pharmingen, San Diego, CA, USA) was used in lane 4; no antibody was added to the immunoprecipitation in lane 3. The largest in vitro translated Rb protein migrates on the protein gel at approximately 90 kDa, although its predicted molecular weight is 105 kDa. The multiple bands underneath are thought to arise from internal initiations at different methionine codons and are usually seen in these in vitro translations of Rb.

the introduction of certain nuclear oncogenes, such as the Adenovirus Ela gene, prior to the temperature shift to 39°C could "rescue the transformation" phenotype of the tsA 14 cells, so that they continued to proliferate at the higher temperature (Jat and Sharp, 1989). Other nuclear oncogenes (for example v-myc) could not compensate for the loss of SV40 large T in this assay. I tested whether EBNA-LP had any immortalisation potential using this system.

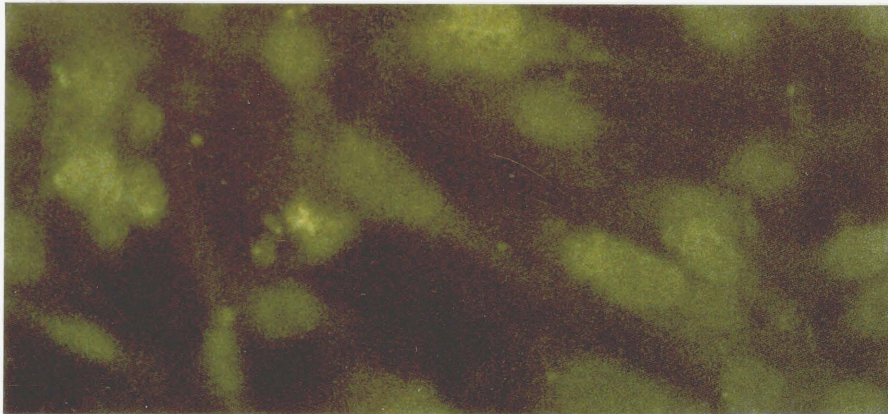
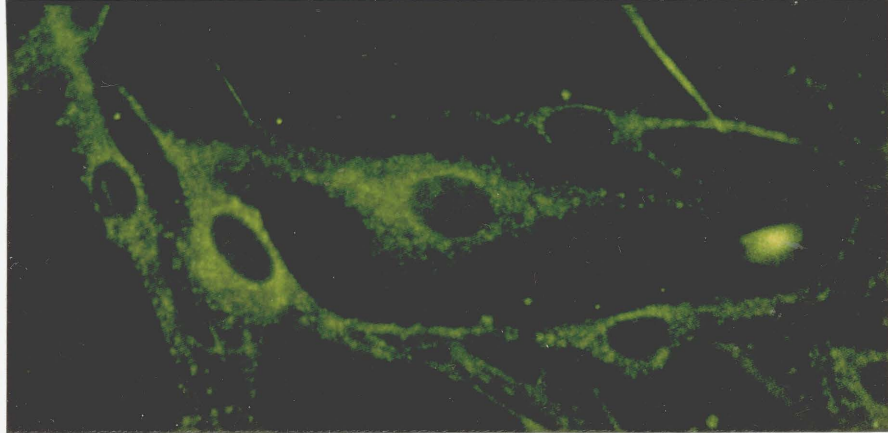
The IB4-WY1 cDNA was subcloned into pUC-MoMlv (described in Chapter 2; Edmonds and Vousden, 1989). This construct (which expresses a 7 repeat leader protein) was cotransfected with pHYG (expressing the hygromycin resistance gene; Sugden et al., 1985) into tsA 14 cells by the calcium phosphate method. The pUC-MoMlv-LP plasmid was added at a 10-fold greater concentration ($\mu\text{g/ml}$) than pHYG in order to ensure that any hygromycin resistant colonies were likely to have the MoMlv-LP sequences integrated into the cellular DNA. The cells were shifted to 39°C the day after the transfection and the hygromycin selection was applied the following day.

Only in one early transfection experiment did colonies grow out at the non-permissive temperature. Two of these colonies were picked and Dr K. Vousden showed by immunofluorescence that these were not revertants containing thermostable large T antigen. The cells were then checked for the expression of EBNA-LP. Initially, this was attempted by immunofluorescence using either the human polyclonal antiserum, RM, or the JF186 monoclonal antibody (Finke et al., 1987) (Fig 5.4 A,B). LP expression could not be detected in this way as the pattern was the same for the drug resistant colonies and the untransfected control. However, Western blot analysis of the cell extracts using the RM antiserum showed that there was a protein of the correct size for a 7 repeat Leader Protein present in the two

Figure 5.4



A. Immunofluorescence of tsA 14 fibroblasts transfected with the LP expressing plasmid pUC-MoMlv-LP. The human polyclonal antiserum, RM (top panel) or the JF186 monoclonal antibody (bottom panel) were used.



B. Immunofluorescence of untransfected tsA 14 fibroblasts using the human polyclonal antiserum, RM (top panel) or the JF186 monoclonal antibody (bottom panel).

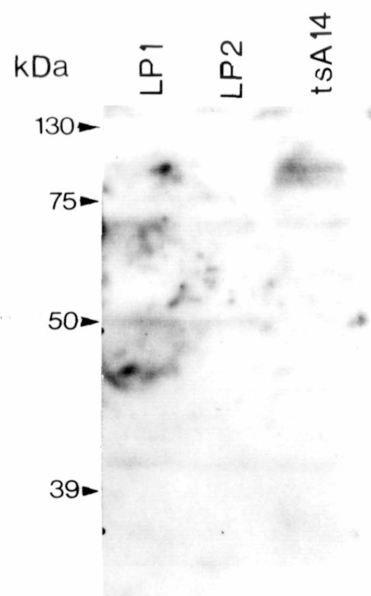
transfected lines, but not in the untransfected control (Fig 5.5). From this it was concluded that these tsA 14 transfectants, which continued to proliferate after the loss of SV40 large T, were expressing EBNA-LP.

This experiment was repeated on several occasions by different people. Unfortunately, it was not possible to obtain any more tsA 14 colonies growing at the non-permissive temperature following transfection of EBNA-LP into the cells, so the significance of the colonies obtained in the earlier experiment is open to question.

In collaboration with Dr L. Banks, ras cooperation assays were also carried out. Rat embryo fibroblasts transfected with the ras oncogene have only limited proliferative ability and they are not tumourigenic when seeded into host animals (Land et al., 1983a). Cotransfections have shown that ras can cooperate with other cellular oncogenes, such as myc, or viral oncogenes, such as polyoma virus large T and adenovirus Ela, to achieve complete conversion of the fibroblasts to tumourigenicity; none of these oncogenes can accomplish this alone (reviewed by Land et al., 1983b). This supports a multistep process of carcinogenesis at the molecular level. Constructs expressing LP and ras were cotransfected into primary baby rat kidney cells. Although the LP transfected cells survived longer than the control cells initially (pers. comm. L. Banks), no transformed colonies arose from this experiment. This shows that EBNA-LP does not cooperate with ras in this transformation assay.

