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THE EPSTEIN-BARR VIRUS NUCLEAR ANTIGEN (EBNA) COMPLEX

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ABSTRACT

Epstein-Barr virus (EBV) has the ability to convert a subpopulation of resting human B lymphocytes *in vitro* into continuously proliferating lymphoblastoid cell lines (LCLs). Viral gene expression in these cells is restricted and cells are described as "immortalized" or "latently infected" by EBV. An EBV associated nuclear antigen (EBNA) complex was first demonstrated in latently infected cells by anti-complement immunofluorescence. It has gradually emerged that viral gene expression in these cells is predominantly derived from a family of genes encoding nuclear proteins, all designated EBNAs. A widely held assumption is that EBNAs play major rôles in the processes of establishing and maintaining both the continuous growth of LCLs and EBV latency. They may also be involved in EBV-associated tumourigenesis. At the onset of this study only EBNAs 1 and 2 had been characterized and mapped on the EBV genome.

This thesis describes the identification, mapping, molecular cloning and partial characterization of an EBNA species designated EBNA6. (Three other antigens, EBNAs 3, 4 and 5 have recently been identified.) In addition it describes the characterization of immunological reagents which were subsequently used to investigate EBNA expression in a variety of EBV infected cells, cell lines and tumours. These studies have included a detailed analysis of the temporal sequence of EBV latent gene expression during the initiation of immortalization (when resting B cells are stimulated to enter and traverse the cell cycle) and in a B cell subset which fails to immortalize. Also the pattern of viral gene expression in various EBV associated tumours was determined. In particular, a very comprehensive study was carried out by constructing and screening a cDNA library made with RNA extracted from a nasopharyngeal carcinoma propagated in nude mice. This revealed an unique pattern of EBV latent gene expression. Finally, seeking an explanation for the highly restricted pattern of latent expression found in this and other tumours, the relationship between EBNA expression and CpG methylation of viral DNA was considered. An inverse correlation between methylation of certain CpG dinucleotides in the EBV large internal (W) repeat and expression of EBNAs 2-6 was revealed.

The results of these investigations, in conjunction with published data, are assessed and discussed. The rôle of the EBNA complex in the EBV life cycle and in EBV associated diseases is considered.

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I INTRODUCTION

1. THE BIOLOGY OF EPSTEIN-BARR VIRUS

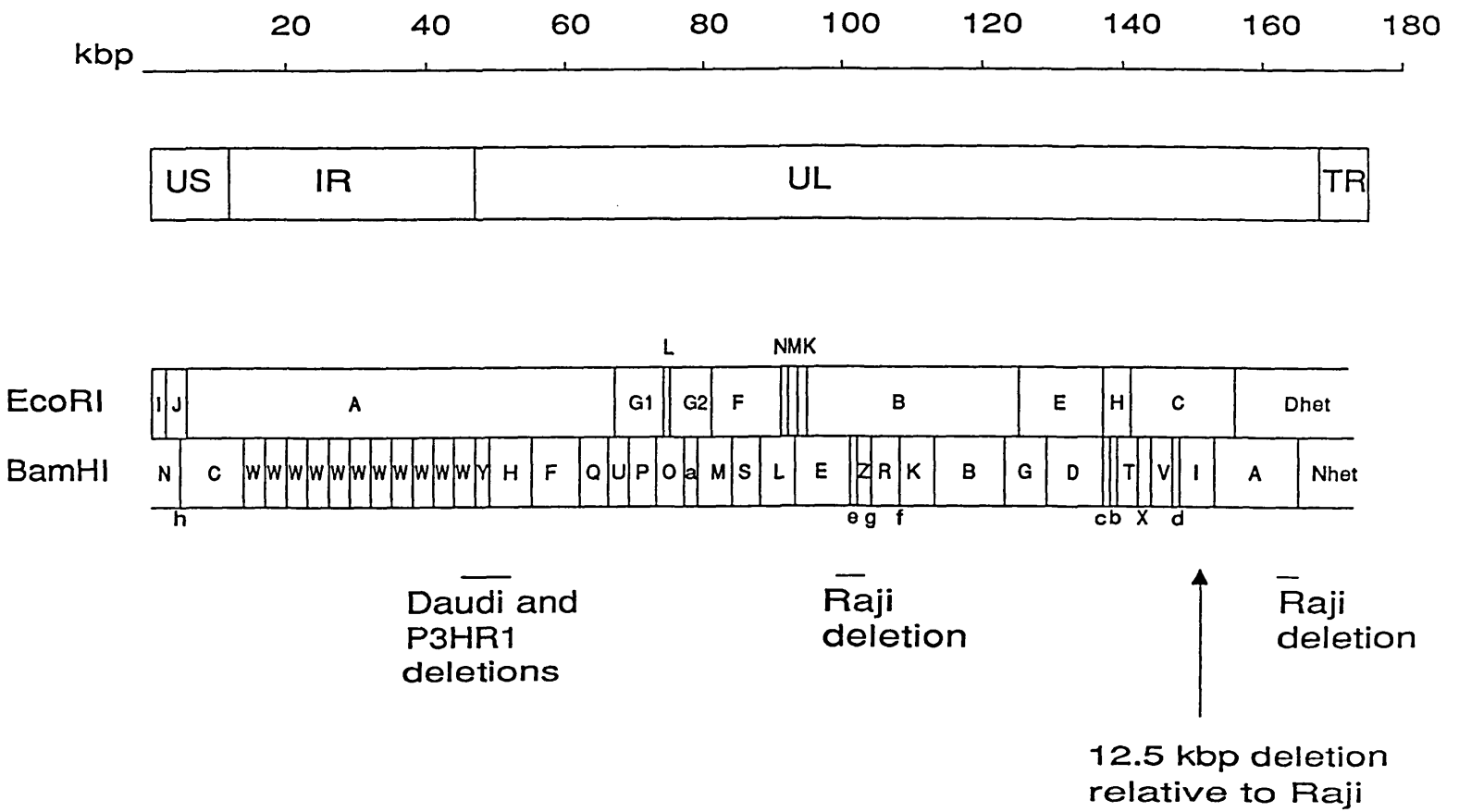
1.1 The Virus

Epstein-Barr Virus (EBV) is a member of the herpes family of DNA viruses which infects 90-95% of the world's human population (Henle and Henle, 1979). Similar to other herpes viruses, the EBV virion is composed of a core consisting of a double stranded DNA molecule (about 172 kbp), an icosahedral capsid and a membrane envelope enclosing the capsid (Epstein and Achong, 1979; Dambaugh *et al.*, 1986a). The linear DNA molecules of virus particles are circularized following infection (Lindahl *et al.*, 1976; Hurley and Thorley-Lawson 1988) to produce covalently closed circular episomes in infected cells. Circularization is thought to involve the interaction of 4-12 copies of a 500 bp terminal repeat (TR) located at each end of the linear molecule (Figure I.1). Multiple tandem repeats of a 3072 bp internal repeat (IR) sequence separate the genome into a short unique region (U_S) and a long unique region (U_L) (see Figure I.1). A number of EBV genomes have been cloned as sets of overlapping restriction fragments after cleavage with the enzymes *Bam*HI or *Eco*RI (for example, Arrand *et al.*, 1981) and the complete nucleotide sequence of the B95-8 strain has been determined (Baer *et al.*, 1984). Deletions and insertions with respect to the B95-8 genome which have been characterised in other virus strains are shown in Figure I.1.

1.2 Viral Tropism and Sites of Replication

A pronounced tropism of EBV for B lymphocytes is reflected in its association with three lymphoproliferative diseases of B-cell origin: acute infectious mononucleosis, African or endemic Burkitt's lymphoma, and lymphomas in immunocompromised individuals (see Section I.2). In addition, EBV infection of some B cells *in vitro* leads to their immortalization with little or no virus replication, and some human B cells harbouring EBV *in vivo* are capable of spontaneous outgrowth when cultivated (see Section I.1.4). This tropism is probably due to the presence of the C3d receptor (CR2, CD21 on B cells, which can act as a receptor for EBV (Fingeroth *et al.*, 1984; Nemerow *et al.*, 1986).