5.4 633 virus analysis

Several attempts had been made to restore the P3HR-1 virus deletion, in order to see whether this would produce transforming virus. This



RM serum

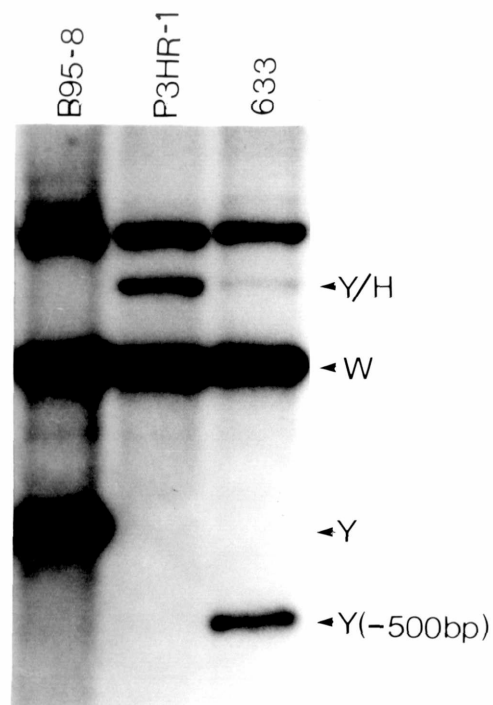
Figure 5.5 Western blot of tsA 14 fibroblasts. The blot was probed with the human polyclonal antiserum, RM. Untransfected tsA 14 cells are in the right-hand lane. LP1 and LP2 are the two EBNA-LP transfected lines which grow at the non-permissive temperature. The positions of molecular weight markers are indicated on the left side. A band of the correct size for a 7-repeat LP (51.4 kDa) is seen in the transfected cells but not in the untransfected control.

was accomplished using an oriLyt construct which contained the deleted sequences from P3HR-1, plus some flanking sequence (Hammerschmidt and Sugden, 1989). These workers used the BZLF1 gene product to induce a burst of plasmid replication via the oriLyt sequences and this optimized the recombination of the EBV sequences into the P3HR-1 viral genome. Transforming virus was produced by recombination, demonstrating that something in the deleted sequences was essential for immortalisation by EBV. The two genes that are deleted in P3HR-1 were mutated to identify whether one or both of them are required for immortalisation. Two derivatives of the aforementioned oriLyt plasmid were constructed (Hammerschmidt and Sugden, 1989). One contained termination codons positioned at one-third of the length of the EBNA 2 open reading frame; the other had the last two exons of EBNA-LP deleted as they are in the P3HR-1 strain. Recombination of these sequences into P3HR-1 showed that restoration of EBNA 2 alone was sufficient to regain immortalising virus, whereas restoration of a full-length EBNA-LP was not (Hammerschmidt and Sugden, 1989). However, it was noted that the efficiency of immortalisation of the viral strain which expresses a truncated LP (633 virus), was approximately 10-fold lower than the P3HR-1 recombinant which has all the EBNA's intact (Hammerschmidt and Sugden, 1989). Therefore, it was concluded that, although the carboxy terminal sequences of EBNA-LP (including the sequence homology to conserved domain 1 of adenovirus Ela) were not required for the immortalisation by EBV, an intact EBNA-LP gene could still contribute to the growth characteristics of EBV-immortalised cells (Hammerschmidt and Sugden, 1989).

To assess the effect of full length LP on the growth of 633 cells, a full-length EBNA-LP gene was reintroduced into the cells. B cells immortalised with 633 virus were kindly provided by Dr B. Sugden.

Southern blot analysis of DNA from the 633 cells verified that the virus contained the characteristic 500bp deletion in the BamHI Y fragment, which removes the last two exons of EBNA-LP (Fig 5.6). The Y fragment is completely deleted in the P3HR-1 genome and is smaller in the 633 virus, which contains a 500 bp deletion in BamHI Y that removes the Y1 and Y2 exons of EBNA-LP. The 633 cells also produce a faint Y/H fusion band, suggesting that there must be some residual P3HR-1 virus in this line. An EBNA-LP expression vector (pSNOC) was constructed, (Fig 5.7), which has the CMV immediate early promoter and the SV40 poly-A sequences at opposite sides of the pUC19 polylinker. This plasmid also contains the neomycin resistance gene and oriP so that it will replicate along with the resident virus in EBV infected cells (the construction of pSNOC is described in detail in Chapter 2). Following transfection of pSNOC or pSNOC-LP into 633 cells, drug resistant cells were selected (150-200 μ g/ml of G418) and eventually checked for their LP status in Western blot analysis (Fig 5.8). Two lines were obtained in this way, one of which expressed only the truncated LP from the resident 633 virus, 633-pSNOC (right hand lane, Fig 5.8), while the other expressed both the truncated LP and the full-length version from the plasmid, 633-pSNOC-LP (middle lane, Fig 5.8). It should be noted that the 633-pSNOC-LP line came through the drug selection considerably faster than the 633-pSNOC line, and could be transferred from wells into flasks several days earlier. The next step was to make a comparison of growth characteristics between these two lines.

The growth curves of the transfected lines were established by plating them out at the same cell density and then counting the cells at appropriate days thereafter (Fig 5.9). It appears that the two lines have essentially the same growth curve, with the 633-pSNOC-LP line reaching a slightly higher cell density. In addition, doubling



probe ; Bam Y

Figure 5.6 The 633 virus deletion. Southern blot of genomic DNA from B95-8, P3HR-1 and 633 cells. The blot was probed with nick-translated BamHI Y DNA from B95-8 virus.

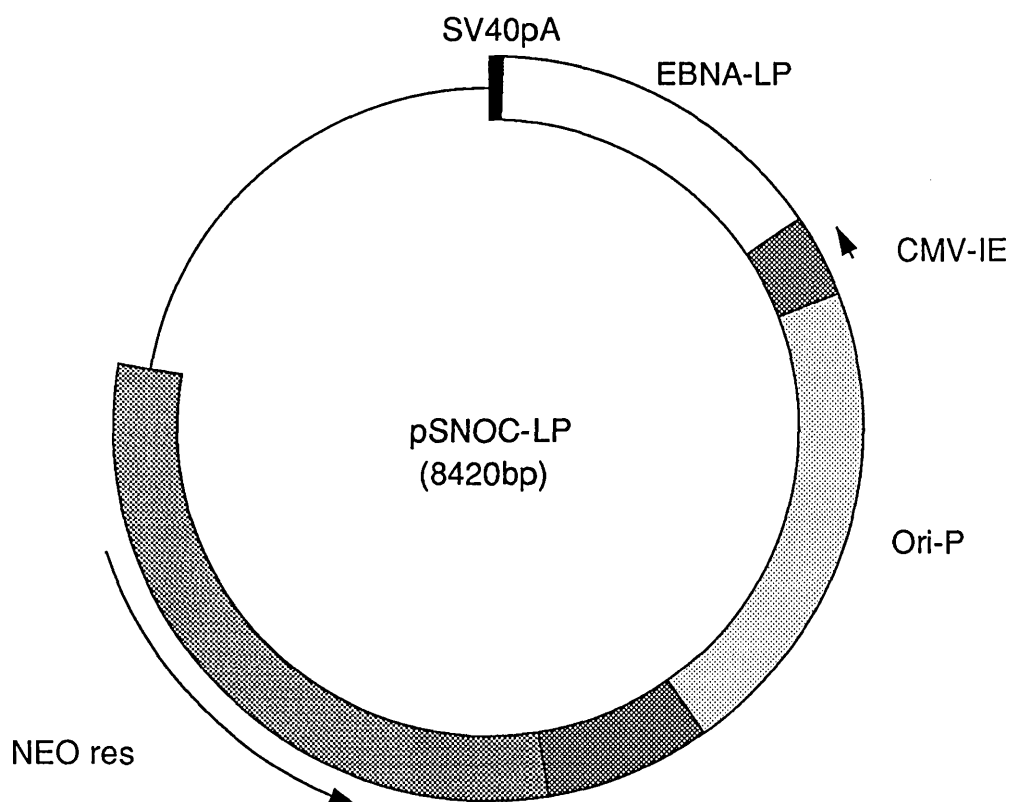


Figure 5.7 The oriP expression plasmid pSNOC-LP. pSNOC is a pBR322 derivative containing the CMV immediate early promoter and the SV40 poly-A sequences, upstream and downstream respectively of the pUC19 polylinker. It also has the EBV origin of latent replication, oriP, and the neomycin resistance gene. The details of the construction of this vector are given in Chapter 2. The unboxed part of the circle is the pBR322 vector sequences. EBNA-LP cDNAs with varying repeat copy numbers were cloned into this plasmid, orientated to be transcribed by the CMV IE promoter.

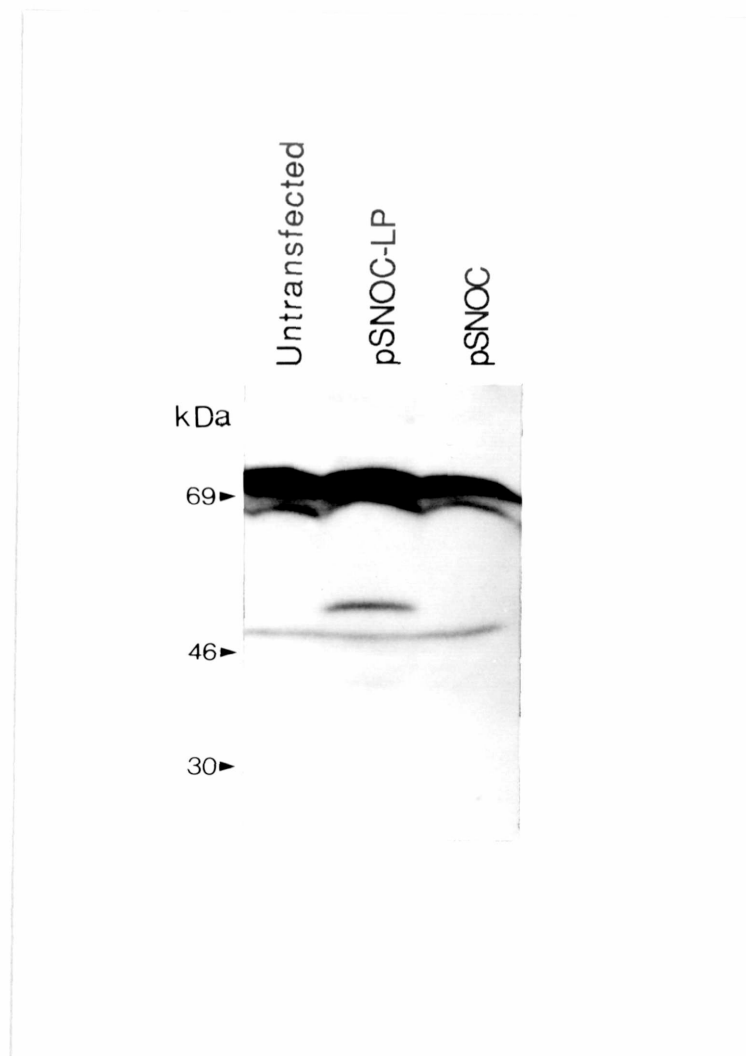


Figure 5.8 EBNA expression in 633 transfectants. Western blot of protein from untransfected 633 cells and two drug-resistant 633 cell lines transfected with either pSNOC-LP or pSNOC. The blot was probed with a human polyclonal serum (RM) which recognises all of the EBNAs. In all three tracks, EBNA 2 is the uppermost band, EBNA 1 is just below, and the lowest common band is thought to be the truncated Leader Protein expressed from the 633 virus. The pSNOC-LP transfected line expresses a full-length (7-repeat) Leader Protein. The positions of molecular weight size markers are indicated on the left hand side.