Figure I.1: Physical Map of the Genome from the B95-8 Strain of EBV. The linear genome can be split into unique short (US), large internal repeat (IR), unique long (UL) and terminal repeat (TR) regions. The linear molecule in EBV virions has terminal repeats at both ends; here they are only shown on the right as conventionally the DNA sequence is numbered from the beginning of US (Baer *et al.*, 1984). *Eco*RI and *Bam*HI restriction endonuclease maps are shown and so are the approximate locations of deletions and insertions (relative to B95-8) which have been found in some other EBV strains (see Text for references).


kbp
20
40
60
80
100
120
140
160
180
US
IR
UL
TR
EcoRI
BamHI
L
NMK
I
J
A
G1
G2
F
B
E
H
C
Dhet
N
C
W
Y
H
F
Q
U
P
O
a
M
S
L
E
Z
R
K
B
G
D
T
V
I
A
Nhet
h
e
g
f
c
b
X
d
Daudi and
P3HR1
deletions
Raji
deletion
Raji
deletion
12.5 kbp deletion
relative to Raji

Although EBV was thought to be a mainly B-lymphotropic virus, many investigators were puzzled as to where and how the virus replicated and was shed. Virus particles can be found in the saliva in most patients with infectious mononucleosis (Golden *et al.*, 1973), at low levels in almost all healthy EBV seropositive individuals (Yao *et al.*, 1985) and at high levels in most of those receiving immunosuppressive therapy (Strauch *et al.*, 1975). *In vitro*, EBV-infected human B cells are poor producers of virus; a very small proportion, probably those cells undergoing terminal differentiation, become permissive for virus replication (Crawford and Ando,1986). It is, however, far from clear how extensive virus replication in B cells is *in vivo*. Viraemia has not been demonstrated (Rickinson *et al.*, 1975) and no circulating B cells containing viral capsid antigen (VCA) have been reported. Although it is possible that a restricted number of terminally differentiating EBV-carrying B cells may release virus particles *in vivo*, such replication seems an inadequate explanation for the observed levels of viral shedding.

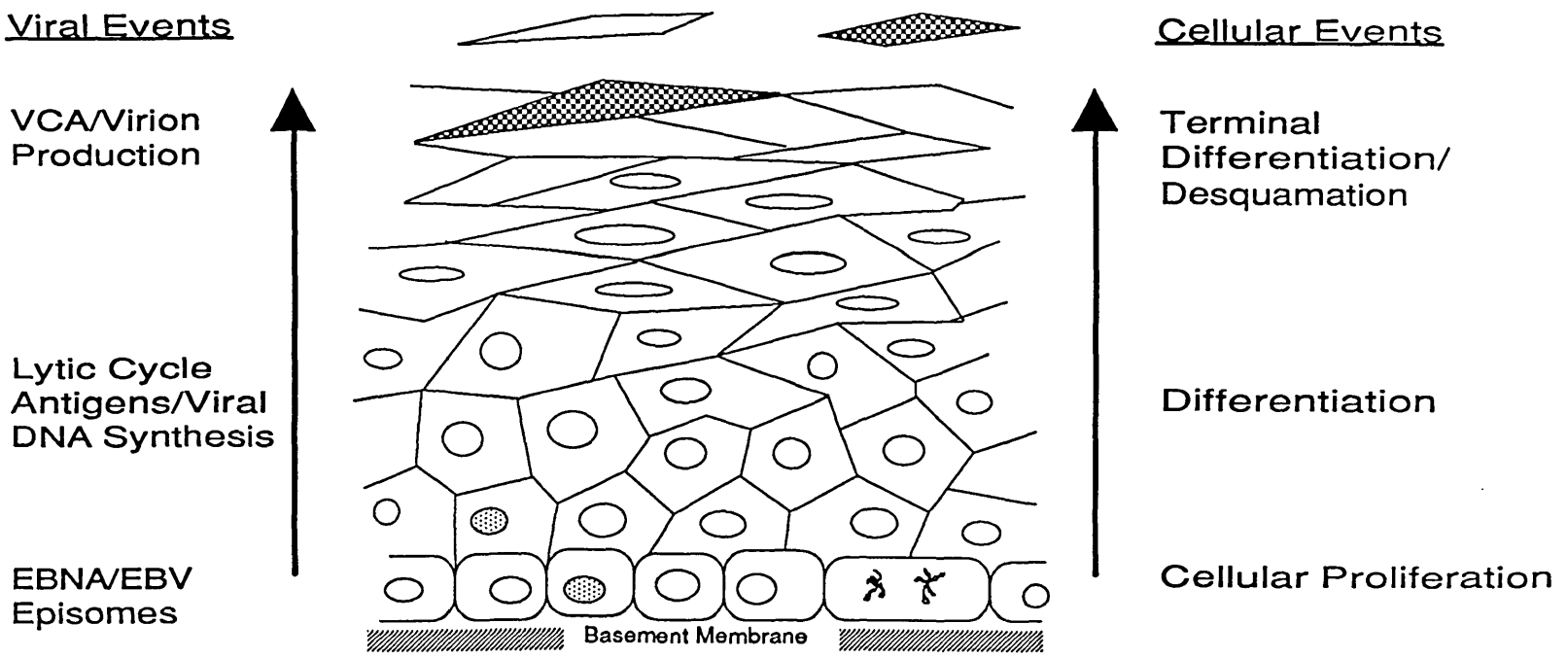
The detection of EBV DNA in the epithelial cell component of undifferentiated nasopharyngeal carcinoma (NPC) (zur Hausen *et al.*, 1970; Wolf *et al.*, 1973) clearly showed that EBV could enter pharyngeal epithelial cells. However, the site and cell type from which the salivary virus particles originate remained obscure until Lemon *et al.*, (1977), then Sixbey and colleagues (Sixbey *et al.*, 1984), demonstrated replicating EBV in desquamated epithelial cells in pharyngeal washings. Wolf *et al.* (1984), also using *in-situ* hybridisation, detected a low level of virus production in parotid duct cells. These findings suggest that epithelial cells in the oropharynx and/or salivary glands represent a primary site of productive infection with B cells becoming secondarily infected during their passage through the pharyngeal lymphoid tissue while virus is being shed. Sixbey and co-workers have also demonstrated EBV replication in cervical epithelial cells *in vitro* (Sixbey *et al.*, 1983) and replication and shedding of virus from cervical epithelium *in vivo* (Sixbey *et al.*, 1986). Moreover, a molecule similar to the CR2 (CD21) found on B cells has been reported on the surface of both pharyngeal and cervical epithelia (Young *et al.*, 1986; Sixbey *et al.*, 1987). This receptor may bind EBV. Replicating EBV has recently been demonstrated in hairy leukoplakia on the lateral margins of the tongue in AIDS patients (Greenspan *et al.*, 1985). Taken together, these data

provide compelling evidence that stratified squamous epithelium includes a cell type which is permissive *in vivo* for virus production.

These studies have revealed something further about the subtle relation between the virus and epithelium. The expression of productive cycle viral antigens (early antigens and VCA) and virion production seems to be restricted to the uppermost layers of cells in the stratified tissue or cells recently desquamated (Sixbey *et al.*, 1983; Sixbey *et al.*, 1984), although receptor expression seems to be confined to the immature cells (Sixbey *et al.*, 1986). Thus the productively infected cells are no longer mitotically active but are mature, terminally differentiated cells (see Figure I.2). The only EBV gene expression detected in the mitotically active cells of the basal and parabasal layers is the nuclear antigen complex (EBNA), as demonstrated by anti-complement-enhanced immunofluorescence (Sixbey *et al.*, 1983). Since there is evidence that EBNA1 antigen alone is sufficient for maintenance of the episomal form of EBV (see section I.4.5), this observation suggests that immature cells are infected with EBV but restrict expression of viral genes to the minimum required for the replication and partitioning of the episomal form of the EBV genome to daughter cells. In this state the cells may be invisible to the immune system.

Such observations led to the hypothesis that the expression of genes associated with replication and the subsequent production of virus is intimately involved with the maturation and differentiation of the infected epithelial cells (Sixbey *et al.*, 1986; Pagano, 1984), and they are consistent with the finding that virus production is linked to maturation in lymphoblastoid cell-lines (Crawford and Ando, 1986). Receptor-positive cells in mucosal sites such as the pharynx and uterine cervix can be infected at an early stage in differentiation. Their progeny mature, terminally differentiate, and when eventually desquamated actively produce virus (Figure I.2), leaving a self-renewing, latently infected stem-cell population in the basal layer of the epithelium. This mechanism is perfect for the persistence and lifelong shedding of virus which is characteristic of EBV infection *in vivo*. Such a strategy for viral replication and spread is reminiscent of that adopted by a variety of epitheliotropic viruses, including other DNA viruses such as Marek's disease virus and the papilloma viruses (Nazerian, 1980; McCance, 1986).

Figure I.2: EBV and Epithelium. Diagrammatic representation of how EBV production in stratified squamous epithelium may be linked to cellular differentiation and the desquamation of terminally differentiated cells. Shedding of virus will occur at the mucosal surfaces of the pharynx and uterine cervix. Light stippling indicates nuclei expressing EBNA; heavy stippling indicates cells producing virus.