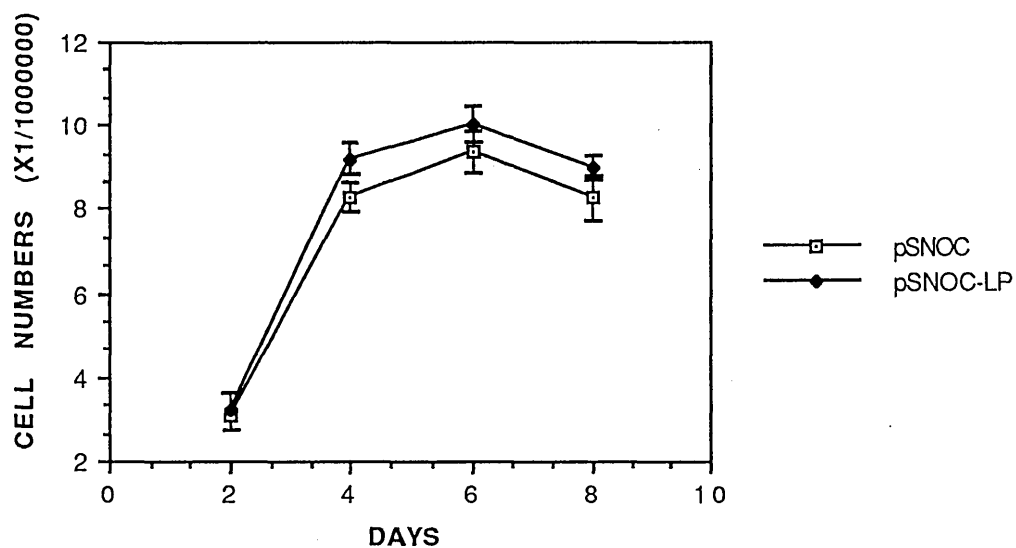
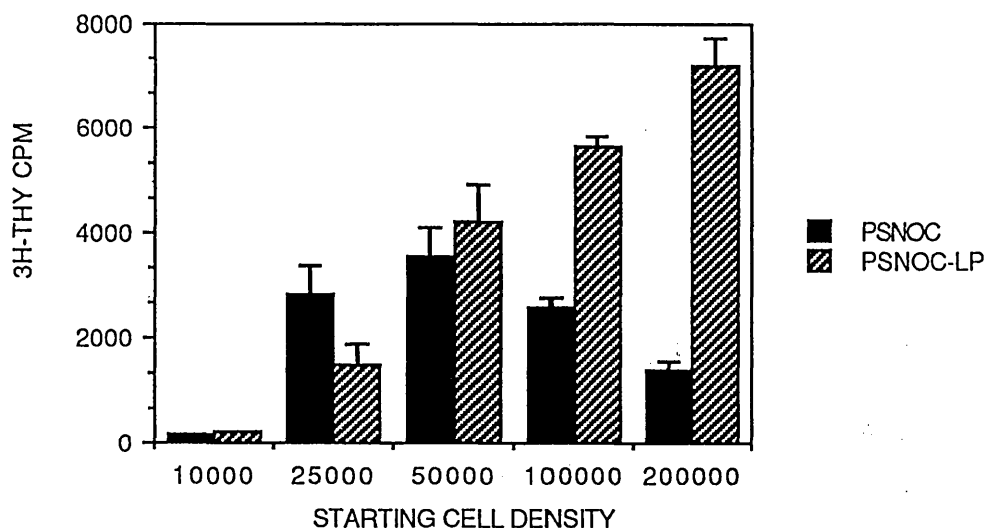


Figure 5.9 Growth Curve. Comparison of the growth rate between stable 633 cell lines expressing only truncated (pSNOC transfectants) or truncated and full-length (pSNOC-LP transfectants) Leader Protein. Cells were seeded at 5×10^4 cells/ml on the first day and aliquots taken 2, 4, 6 and 8 days later for cell counts. Cell counts were averaged from triplicate Coulter counter measurements (standard deviations are indicated).

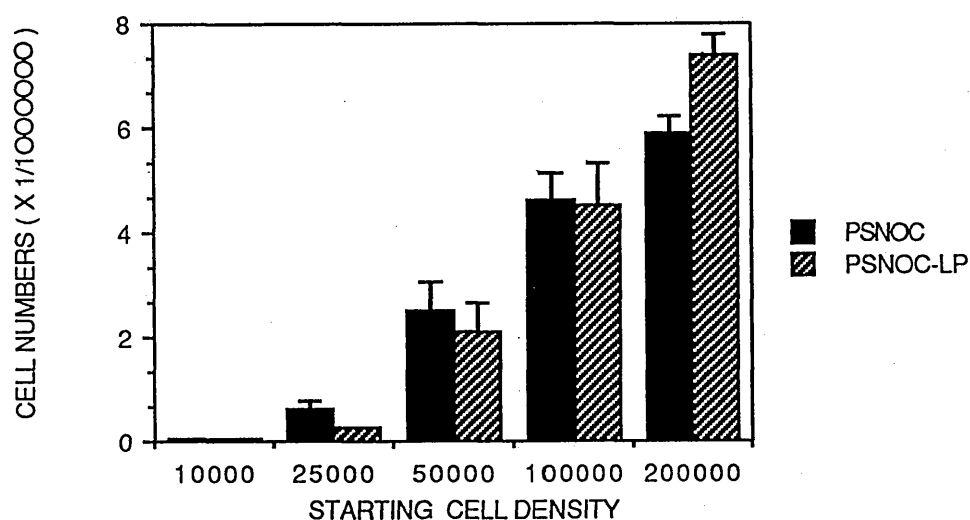
dilutions (from 2×10^5 down to 1×10^4 cells/ml) were set up in 96-well plates. These plates were then incubated for one week before counting the cells and measuring the incorporation of ^3H -thymidine (Fig 5.10). The cell counts were again essentially the same for the two lines. However, it was noticed that at the highest starting cell density, there was several fold more incorporation of ^3H -thymidine in the 633-pSNOC line (right hand side of Fig 5.10 A). Even when this data is normalised by dividing the counts by the cell numbers, to get counts per minute per cell, there was still approximately 3-4 fold more incorporation of label in these cells (right hand side of Fig 5.10 C). This experiment was repeated several times, and the same result was observed each time. A further example is shown in Figure 5.11 (note the difference between the two lines in the right hand side of 5.11 D). In these experiments the tritiated label was added to the wells four hours prior to harvesting in an equivalent volume of fresh growth medium, thus diluting the cells 2-fold. It seemed possible that this effect might be due to a different response to serum induction in the most saturated 633-pSNOC-LP cells.

This hypothesis was tested further by changing the conditions of the ^3H -thymidine incorporation experiments. This time the two lines were plated out at the same cell density, and left for approximately one week until they were both saturated. At this stage, the cells were counted and were again found to be at very similar cell densities (approximately 2×10^6 cells/ml). They were then diluted 2-fold in fresh growth medium and label added to aliquots taken at two hour time intervals following this. It was found consistently that, on serum induction, the line which contained full-length EBNA-LP, 633-pSNOC-LP, had a 3-4 fold greater increase in ^3H -thymidine incorporation than the line which only expressed the truncated LP, 633-pSNOC (Fig 5.12).

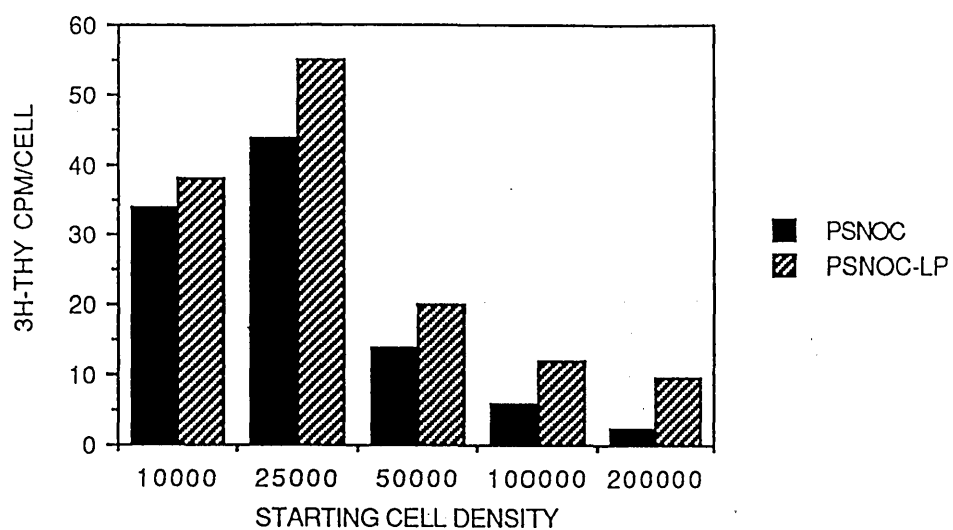
Figure 5.10 Doubling dilutions from 2×10^5 down to 1×10^4 cells/ml were made in duplicated 96 well plates, for both the 633-pSNOC and 633-pSNOC-LP cell lines. In each plate the dilutions were set up in triplicate. The plates were incubated for a week.