Viral Events
VCA/Virion Production
Lytic Cycle Antigens/Viral DNA Synthesis
EBNA/EBV Episomes
Basement Membrane
Cellular Events
Terminal Differentiation/ Desquamation
Differentiation
Cellular Proliferation

1.3 Immortalization of B Cells

In vitro infection of B cells with EBV results in their immortalization to produce continually growing lymphoblastoid cell lines (LCLs). However, limiting dilution analysis has revealed that only 1-10% of the cells that are infected become immortalized (Katsuke and Hinuma, 1976; Henderson *et al.*, 1977; Katsuki *et al.*, 1977; Tosato *et al.*, 1985). It has been shown that small, dense B cells which have the highest concentration of CD21 (Aman *et al.*, 1984) are most likely to become immortalized. In addition, more mature surface IgM bearing B cells (Steel *et al.*, 1977; Chan *et al.*, 1986), less mature pre-B cells (Kubagawa *et al.*, 1988) and even foetal pro-B cells (Gregory *et al.*, 1987a) can be successfully infected and immortalized. Infection of leukaemic cells has shown that whereas chronic lymphocytic leukaemic (CLL) cells appear refractory to the immortalizing effects of EBV (Rickinson *et al.*, 1982) the more mature prolymphocytic leukaemic (PLL) cells are readily immortalized (D.H. Crawford personal communication; this study). Recent studies undertaken to isolate the immortalized cells after EBV infection have used the CD23 antigen as a marker (Thorley-Lawson and Mann, 1985; Azim and Crawford, 1988; Section I.1.4). Separation of the CD23-positive and negative B cells 2 or 3 days after infection has shown that immortalization is restricted to the CD23-positive fraction; these cells are also predominantly EBNA positive (Thorley-Lawson and Mann, 1985; Azim and Crawford, 1988). The CD23-negative cell fraction, on the other hand, contains both EBNA-positive and negative cells but no cells are immortalized; the EBNA-negative population appears to include cells which terminally differentiate and secrete high levels of immunoglobulin (Azim and Crawford, 1988). Thus although the EBNA complex (see Section I.4) is expressed 12-24 hours after infection and appears to be necessary to drive cells into blastogenesis and DNA synthesis (Einhorn and Ernberg, 1978; Thorley-Lawson, 1988) its expression is not always associated with immortalization. The observation that early after infection the percentage of cells expressing EBNA far exceeds the predicted incidence of immortalization (Zerbini and Ernberg, 1983) is consistent with these more recent observations.

Circularization of the EBV genome appears to be important for immortalization to occur; circular genomes have not been found in the CD23-negative cellular fraction. Analysis of the CD23-positive fraction suggests that only a single EBV genome needs to circularize in order for immortalization to proceed and latency to be established (Hurley and Thorley-Lawson, 1988). The single circular genome is then amplified (to around 50 copies/cell) about 1-2 weeks after infection, apparently when all the linear molecules have been lost (Hurley and Thorley-Lawson, 1988).

At least four strains of EBV have been identified which are incapable of immortalizing B cells: P3HR1 (Miller *et al.*, 1974; Rabson *et al.*, 1982); Daudi (Jones *et al.*, 1984; A.A. McBride personal communication) Naliaki and Jijoye-nude (Ernberg *et al.*, 1986). All four viruses carry genomes with a deletion in the *Bam*HI WYH region which removes the coding sequence for EBNA2 (see Figure I.1).

1.4 The Lymphoblastoid Cell Line (LCL)

Lymphoblastoid cell lines (LCLs) can be established from normal blood or lymphoid tissues by infection *in vitro* with immortalizing strains of EBV or they may occur by spontaneous outgrowth from the blood of EBV-positive individuals. They are generally polyclonal; the cells are diploid, have an irregular "spikey" appearance and usually grow in tight clumps. LCLs express some normal B cell markers including HLA Class I and II antigens, CD19, CD20 and surface Ig with or without cytoplasmic Ig and secretion (Walls and Crawford, 1987). Antigens characteristic of activated lymphocytes [such as CD23, AC2 (CD39), Ki-1 (CD30), Ki-24], cell surface adhesion molecules (such as LFA1, LFA3 and ICAM1) and receptors for Interleukin-2 (IL-2) and transferrin are also expressed (Rowe *et al.*, 1985; Gregory *et al.*, 1988a; Crawford and Ando, 1986).

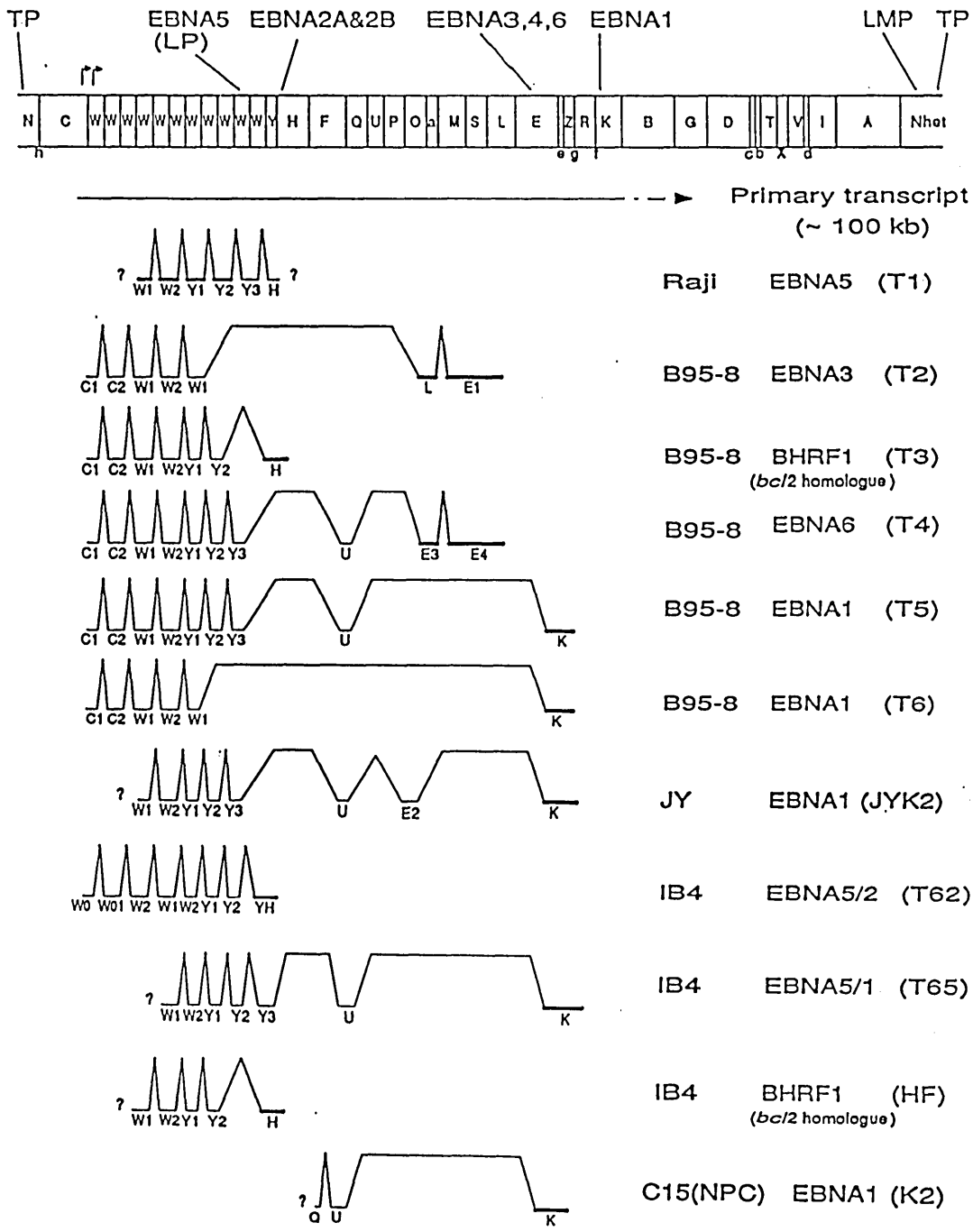
CD23 (also known as Blast-2) is of particular interest. It is a 45 kD membrane-associated glycoprotein (Thorley-Lawson *et al.*, 1986) which may act as receptor for low molecular weight B cell growth factor (BCGF) (Gordon *et al.*, 1986). CD23 is also rapidly shed from the surface of EBV immortalized B cells as a 25 kD polypeptide complexed to a 12 kD protein of unknown origin. This complex

called sCD23 (and also the 25 kD component alone) can stimulate and support the growth of EBV immortalized B cells under conditions of low serum and low cell density (Swendeman and Thorley-Lawson, 1987; Callard *et al.*, 1988). Thus sCD23 appears to be a major EBV-induced autocrine growth factor necessary for the independent growth of LCLs (Thorley-Lawson, 1988). This is consistent with the results of experiments which suggest LCLs produce and respond to their own BCGF (Blazar *et al.*, 1983; Gordon *et al.*,1984 a and b).