A. Uptake of ^3H -Thymidine. After 7 days the wells in one set of plates were diluted 2-fold in fresh RPMI-1640 containing the ^3H -Thy. These plates were incubated for a further 4 hours before harvesting the cells and measuring the incorporation of label.

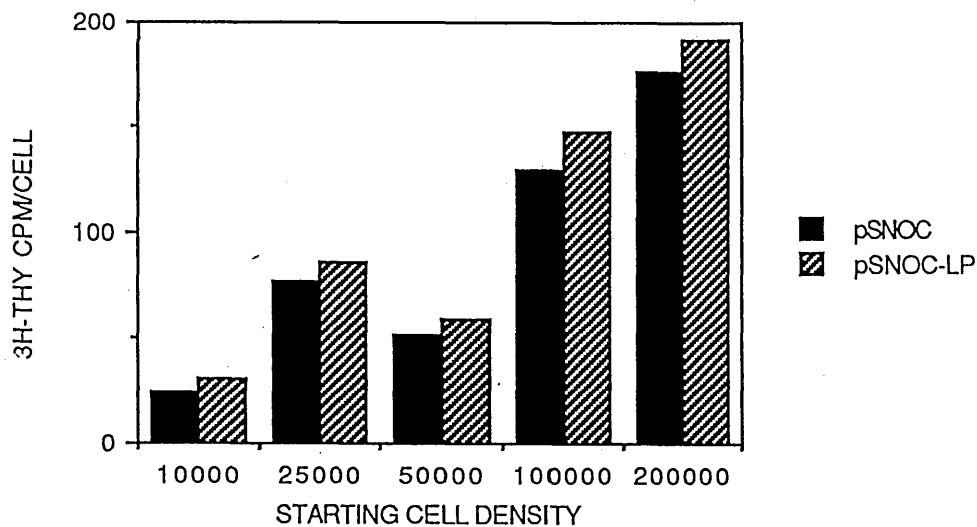


B. Cell Counts. The cells in the other set of plates were resuspended in isotonic buffer and counted using a coulter counter.

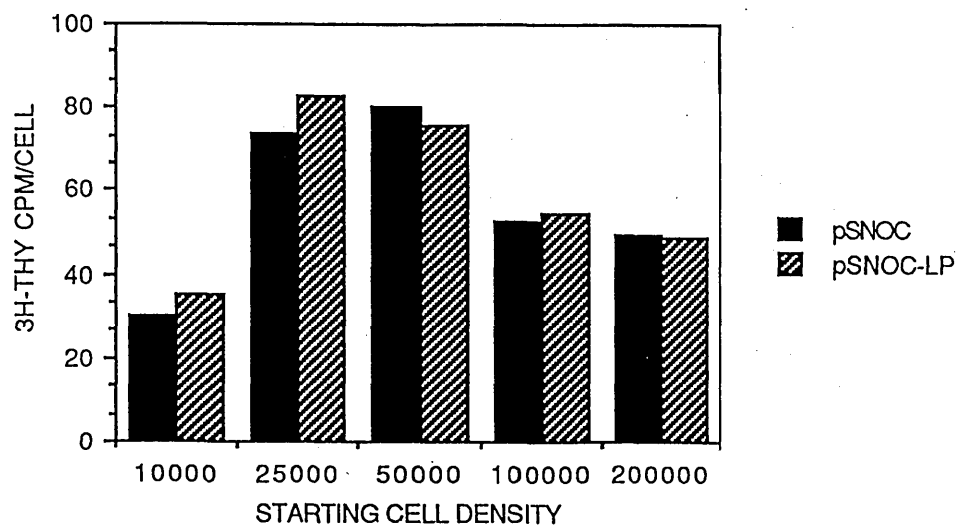


C. Normalised Data. The counts per minute of ³H-thymidine are divided by the cell numbers to give the c.p.m. per cell.

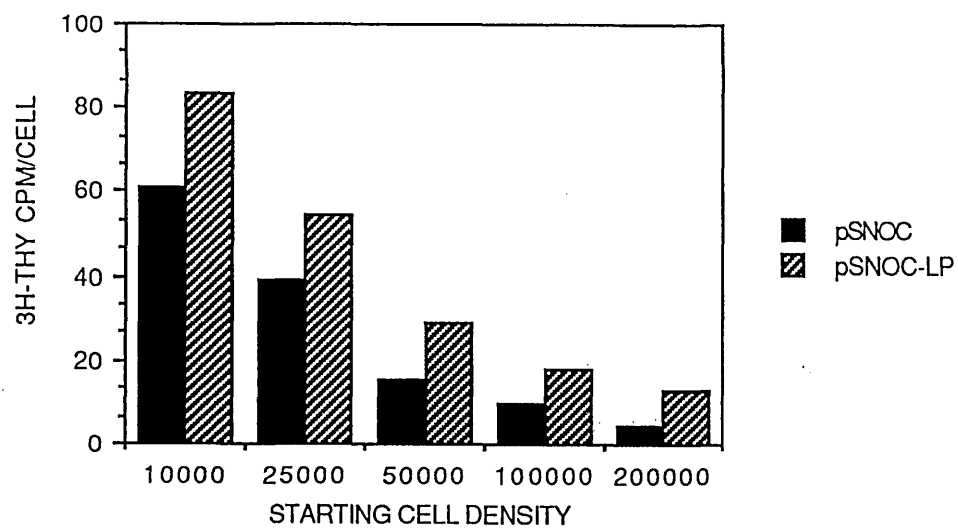
Figure 5.11 Doubling dilutions from 2×10^5 down to 1×10^4 cell/ml were made in duplicated 96 well plates, for both the 633-pSNOC and 633-pSNOC-LP cell lines. In each plate the dilutions were set up in triplicate. The plates were incubated for 2, 4, 6 or 8 days. As described before, the cells were counted in one set of plates and the incorporation of ^3H -Thy was measured in the other set. The data was normalised by dividing the counts per minute by the cell numbers to give the c.p.m. per cell.