The EBV genome in LCLs is generally maintained as multiple copies (5-500 per cell) of an extrachromosomal circular episome (see Section I.1.3); viral gene expression from this (in non-lytic cells) is highly restricted. The cells are described as "latently infected" and "growth transformed" by EBV; the latter term is misleading, however, as the cells are not tumourigenic after subcutaneous inoculation in nude mice. EBV encoded antigens identified in LCLs include the latent membrane protein (LMP) and EBNA complex (EBNAs 1-6; see Section I.4 for details); other virus-encoded proteins such as early antigens (EA) and viral capsid antigen (VCA) are only expressed during lytic infection (see Section I.1.2 and I.1.5). Under standard culture conditions not more than about 5% of the B cells in virus-producing LCLs are undergoing lytic infection. Many lines established from foetal cord blood lymphocytes, for example IB4, (King *et al.*, 1980), contain no virus producing cells (or at least <0.01%); these lines are described as being "tightly latent".

EBV transcription in tightly latent lines (such as IB4) is derived from regions spread throughout the EBV genome. Data from northern blot analyses have revealed a number of very low abundance poly(A)$^+$ mRNAs (<5 copies/cell) transcribed from left to right (on the conventional EBV genome map, Figure I.1) corresponding to the *Bam*HI WYH, E and K regions (reviewed by Dambaugh *et al.*, 1986a; Figure I.3). Over the past five years it has gradually emerged that these transcripts encode the proteins of the EBNA complex (see Section I.4). A more abundant transcript (60 copies/cell), which encodes LMP, is transcribed right to left from the *Bam*HI N_{het} region (see Dambaugh *et al.*, 1986a; Figures I.1 and 3). In addition, two small non-polyadenylated RNAs (Rymo, 1979) transcribed by RNA polymerase III (Jat and Arrand, 1982) are encoded within the *Eco*RI J fragment. These EBERs (EBV encoded RNAs) are similar to the VA RNAs of adenoviruses in that they form a

Figure I.3: Complex Structure of EBV Latent Transcripts. Analysis of cDNA clones has revealed that mRNAs encoding the EBNA family and the *bcl2* homologue (BHRF1) are differentially spliced from primary transcripts of up to 100 kb long. Exon maps are shown with respect to the *Bam*HI restriction endonuclease map of the B95-8 strain of EBV. The cDNA clones are named according to the cell line or tumour from which they were derived. The 3' coding exon(s) are indicated by heavy lines and proteins they encode are shown. Exon structures are taken from the following references: Raji-T1 (Bodescot *et al.*, 1984); B95-8-T2 (Bodescot *et al.*, 1986); B95-8-T3, T4, T5, T6 (Bodescot and Perricaudet, 1986); JY-K2 (Speck and Strominger, 1985); IB4-T62, T65 (Sample *et al.*, 1986); IB4-HF (Austin *et al.*, 1988); C15-K2 (Hitt *et al.*, submitted).


TP
EBNA5 (LP)
EBNA2A&2B
EBNA3,4,6
EBNA1
LMP
TP
N C W W W W W W W W W W Y H F Q U P O M S L E Z R K B G D T V I A Nhet
h
e g f
c b X d
Primary transcript (~ 100 kb)
? W1 W2 Y1 Y2 Y3 H ?
Raji EBNA5 (T1)
C1 C2 W1 W2 W1 L E1
B95-8 EBNA3 (T2)
C1 C2 W1 W2Y1 Y2 H
B95-8 BHRF1 (T3)
(bcl2 homologue)
C1 C2 W1 W2Y1 Y2Y3 U E3 E4
B95-8 EBNA6 (T4)
C1 C2 W1 W2Y1 Y2Y3 U K
B95-8 EBNA1 (T5)
C1 C2 W1 W2 W1 K
B95-8 EBNA1 (T6)
? W1 W2Y1 Y2Y3 U E2 K
JY EBNA1 (JYK2)
W0 W01 W2 W1W2Y1 Y2 YH
IB4 EBNA5/2 (T62)
? W1W2Y1 Y2 Y3 U K
IB4 EBNA5/1 (T65)
? W1 W2Y1 Y2 H
IB4 BHRF1 (HF)
(bcl2 homologue)
? Q U K
C15(NPC) EBNA1 (K2)

complex with the cellular protein "La" (Lerner *et al.*, 1981). Their function, however, has yet to be determined.

Recently a number of groups have investigated latent EBV expression by cDNA cloning experiments. This has revealed that very long primary transcripts (>100 kbp) are initiated from one of two promoters located in the *Bam*HI C or W regions (Bodescot *et al.*, 1987) and then by a process of differential splicing mature messages (which in some cases are bicistronic) are generated. These in the main encode the components of the EBNA complex (see Figure I.3 and legend for details and references).

1.5 Viral Replication

Study of the viral productive cycle (lytic cycle) and its proteins has been hampered by the lack of a permissive cell system. Although EBV replicates in epithelial cells *in vivo* it has not yet been possible to study replication in this type of cell *in vitro*. This is due to the inefficiency of infection and presumably the requirement for an ordered pattern of cell differentiation. Our knowledge of EBV replication and the proteins of the replicative cycle has thus been derived primarily from studies of a few BL-derived cell lines and the EBV-immortalized marmoset B cell line, B95-8 (Miller *et al.*, 1972). In these lines usually 1-5% of cells express early and late antigens. Interestingly agents which alter the stage of differentiation of B cells, such as phorbol esters, induced a higher percentage of cells to enter the lytic cycle (zur Hausen *et al.*, 1978). This is consistent with the cell maturation-linked viral replication observed in both epithelial and B cells (see Section I.1.2).

A family of viral nuclear proteins (30-40 kD), apparently encoded by a single gene, is induced when cells enter the lytic cycle (Grogan *et al.*, 1987; Marschall *et al.*, 1989). When plasmids containing the gene which encodes these proteins (EBV open reading frame, BZLF1; Baer *et al.*, 1984) are transfected into tightly latent cell lines (<0.01% lytic) viral replication is induced in up to 8% of the cells (Grogan *et al.*, 1987). Furthermore, the proteins (designated ZEBRA or EBI, Countryman and Miller 1985; Chevallier-Greco *et al.*, 1986) transactivate a number of other genes associated with the lytic cycle including another transactivator of EBV genes (EB2,

encoded in *Bam*HI S, Chevallier-Greco *et al.*, 1986). Recently a cis-acting element in the EBV genome has been identified which mediates lytic phase DNA replication; this has been termed *ori Lyt*. The products of BZLF1 are involved in the activation of *ori Lyt*, however, it is not clear whether this is by direct transactivation or mediated through other viral or cellular genes. (Hammerschmidt and Sugden, 1988). Neither is it known what normally induces the expression of ZEBRA/EBI and initiates the replication pathway. Nevertheless, by analogy with other herpes viruses, it seems that a cascade of events occurs leading to sequential expression, first of "immediate early" (eg ZEBRA/EBI), then "early" and finally "late" gene products. The distinction between early and late is operationally defined by whether or not viral DNA synthesis is required for production of a protein. Although the exact number and function of all the early antigens is unknown it is assumed their major role is in replication of viral DNA. The late gene products are required for packaging viral DNA and forming the structural components of the virions (reviewed by Dillner and Kallin, 1988).

1.6 Control of EBV *in vivo* - the LYDMA Concept

It has not been possible to study the immune response to EBV during a silent primary infection and the primary response during infectious mononucleosis IM is complex and incompletely understood. Although there is a humoral component, cell mediated activity is generally assumed to represent the effective response which limits EBV-driven B cell proliferation. Suppressor T cells, NK cells and both EBV specific and non specific cytotoxic T cells, together with interferon, all play a role in control of primary infection; the details are, however, unclear (Rickinson, 1986). With the profusion of cell mediated activity it is not surprising that the proliferation of atypical lymphocytes in IM is composed mainly of T cells reactive to the viral infection (Sheldon *et al.*, 1973).