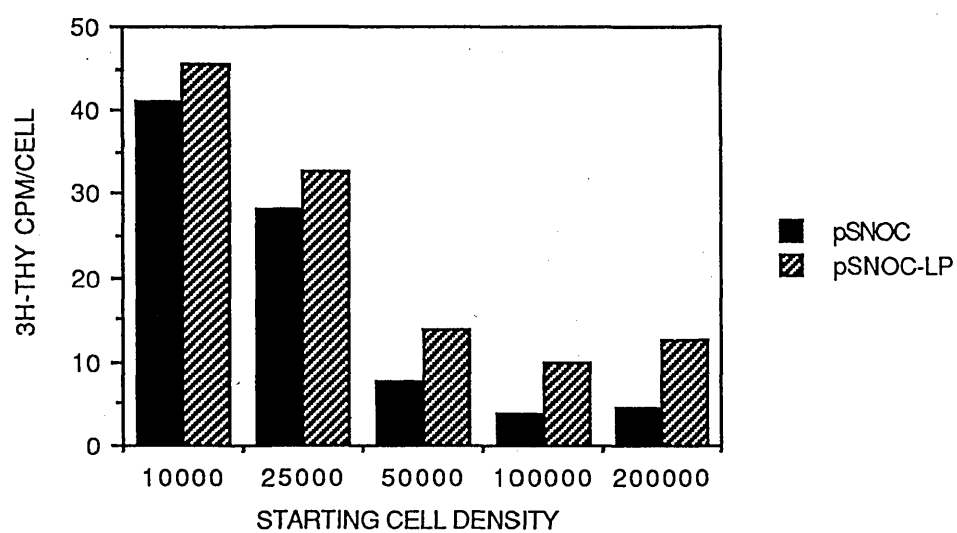
A. 2 Days Incubation



B. 4 Days Incubation



C. 6 Days Incubation



D. 8 Days Incubation

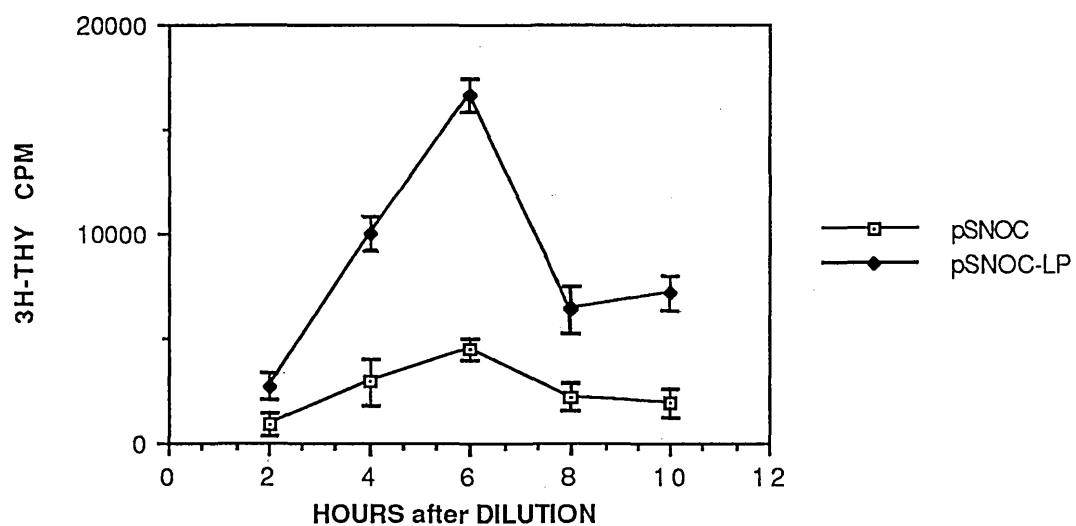


Figure 5.12 Growth of cells after saturation arrest. 633-pSNOC and 633-pSNOC-LP cells were left to grow for 7 days until the cells were saturated (at comparable cell numbers, $\approx 2 \times 10^6$ cells/ml). The cells were then diluted 2-fold in fresh RPMI-1640 medium with 10% FCS. Cells were then pulse labelled with ^3H -Thymidine from 0-2 hrs, 2-4 hrs, 2-6 hrs, 6-8 hrs and 8-10 hrs after dilution.

5.5 Discussion

The attempt to reduce EBNA-LP expression using antisense oligonucleotides containing repeat sequences may have failed because of the unusual nature of EBNA expression in EBV. There is evidence that antisense oligonucleotides exert their effect by hybridising to a specific mRNA, which then gets cleaved by RNase H (Shuttleworth and Colman, 1988). An antisense oligonucleotide containing W1W2 repeat sequences would therefore affect not only monocistronic EBNA-LP transcripts, but also those for the other EBNA's which have LP coding sequences in their 5' region (in certain circumstances EBNA 1 may utilise a separate promoter; pers. comm. S. Speck). This could explain the slightly lower levels of EBNA 1 and EBNA 2 expression that were observed when the cells were grown with higher concentrations of S-oligonucleotides that had antisense W2 sequences (Fig 5.2, 25 μ M LP and 50 μ M LP). Oligonucleotides that are antisense to the region 5' of the start site and overlapping the initial methionine or those beginning at the initial methionine and extending over the first few additional codons appear to be the most successful (reviewed by Marcus-Sekura, 1988). In addition, sequences that are antisense to splice sites have also proved effective (Smith *et al.*, 1986). Therefore, in these experiments it might have been better to employ antisense oligonucleotides which bridged the C2W1' or W0W1' splices in the EBNA-LP open reading frame.

The limited homology (Fig 5.1) between the unique carboxy terminal sequences of LP and conserved domain 1 of the Adenovirus transforming protein, Ela (Huen *et al.*, 1988) has already been discussed. Although it is not clear whether this homology reflects a common function, these sequences are important for the transformation by Ela. Mutational analysis of the Adenovirus Ela gene product has shown that

there are two conserved domains which are required for transformation (Whyte et al., 1988a). These same two regions are also important in the binding of Ela to the retinoblastoma gene product, Rb, and mutations which reduce Rb binding also abrogate the transforming activity of this viral oncogene (Whyte et al., 1988b; Whyte et al., 1989). Rb is now believed to be a tumour supressor which blocks the initiation of DNA synthesis (Buchovich et al., 1989; Chen et al., 1989b; DeCaprio et al., 1989), so that its binding by Ela would then permit the entry into S phase. The SV40 large T antigen and the HPV E7 protein also bind to Rb (DeCaprio et al., 1988; Dyson et al., 1989) and this is likely to be an important part of the way in which these viruses are able to circumvent the cell cycle control of the infected cells. The immunoprecipitation experiment with in vitro translated LP and Rb failed to show that EBNA-LP of EBV does not directly interact with Rb (Fig 5.3). This is supported by work from other laboratories (pers. comm. L. Young). However, in retrospect the experiment I performed on LP/Rb interaction is not conclusive, because a faint LP ³H signal might have been difficult to see against the multiple species of Rb. An obvious positive control would be to immunoprecipitate with an antibody to LP (we did not have JF186 at this point). There is now some debate as to whether both conserved domains of Ela are actually involved in binding Rb, or whether conserved domain 2 alone is required for this (pers. comm. K. Vousden). Although there is no apparent sequence homology between conserved domain 2 of Ela and any other EBV latent cycle protein, it is still conceivable that interaction between an EBNA and Rb is an important step in the immortalisation potential of EBV.