After the initial acute infection (whether it is silent or produces IM) the vast majority of individuals develop an asymptomatic persistent infection in which there is no overt sign of EBV associated lymphoproliferation. There is very good evidence that EBV-specific cytotoxic T-lymphocyte (CTL) memory plays the major role in regulating this viral persistence and preventing uncontrolled proliferation of

EBV-infected B cells (Moss *et al.*, 1978; Rickinson *et al.*, 1981a; Sections I.1.7). The concept of LYDMA (lymphocyte detected membrane antigen) was proposed to describe the target(s) expressed on EBV immortalized B cells *in vitro* and infected cells *in vivo* which elicited a virus specific T cell response. It was first postulated in order to explain what was thought to be an EBV specific T cell response in IM (Svedmyr and Jondal, 1975; Klein *et al.*, 1976a); subsequently it was adopted to describe the target(s) for the cells which establish CTL memory in persistently infected individuals (Rickinson *et al.*, 1981 b). This response was soon shown to display HLA class I restriction (Wallace *et al.*, 1982) but target structures have only recently been identified.

DNA-mediated transfection studies using recombinant plasmids encoding LMP (see Section I.4.1) suggest that this membrane protein (and in particular epitopes derived from the N-terminal region) provides a target(s) for an EBV-induced CTL response (Murray *et al.*, 1988a). This is consistent with a report that a 10 amino acid polypeptide corresponding to a region of LMP expressed on the cell surface is recognised by, and induces proliferation in, both HLA restricted CTL and also the anomalous or natural killer cells of acute phase IM (Thorley-Lawson and Isrealson, 1987). Further studies have suggested that EBNA2 also acts as a target for EBV specific CTL recognition (Moss *et al.*, 1988). These results are consistent with the demonstration that a processed influenza nuclear protein can be a target for CTLs (Townsend *et al.*, 1986); however, in this particular investigation the experiments were performed with EBNA2A and 2B strains of virus (see I.4.6), rather than isolated genes. It is possible, therefore, that significant variations might exist in gene products other than EBNA2 (for instance LMP or the other nuclear antigens) and these could contribute to the apparently EBNA2-specific responses observed.

1.7 The Nature of Viral Persistence

How the virus/host balance is controlled in normal seropositive individuals without the complete elimination of the virus from the body is obscure and is only partly explained by a model such as that outlined above (Sections I.1.2 and 1.6.). However, various conditions in which levels of viral shedding are increased have thrown some light on the mechanisms that control this persistent infection. Renal

transplant recipients, who are iatrogenically immunosuppressed for long periods, have been studied in detail. Immunosuppressive drugs such as cyclosporin A (CSA) have a profound effect on cell-mediated immune function but little on antibody-mediated mechanisms (Leoni *et al.*, 1978). CSA suppresses the function of EBV-specific T cells both *in vivo* (Crawford *et al.*, 1981) and *in vitro* (Bird *et al.*, 1981). Thus the increase in viral shedding from the pharynx which is regularly found in renal transplant recipients suggests that control of EBV replication is largely T-cell mediated, and the raised serum antibody titres to EBV lytic-cycle antigens merely reflect, rather than control, these events.

The numbers of circulating EBV-infected B cells are also increased in immunosuppressed patients (Crawford *et al.*, 1981), and it has been assumed that this is due to a dampening of immunosurveillance mechanisms. Specific HLA restricted cytotoxic T cells may be recovered form the peripheral blood of healthy virus carriers (at least 100-1000 LYDMA-specific CTL precursors per 10^6 circulating T cells; Rickinson *et al.*, 1981a). These T cells are assumed to be responsible for clearance of the infected B cells. However, the effect of CSA on oropharyngeal shedding leads one to conclude that a critical target for T cell immunity must also be the epithelial cells in which the virus is replicating. This conclusion is consistent with the correlation that exists between severely declining T cell immunity in AIDS patients and the development of EBV-associated oral hairy leukoplakia (see Section I.2.8; Greenspan *et al.*, 1987; Redfield and Burke, 1988). It has not been clearly established whether there are cells (either epithelial or B lymphocytes) which contain the EBV genome and are long-lived because they are invisible to the immune system. However, several recent studies suggest that such cells may reside in the B cell population. Rickinson and colleagues (Yao *et al.*, 1989a; Yao *et al.*, 1989b) and Tosato (1987) have reported that EB virus infected B cells persist, and can be recovered from the circulation of acyclovir-treated carriers. These data suggest that virus carrying B cells survive in fully immunocompetent hosts for at least a month (the period during which oral virus replication and shedding was blocked by the action of acyclovir). Such observations are consistent with another report (Gratama *et al.*, 1988) in which it was demonstrated that EBV-carrying cells were completely eliminated by the pre-transplant chemotherapy and total body irradiation given to a recipient of an allogeneic bone marrow. Because the haemopoietic system is

particularly sensitive to such treatment it was assumed that the EBV infected cells must have been resident in this compartment.

2. DISEASES AND SYNDROMES ASSOCIATED WITH EBV

2.1 Acute Primary Infection and Infectious Mononucleosis (IM)

If infection with EBV occurs during the early years of life it is usually asymptomatic. Approximately fifty percent of those who first encounter the virus during adolescence, however, develop IM. This is a generally self limiting disease characterised by polyclonal proliferation of EBV-infected B cells, followed by the appearance of characteristic, atypical (anomalous) cytotoxic T lymphocytes. These are the "mononuclear" cells that give rise to the name of the disease. However, the typical IM syndrome (fever, lymphadenopathy, pharyngitis, skin rash, splenomegaly, etc.) usually lasts about three weeks then symptoms resolve and this is followed by life-long seroconversion and asymptomatic persistent infection (reviewed by Chang, 1980). During and directly following infection, antibodies (IgM and IgG) to VCA and early antigens (EA) are detectable. After this IgG to VCA is generally produced for life; titres tend to reflect the level of virus shed in the oropharynx (Henle *et al.*, 1974; Henle and Henle, 1979). IgG to one or more of the EBNA species (see Section I.4) appears 30-50 days after infection and titres against these antigens seem to remain essentially stable over many years (Henle *et al.*, 1974; MJA unpublished observations). T cell immunity has been discussed above (Section I.1.6 and 7).

2.2 Fatal Lymphoproliferation

Although IM involves a characteristic B cell proliferation, this virtually always resolves; extremely rarely it does not. This is usually in individuals with a genetically determined immunodeficiency, XLPS (X-linked lymphoproliferative syndrome) and is rapidly fatal (Purtilo *et al.*, 1979). Although the precise nature of the syndrome is unclear, lymphoproliferation and infiltration of tissues appears to go unchecked. It has been reported in one case that the spontaneously derived LCLs (from an XLPS child) were resistant to HLA restricted CTL killing (Ando *et al.*, 1986). Abnormalities of NK cell activity have also been reported (Sullivan *et al.*,

1983). It has been suggested that the cause of death is due to the uncontrolled proliferation of the atypical killer cells associated with IM infiltrating and damaging vital tissues such as the liver (Weisenbergen and Purtilo, 1986). Lymphocytic infiltrates have been shown to include EBNA positive B cells, but these generally only represent a minor population (Ando *et al.*, 1986). Occasionally, in addition to the characteristic diffuse infiltrates, multiple EBNA-positive tumour-like nodules can develop (Morgan-Capner *et al.*, 1986; Thomas *et al.*, 1989).

2.3 "Chronic" Mononucleosis

A rare sequel to IM is a chronic illness manifested as fatigue, recurrent fever, sore throat, lymphadenopathy, muscoskeletal pains, headache and occasionally more severe abnormalities such as interstitial pneumonitis and hepatosplenomegaly (Schooley *et al.*, 1986). This is generally called "chronic mononucleosis" and is thought to be due to a chronic active EBV infection. The disease process is very poorly understood; however, an interesting, but completely unexplained observation has been that these patients produce either no anti-EBNA1 antibodies (Miller *et al.*, 1987) or very low titres of anti-EBNA1 relative to anti-EBNA2 titres (Henle *et al.*, 1987).

2.4 Burkitt's Lymphoma

i. Introduction:

The tumour now called Burkitt's lymphoma (BL) was first described as an outgrowth of rapidly dividing undifferentiated lymphoid cells, often localising in the jaw, which occurred at high frequency in children from central Africa (Burkitt, 1958, 1962). It is now classified as a non-Hodgkin's lymphoma, a high-grade malignant lymphoma of the small non-cleaved cell group, a monoclonal proliferation of a B cell subset (Rosenberg, 1982). The areas where BL is found at high incidence are distributed across equatorial Africa and in Papua New Guinea, generally at altitudes below 1500 metres; this distribution is coincident with that for holoendemic malaria (Burkitt, 1962).

EBV was first discovered in cultured BL biopsies by Epstein and colleagues 25 years ago (Epstein *et al.*, 1964). Subsequently 96% of all the endemic BL (eBL) from the high incidence regions have been shown to contain EBV DNA and also express EBNA (zur Hausen and Scholte-Holthausen, 1970; Reedman and Klein 1973; Lindahl *et al.*, 1974). The remaining 4% (referred to as sporadic or sBL) contain no detectable EBV DNA or antigens; the frequency of this tumour in tropical Africa is about the same as it is throughout the rest of the world (Lenoir, 1986). Unlike eBL, sBL is not generally restricted to children between 6-10 years of age but occurs mainly in adults and often localizes in abdominal tissues but rarely in the jaw (Ziegler 1981). A large prospective study performed by the WHO showed that if an African child has an elevated EBV VCA titre he/she has an increased risk of developing eBL (>30 times the normal population) (de-Thé *et al.*, 1978). EBV is always present in eBL and the epidemiological data suggest it plays an important role in tumourigenesis. The virus is however extremely ubiquitous and sBL can develop in its absence, therefore EBV clearly cannot be considered the sole aetiological agent in BL.

ii. Chromosomal Translocations:

All unequivocally identified BL tumour cells (whether eBL or sBL) contain the characteristic reciprocal chromosomal translocations, first described by Manolov and Manolova (1972; 1979) and recently reviewed by Lenoir (1986). Three such translocations have been identified and all involve a breakpoint in the long arm of chromosome 8 that occurs consistently at the 8q24 locus. The most frequent, t(8:14)(q24:q32), transposes the telomeric region of chromosome 8 to chromosome 14. In the less common, "variant translocations" t(2:8)(q11:q24) and t(8:22)(q24:q11), material is transposed from chromosome 2 to 8q24 and from 8q24 to chromosome 22 respectively, (see Lenoir, 1986). Molecular analysis has revealed that the breakpoint on chromosome 8 is consistently adjacent to or within the *cmyc* oncogene and that chromosomal translocation results in a juxtaposition of *cmyc* with one of the immunoglobulin gene complexes; heavy chains on chromosome 14, *k*-light chains on 2 and λ-chain on 22. In recent studies chromosomal breakpoints have been analysed by molecular cloning and DNA sequencing. These have revealed a remarkable heterogeneity of the precise loci involved; different tumours all seem to have different breakpoints. However, a pattern appears to be emerging which suggests

that different regions within the immunoglobulin loci are involved in translocations in the distinct forms of BL (see Table I.1). Although the translocations in eBL and sBL are similar they probably occur by different mechanisms. This suggests the tumours may derive from lymphocyte precursors at different stages along the B cell differentiation pathway (Table I.1 and Figure I.4). In eBL the translocations predominantly involve immunoglobulin heavy-chain J-regions [or kappa light-chain J regions in t(2:8) variants]. In sBL, on the other hand, the breakpoints occur within or near the immunoglobulin heavy-chain μ switch-region (sμ), (Pellici *et al.*, 1986; Haluska *et al.*, 1986; Hartl and Lipp, 1987; Neri *et al.*, 1988). Thus sBL tend to secrete IgM and represent more mature "activated" B cells; in eBL all the data suggest that the translocation occurs early during B cell differentiation, when functional Ig genes are formed (Alt *et al.*, 1986; Haluska *et al.*, 1986). Furthermore, when the c*myc* gene is not directly disrupted by the translocation (that is in most eBL and a few sBL; see Table I.1) there are invariably point mutations and/or deletions in the 3' region of exon 1 (Cesarman *et al.*, 1987; Hartl and Lipp. 1987; Morse *et al.*, 1989). These alterations may play an important role in the activation of the c*myc* gene by disrupting the premature termination of transcription that normally occurs near the end of exon 1 (Cesarman *et al.*, 1987; Eick *et al.*, 1987). Additionally such mutations sometimes prevent the synthesis of the 68 kD form of c*myc* protein (c-Myc-1) which initiates at a CUG codon in exon 1 (Hann *et al.*, 1988). This is consistent with the observation that most BLs only synthesise the 65 kD protein (c-Myc-2) (Hann *et al.*, 1988). Activation of c*myc* therefore involves both translocation to an immunoglobulin locus and disruption of transcriptional and translational control signals in exon 1 resulting in a gene that cannot be "switched off". It is unclear precisely what role the translocation plays - a favoured notion is that c*myc* is brought into a region of "active chromatin" possibly placed under the influence of an Ig enhancer element or dominant control region (DCR) (Lenoir and Bornkamm, 1987; Grosveld *et al.*, 1987).

iii. c*myc* as an Oncogene:

In DNA transfection studies, using rat embryo fibroblasts, constitutive c*myc* expression induces continuous proliferation of cultured cells (immortalization) and reduces the requirement for growth factors (Keath *et al.*, 1984; Land *et al.*, 1983; Mougneau *et al.*, 1984). These cells are not, however, tumourigenic; c*myc* can

Table I.1: Molecular Analysis of BL Translocations

	Endemic BL (eBL)	Sporadic BL (sBL)	AIDS-BL
EBV	+	-	+/-
Breakpoint w.r.t.‡ *cmyc*	5' distant†; or 3' to gene (in 8:22 variants)	5' Flanking region; or within Exon 1 or 3' to gene (in 8:22 variants)	as sBL and eBL
Mutations in *cmyc* exon 1	+	+/-*	+/-
Translocation involving J_H region	+	-	+/-
Translocation involving S_μ region	-	+	+/-
IgM secretion	-	+	+/-

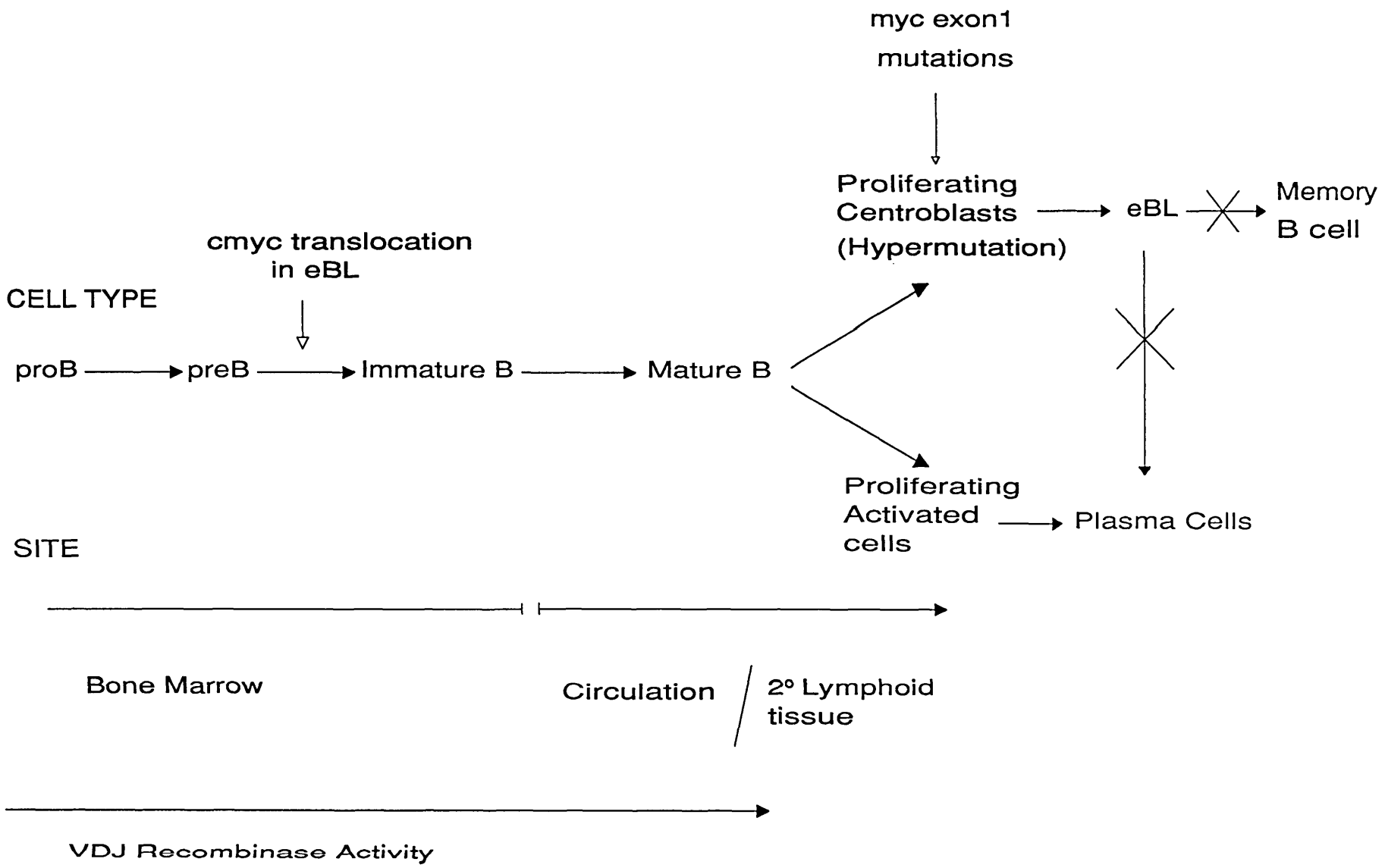
† The breakpoint may be more than 20 kbp 5' to *cmyc*