It has been shown that Ela is still able to rescue transformation in tsA 14 cells that are at 39°C, when conserved domain 2 is mutated. However, mutation of conserved domain 1 results in Ela being unable

to complement the loss of SV40 large T in this assay (Riley et al., 1990). This has led to the speculation that conserved domain 2 may be involved in the initiation of immortalisation by Ela, while conserved domain 1 plays a part in its maintenance. In addition, conserved domain 1 is required for the binding between Ela and a 300kDa cellular protein (van Dam et al., 1989). It has since been confirmed that it is this binding which enables Ela to operate in the tsA 14 system (pers. comm. K. Vousden). In a recent analysis of Ela it was shown that to permit uncontrolled growth associated with transformation, the Ela protein has to be able to bind to both Rb and the 300kDa protein (Howe et al., 1990). This work also included data showing that for Ela to induce DNA synthesis, it only has to bind to either Rb or the 300kDa protein. However, this is contradicted by other work which demonstrates that binding of the 300kDa protein is absolutely required for induction of DNA synthesis by Ela (Stein et al., 1990). It may be that the 300kDa protein also has a tumour suppressor like function, which provides an alternative control to that of Rb at the G1/S boundary in the cell cycle.

The experiments looking at transformation rescue by EBNA-LP in the tsA 14 fibroblasts are inconclusive. On the one occasion where colonies grew out at 39°C, the cells were subsequently shown not to be large T revertants, and to be expressing a protein of the correct size for a 7-repeat LP. At first sight this indicates a function for LP in the growth of these cells. However, the failure to reproduce this experiment casts doubt on whether these cells have retained their transformed phenotype as a result of their LP expression. An alternative explanation is that the plasmid DNA may have disrupted important cellular sequences when it integrated, allowing the cells to grow in the absence of the t.s. large T. Nevertheless, the amino acid sequences which are homologous between EBNA-LP and Ela are the

same ones which enable Ela to function in this assay (Riley et al., 1990). It may be worthwhile to repeat these experiments, perhaps using a different plasmid expressing EBNA-LP. Since this assay is based on binding to the 300kDa protein experiments have been started in this laboratory to determine whether there is a similar interaction between this important cell cycle regulatory element and LP.

The 633 EB virus provides an good system for analysing the contribution of a full-length EBNA-LP to the growth characteristics of EBV-immortalised cells. The conclusion that EBNA-LP does not contribute to the initiation of immortalisation by EBV (Hammerschmidt and Sugden, 1989) may be premature, since this ignores the fact that the efficiency of immortalisation is poor for 633 virus. In addition, the repetitive N-terminal region of LP appears to be stable within 633 cells, as the truncated protein can still be detected with the monoclonal antibody, JF186, which recognises an epitope in the W1 segment. The antibody fails to recognise the W1 epitope in the P3HR-1 LP (Finke et al., 1987), but it will recognise the B95-8 repeat sequences that were recombined into the P3HR-1 genome (Hammerschmidt and Sugden, 1989). In addition, the subnuclear localisation of the truncated LP is the same as that of full-length protein (pers. comm. B. Parker). This suggests that any function that the LP repeats could have which is separate from that of the carboxy terminal region, may still operate in the immortalisation of B cells by 633 virus. The construction of a truly EBNA-LP negative viral genome would require the removal of the major internal repeat region. This may not only be technically difficult (or impossible), but the many possible effects on any resulting virus would be hard to interpret.

Once the lines were established which expressed either the truncated LP alone or the truncated and full-length protein, it was hoped that the effect of the intact gene on the growth properties of EBV immortalised B cells could be assessed. There are some fundamental problems with this experiment which will be discussed now. The original B cells immortalised with 633 virus grew slowly and were feeder cell dependent. After a few months of passage in this laboratory, the growth of the cells improved markedly and they could be grown without feeder cells. The viral DNA in these rapidly growing cells was analysed and was found to still contain the 500 bp deletion in the BamHI Y fragment, which is characteristic of 633 virus (Fig 5.6). This also revealed that there was a faint Y/H fusion band, suggesting that there must be some residual P3HR-1 virus in the 633 cells. This is not surprising, as the only selection in the original recombination experiment was for immortalising virus (Hammerschmidt and Sugden, 1989), and there would be nothing to prevent unaltered P3HR-1 virus from co-infecting the primary B cells. However, the presence of P3HR-1 virus in these cells should have no bearing on a comparison between truncated and full-length EBNA-LP. The change in growth of the 633 cells prior to transfection of the pSNOC constructs is more problematic. When 633 virus was extracted from these cells and used to reinfect primary B cells, the efficiency of immortalisation was again low and the colonies could only be grown out on feeder cells. This suggests that any alteration that may have occurred in the original line must be cellular. To see the full effect of restoring EBNA-LP, it may have been better to transfect into the original cells that were still growing poorly. Unfortunately, it was only after the improvement in growth that it was possible to grow up enough cells for the transfections.

At first it seemed disappointing that the growth curves should be the same between the 633-pSNOC and 633-pSNOC-LP lines. The latter consistently reached a slightly higher cell density, but this was not a large difference. It was only when density saturated cells were diluted 2-fold in fresh medium and the incorporation of ^3H -thymidine was measured thereafter, that any significant effect was observed (Fig 5.10 A,C, right hand side; Fig 5.11 D, right hand side; Fig 5.12). I only had time to repeat these experiments with the two original cell lines 633-pSNOC and 633-pSNOC-LP. In order to establish whether the effect in these lines is artefactual or a result of the different LP status in the cells it will be necessary to repeat the experiments in several other 633 virus derived lines. The pSNOC and pSNOC-LP plasmids have not only been transfected into the 633 cells provided by Dr B. Sugden, but also into cells which were immortalised with 633 virus in our laboratory and several transfected lines have recently been produced (including some which express LPs with different numbers of repeats). My results suggest that the increased DNA synthesis in saturated 633-pSNOC-LP cells on serum induction is a result of full-length EBNA-LP being present in these cells.