+/- indicates that either may occur

* Not if breakpoint within exon 1

‡ w.r.t. = with respect to

Figure I.4: Activation of *cmyc* in Endemic Burkitt's Lymphoma (eBL). Vertical arrows indicate the two points in B cell ontogeny at which molecular lesions leading to deregulation of *cmyc* are thought to occur (based on Haluska *et al.*, 1987 and Ling *et al.*, 1989).


myc exon1
mutations
Proliferating
Centroblasts
(Hypermutation)
eBL
Memory
B cell
cmyc translocation
in eBL
CELL TYPE
proB
preB
Immature B
Mature B
Proliferating
Activated
cells
Plasma Cells
SITE
Bone Marrow
Circulation
2° Lymphoid
tissue
VDJ Recombinase Activity

cooperate with other factors, (for instance activated *ras* and polyoma middle T antigen) to render these primary cells fully malignant (Land *et al.*, 1983; Mougneau *et al.*, 1984). Studies in transgenic mice and chick embryos infected with *myc*-retroviruses suggest that deregulation of c*myc* alone is insufficient to induce fully malignant tumours but that it can induce a premalignant polyclonal proliferation at a particular stage of differentiation, that is, in pre-B cells in transgenic mice and pre-B/immature B-cells in the chick embryos (Langdon *et al.*, 1986; Thompson *et al.*, 1987). These cells are presumably prevented from entering the next G_o in B cell ontogeny (when they would become mature resting B cells) by the constitutively expressed c*myc* protein. Tumours which subsequently develop in these animals are monoclonal; this is consistent with other factor(s) cooperating with c*myc* and contributing to tumourigenesis.

A recent study, which attempted to mimic eBL, suggested that constitutive expression of c*myc* from stably transfected plasmid DNA in human B cells (LCLs) previously immortalized by EBV induced a tumourigenic phenotype. This was judged by growth in soft agar and tumour production in athymic nude mice (Lombardi *et al.*, 1987). The significance of these data is, however, controversial. Similar experiments performed in our laboratory have produced cells which constitutively express c*myc*, have altered growth properties and a modified cell surface phenotype but remain non-tumourigenic (Hotchin *et al.*, submitted for publication).

iv. Multistep Carcinogenesis and the Aetiology of eBL:

Neither EBV nor activation of c*myc* alone seem to induce eBL. Two hypotheses have been proposed to explain the aetiology of BL (Klein 1979 and 1987; Lenoir and Bornkamm, 1987). In his original model Klein proposed that in high incidence areas, children carry a very large number of EBV-infected cells. This is due to immunosuppression of T cell responses to EBV-infected cells caused by malarial (*P. falciparum*) infection (see Whittle *et al.*, 1984). He argued that the presence of this large pool of mitotically active EBV-carrying B cells increased the possibility of a random second event - the translocation involving c*myc*. The observation that EBV immortalized B cells (LCL) never appear to develop these specific *myc* translocations has always been considered inconsistent with this view.

The model has therefore been reappraised and updated in the light of more recent findings by Lenoir and Bornkamm (1987). They suggested that the c-*myc*/immunoglobulin gene translocation, because it is clearly non-random (being associated with an aberration of normal B cell development), occurs at a regular but low frequency when B cells are actively rearranging their immunoglobulin genes. Immunosuppression due to chronic malaria is not considered particularly important in this model. Polyclonal B cell activation by *P. falciparum* is, on the other hand, critical. This would increase the pool of B cells, recruited from the bone marrow, with their Ig genes undergoing rearrangement and therefore likely to develop a translocation. These cells would usually die unless they are infected by EBV and the deregulated *cmyc* is complemented by an, as yet unidentified, EBV function. In favour of the Lenoir and Bornkamm proposal is the observation that the BLs which are an increasingly common feature of AIDS are associated not so much with the chronic immunosuppression of the late stages of the disease, as with the polyclonal B cell activation which produces the chronic reactive lymphadenopathy in the early stages (see Section I.2.5). The Lenoir and Bornkamm scenario also provides an explanation for the rare sBL where another genetic lesion could replace EBV infection and provide the final stimulus for neoplastic transformation. The cooperation of *ras* and *cmyc* oncogenes in the malignant transformation of murine B lymphocytes suggests that *ras* gene activation may substitute for EBV infection in sBL (Schwartz *et al.*, 1986). Consistent with this hypothesis is the observation that an sBL derived cell line, Ramos, contains an activated N-*ras* gene (Murray *et al.*, 1983).

v. The BL Cell Phenotype:

The use of monoclonal antibodies to study the surface of cells in BL biopsy material and newly cultured BL cells has revealed that the tumour cells have a consistently uniform phenotype. Antigens expressed in addition to the expected B cell markers (such as CD19 and CD20) include the common acute lymphoblastic leukaemia antigen (CALLA, CD10) and the BL-associated antigen, BLA (Rowe *et al.*, 1987; Rowe and Gregory, 1988). These cells do not, however, express the B cell "activation" antigens found on all LCLs and most normal B cells following activation, for example CD21, CD23, Ki24, CD30, CD39 etc (Rowe *et al.*, 1987; Rowe and Gregory, 1988; Section I.1.4). Neither do BL biopsies and early passage (<20) BL

cells express the adhesion molecules LFA1, ICAM1 and LFA3 (Gregory and Rickinson, 1988; Gregory *et al.*, 1988a) which are also found on most LCLs. Both sBL and eBL cells display the phenotype described above. However, many EBV-positive eBL lines have a capacity to "drift" towards the LCL-like phenotype within the first 20 or so passages *in vitro* (Rowe *et al.*, 1987). A nomenclature was proposed in which the BL biopsy phenotype is called group I and the LCL phenotype group III. Intermediates in the phenotypic drift are termed I/II and II (Rowe *et al.*, 1987; Rowe and Gregory, 1988). BL derived cells, unlike LCLs are generally tumourigenic in nude mice assays, (Nilsson *et al.*, 1977). sBL, which generally maintains the group I phenotype *in vitro*, can be induced to express the activation markers CD21 and CD23 (characteristic of the "drifted" group III cells) by conversion with the immortalizing B95-8, but not the non-immortalizing P3HR1 strain of EBV (Calender *et al.*, 1987). This suggests that the "drift" may be driven by an increase in the number of EBV genes expressed - in particular EBNA2 (see below and Section I.4.6).

Recently Gregory and colleagues have identified a normal B cell with essentially the same phenotype as BL cells. These normal cells appear to be rapidly proliferating centroblasts located in the germinal centres of lymphoid follicles in the tonsils (Gregory *et al.*, 1987b; Gregory *et al.*, 1988b). It has been proposed that the malignant BL cell *in vivo* is blocked at a germinal centre B cell phenotype and that the final genetic lesion(s) (translocation and/or point mutations/deletions) in the c*myc* gene occur in such cells (Ling *et al.*, 1989; Rowe and Gregory, 1988). As isotype class switching is thought to occur in proliferating germinal centre B cells, (MacLennan and Gray, 1986) this would provide a satisfactory explanation for the switch-region translocations in sBL. Similarly, as the period of hypermutation in the maturation of the immune response (selecting high affinity immunoglobulins) is also thought to occur in germinal centres (Berek and Milstein, 1987; MacLennan and Gray, 1986), point mutations or deletions in an already translocated c*myc* locus may produce the final genetic abberation leading to neoplastic transformation in eBL (Ling *et al.*, 1989; Figure I.4).

vi. EBV Expression in eBL:

The pattern of EBV antigen expression in latently infected eBL cells is different from that found in LCLs (see Sections I.1.4 and I.4). Significantly,

although EBNA1 is invariably present, EBNAs 2-6 and LMP have not been detected (Rowe *et al.*, 1986; Rowe *et al.*, 1987 and this study). When EBNA2 and LMP are expressed in B cells they become targets for EBV-specific cytotoxic T lymphocytes (that is, they are components of LYDMA; see Section I.1.6). Thus, in relatively immunocompetent individuals there is strong immunoselection against their expression. Expression in tumour cells would result in their elimination and inhibition of tumour development. The failure to detect these antigens in eBL is therefore consistent with the observed resistance of group I cells to T-cell killing (Rooney *et al.*, 1985; Rowe *et al.*, 1986). It is unclear, however, from currently available data what restricts the expression of EBV latent genes in these cells. Interestingly, when BL cells are grown *in vitro*, and immunoselection removed, cells expressing the full range of latent proteins appear in the culture; these have a selective advantage and outgrow the remainder (Rowe *et al.*, 1986; Rowe *et al.*, 1987). This expansion of EBV gene expression to include EBNA2 and LMP probably accounts for the phenotypic drift from group I to group III. In addition to EBNA1, the small untranslated EBERs are always expressed in eBL cells (Rymo *et al.*, 1979; Rowe *et al.*, 1987). It is not known whether other, as yet unidentified, EBV gene products are also present in eBL cells and contribute to the malignant phenotype.