The EBNA-LP phenotype described here in 633 immortalised cells should provide a basis for further study on LP function. This increased DNA synthesis may reflect there being easier entry into S-phase in the 633-pSNOC-LP cells. This is reminiscent of an interaction with a tumour suppressor, such as Rb, which exerts its effect at the G1/S boundary. Adenovirus Ela mutants which can no longer bind Rb, but bind the 300kDa protein, still overcome a block at G1/S and promote several rounds of DNA synthesis before the cells arrest in G2 (pers. comm. E. Moran). This supports other work which shows that binding of the 300kDa protein is sufficient to promote entry into S-phase (Howe *et al.*, 1990; Stein *et al.*, 1990). It is conceivable that EBNA-LP may

interact with the 300kDa protein or another control element at the G1/S boundary, thereby removing a cellular block to DNA synthesis. The importance of the repetitive structure of EBNA-LP in the 633 system may also be assessed, using the new lines which express LPs with different numbers of repeats.

General Conclusions

The structure of the EBNA-LP gene of Epstein-Barr virus is unusual in that it is primarily composed of multiple copies of repeated exons within the major internal repeat region (IR-1) of the virus. Consequently the protein product is made up of a number of 66 amino acid repeats, followed by 45 amino acids of unique carboxy terminal sequence. A distinctive ladder of Leader Proteins (LP) with a regular spacing of 6 to 8 kDa is frequently observed in EBV immortalised cells (Finke et al., 1987; Rowe et al., 1987). This could result from alternative splicing in the LP coding regions, or use of different internal W promoters. If the repetitive structure of LP is central to its function/s, it is interesting to speculate whether more or less repeats are an advantage to the virus.

The repeat copy number of IR-1 was determined in a variety of EB viral isolates by sizing large DNA fragments containing the repeats on Field Inversion gels. This demonstrated that the B95-8 genome contains 8.6 repeats, while the mean copy number of all the strains studied was 6. The analysis also determined that the naturally occurring deletion in the P3HR-1 viral strain, which renders this virus non-immortalising, only extends into the most 3' IR repeat and is therefore 6806bp in size. Overall a strong correlation between repeat copy number and the size of LP was not observed, although there was a general trend for smaller proteins to be made from genomes with fewer repeats. More problematic is the presence of LPs which are much larger than the theoretical size range determined from the repeat copy number. Throughout this study there was no evidence

for anomalous gel migration of in vitro translated LPs of those expressed in vivo by transfection. Other explanations are required to account for these large LPs, such as splicing between IR-1 sequences and other open reading frames downstream.

The FIGE analysis also revealed that in certain circumstances the number of repeats can alter. During virus induction and reinfection it appears quite common for the dominant number of repeats to change, whereas in latency the repeat copy number is relatively stable.

EBNA-LP can be expressed from both the C and W promoters of EBV (Woisetschlaeger et al., 1989). A Wp start requires a specific splice (W0 to W1') in order to create an initiator methionine codon for the LP open reading frame. S1 protection analysis from a different laboratory (Rogers et al., 1990) and the cDNA analysis presented here both demonstrate that an LP start codon is created by a very similar splice (C2 and W1') when the C promoter is active. In different systems it has been shown that translation initiation can sometimes occur from CUG codons (Clements et al., 1988; Hann et al., 1988; Prats et al., 1989). Although there are two such codons in the non-coding C2 exon, it appears that they cannot operate in the initiation of LP translation. Therefore, the expression of EBNA-LP is dependent on the detailed mRNA splicing, and this may be regulated in some way. Where the LP AUG is formed by splicing, translational polarity can lead to reduced expression of any other EBNA encoded in the 3' region of a bicistronic mRNA.

LP expression alone has no effect on the activity of the C promoter, as seen in CAT assays. In my hands, EBNA 2 by itself had only a modest effect on Cp compared to experiments where both EBNA 2 and LP may potentially be expressed. These two EBNAs may cooperatively

transactivate the C promoter, but further work is required to clarify this.

EBNA-LP has no known homologues in other viruses and little can be inferred about its function from its primary amino acid sequence. Repetitive peptide sequences have been observed in a diverse array of proteins, including structural proteins, secretory proteins, and proteins associated with the cell surface (Bressan et al., 1987; Klickstein et al., 1987; Moore et al., 1987; Sharp and Li, 1987). Within each of the LP repeats there are two possible amphipathic helices, which may be able to pack together. However, the rest of each repeat, being proline rich and otherwise hydrophilic, should have no structural stability, and because of this, it was suggested that the repeats could only function in an inert sense, such as the recognition and binding of some other protein (pers. comm. T. Gibson). There are none of the recognised DNA binding motifs (Mitchell and Tijan, 1989) within the repeat sequences.

EBNA-LP is implicated in the immortalisation potential of EBV, because of the loss of its unique 3' sequences in the non-transforming viral strain P3HR-1 (Miller et al., 1974; Menezes et al., 1975; Raab-Traub et al., 1978; Delius and Bornkamm, 1978; Rabson et al., 1982). It is not clear whether the limited homology between conserved domain 1 of the Adenovirus transforming protein, Ela and the unique carboxy terminal sequences of LP (Huen et al., 1988) reflect a common function, but it is interesting that these sequences in Ela are absolutely required for Ela mediated transformation (Zerler et al., 1987; Subramanian et al., 1988; Whyte et al., 1988a). For these reasons, my analysis of LP function was primarily directed at the possible role of EBNA-LP in immortalisation by EBV.

Transfection of an LP expressing plasmid into the tsA 14 fibroblasts did, on one occasion, result in colonies growing at the non-permissive temperature for the t.s. SV40 large T antigen. However, the failure to reproduce this effect casts doubt on whether LP can truly function in this assay. It is now known that the assay is based on an interaction between the transfected protein and the 300 kDa cellular protein (pers. comm. K. Vousden) and experiments are being undertaken to see if EBNA-LP binds to this important cell-cycle regulatory protein. In order to be sure that LP does not bind to the retinoblastoma gene product, Rb, more controls will have to be introduced into the LP/Rb co-immunoprecipitation experiments; such as using the LP specific monoclonal antibody, JF186 (Finke et al., 1987).

The 633 EB virus can only express a truncated EBNA-LP, which lacks its carboxy terminal sequences. This virus has a low efficiency of immortalisation and 633 virus immortalised cells grow poorly (Hammerschmidt and Sugden, 1989). As this phenotype may reflect the LP status in the cells, I decided to investigate this by reintroducing a full-length EBNA-LP into 633 cells. Drug resistant lines were established which expressed either the truncated LP alone or both the truncated and full-length version of the protein, named 633-pSNOC and 633-pSNOC LP respectively. Comparison of these lines revealed that they had the same growth curve. However, my results suggest that the presence of a full-length EBNA-LP in growth saturated cells leads to increased DNA synthesis on serum induction. It may be that entry into S-phase is facilitated in cells expressing an intact LP. One hypothesis would be that LP interacts with a tumour suppressor, such as Rb or the 300 kDa protein, thereby removing a block at the G1/S boundary of the cell cycle.

The work presented here on the structure and expression of EBNA-LP has characterised more fully this important Epstein-Barr viral antigen. The assay for LP function based on 633 EB virus describes for the first time a phenotype for this protein. This should provide a basis for further study on the role of EBNA-LP in EBV infected cells.

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