2.5 Post-Transplantation Lymphomas Associated with EBV

Recipients of allografts such as kidneys, hearts and heart/lungs are generally treated, post transplantation, with immunosuppressive drugs such as cyclosporin A (CSA); this produces profound changes in the T cell mediated responses to EBV infected cells (see Sections I.1.6 and 7). If immunosuppression is prolonged, EBV is shed at an increased rate in the oropharynx and virus infected B cells can in some cases proliferate unchecked and give rise to a spectrum of lymphoproliferative disorders. At one end of this spectrum are EBNA-positive polyclonal B cell hyperplasias similar to those seen in acute IM (and fatal IM). These cells are cytogenetically normal and the lesions regress on removal of immunosuppression (Starzl *et al.*, 1984) and may also regress after acyclovir treatment (Hanto *et al.*, 1982). Acyclovir blocks viral replication by inhibiting EBV DNA polymerase activity, but has no effect on the proliferation of latently infected B cells (Collins,

1983). This suggests, therefore, that at this polyclonal stage of the disease viral replication contributes to tumour progression: viral replication in pharyngeal epithelial cells, with secondary infection of B cells, may be a necessary link in the disease. At the other end of the spectrum are life-threatening malignant lymphomas (mainly non-Hodgkin's lymphomas of the large cell type, Rosenberg, 1982). These are again EBNA-positive, monoclonal, do not respond to either removal of immunosuppressive therapy or acyclovir treatment and generally contain cytogenetic abnormalities (but not of the characteristic BL-type) (Hanto *et al.*, 1981; Cleary, 1986). It has been reported that progression of these tumours from poly- to monoclonality can occur (Hanto *et al.*, 1981; Shearer *et al.*, 1985). Generally, however, the disease presents as discrete tumour nodules which are already monoclonal. If there are multiple tumours at different sites they usually represent different clones (Cleary, 1986a). It appears then that the evolution of these lymphomas is a multistep process in which both EBV infection and another genetic lesion (or unidentified epigenetic factor, such as microenvironment) is necessary for complete neoplastic transformation and the development of a monoclonal proliferation. An assumption that such a "second event" is uncommon would provide an explanation for the rarity of these tumours (1-2% of transplants; D.H. Crawford, personal communication). At least two studies (Ho *et al.*, 1985; Thomas *et al.*, 1989) suggest that primary infection after transplantation is a considerable risk factor for the development of such lymphomas. It is even possible that all primary EBV infections occuring during the period of immunosuppression after transplantation lead to the development of life threatening tumours. This suggests that EBV seronegative recipients may be significantly more at risk than seropositive individuals.

Malignant lymphomas have also been reported in bone marrow transplant recipients. These tumours generally develop in cases of mismatch (histoincompatability between donor and recipient) where the donor marrow has been *ex vivo* T cell depleted (to reduce the likelyhood of graft-versus-host disease) and high-doses of anti-thymocyte globulin (ATG) have been used post-transplantation. The tumours are usually of donor-cell origin (Shapiro *et al.*, 1988).

2.6 Non-Hodgkin's Lymphomas Associated with AIDS

Human immunodeficiency virus (HIV) seropositive individuals are at increased risk of developing malignant lymphomas. The latter have been reported so frequently that the occurence of non-Hodgkin's lymphoma is now included as a manifestation of AIDS (Ziegler *et al.*, 1984; U.S. Centre for Disease Control, 1985). The lymphomas developing in AIDS patients appear to fall into two categories. The first, histologically defined as large cell or histiocytic lymphoma, often presents as multicentric brain lymphomas. These EBV-positive tumours are generally found in patients with late stage AIDS (Walter Reed staging 4, 5 and 6) involving profound T cell dysfunction and characterised by multiple opportunistic infections (Kalter *et al.*, 1985). The tumours closely resemble those found in immunosuppressed transplant patients and usually show a poor response to chemotherapy (Kalter *et al.*, 1985). The second, diffuse undifferentiated lymphoma, usually present as abdominal tumours which are histologically and clinically similar or identical to Burkitt's lymphoma. These BL-like lymphomas are associated with the generalised reactive lymphadenopathy and less severe immune dysfunction found in the early stages of AIDS (Walter Reed staging 1, 2 and 3) (Ziegler *et al.*, 1982 and 1984; Kalter *et al.*, 1985) and patients with these have a better prognosis because, like eBL, they respond well to chemotherapy (Kalter *et al.*, 1985). Most studies of these BL-like tumours have not considered histology, translocation breakpoints and EBV involvement in the same series of tumours. However, the picture which has emerged from a number of separate studies is that within this group of tumours, with similar features to either lymphomas eBL or sBL can be found. There are reports of both EBV-positive and EBV-negative tumours (Haluska *et al.*, 1987; G. Lenoir, personal communication) and, although most of the Ig/*cmyc* translocations from AIDS-BL so far analysed are of the sBL type (that is involving sμ region), translocations similar to those found in eBL have also been reported (Neri *et al.*, 1988; Haluska *et al.*, 1987; C. Croce, personal communication). The chronic reactive lymphadenopathy, recruiting B cells from bone marrow and increasing the number of cells undergoing Ig gene rearrangements, appears to be a predisposing factor for both eBL and sBL in HIV-positive individuals.

2.7 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma occurs at a very high frequency in Southern China and to a lesser extent in northern and eastern Africa and among Eskimos. The evidence linking EBV with NPC is compelling. The association between EBV and anaplastic (poorly differentiated) NPC is virtually 100%, irrespective of geographical or ethnic origin (Klein, 1979). All the malignant cells carry multiple copies of circular EBV DNA (zur Hausen *et al.*, 1970; Nonoyama and Pagano, 1973; Wolf *et al.*, 1973; Raab Traub *et al.*, 1986) and express EBNA (as judged by anti-complement immunofluorescence, ACIF) (Klein *et al.*, 1974). Various cofactors, which presumably complement EBV in the aetiology of NPC, have been proposed. These include a predisposing HLA phenotype (Lanier *et al.*, 1980) and environmental carcinogens or tumour promoters in foods, medicines or drinks (de Thé and Zeng, 1986; Poirier *et al.*, 1987). As in BL, antibody titres to VCA are elevated and antibodies to EA are regularly detected in NPC sera (Henle and Henle, 1979). The production of high titre IgA antibodies to VCA and EA appears to be unique to NPC patients and their detection has therefore been very useful in the early diagnosis of the disease (Zeng and de Thé, 1985; de Thé and Zeng, 1986). The serology is consistent with a strong aetiological association between EBV and NPC.

Although the malignant cells within the tumour are epithelial in origin, there are always heavy infiltrates of T lymphocytes (Thomas *et al.*, 1984) which remain uninfected by EBV (Wolf *et al.*, 1973). It has been reported that some NPCs secrete interleukin-1α (Busson *et al.*, 1987; Busson *et al.*, 1988) and suggested that this lymphokine production may account for the T-cell infiltration. Because the epithelial cells from NPCs cannot generally be cultured *in vitro* little is known about these cells. No consistent cytogenetic abnormalities have been detected (Mitelman *et al.*, 1983) and until recently the information on EBV transcription in tumour cells was scarce and poorly defined. The regions apparently expressed correspond to *Bam*HI fragments W, Y, H, K and N_{het} and the *Eco*RI J fragment (Raab Traub *et al.*, 1983). Transcription of the *Eco*RI E, *Bam*HI I and A regions has also been reported in some tumours (Tugwood *et al.*, 1987). However, neither of these reports included details of the transcripts or associated proteins, merely specific hybridization.

NPC can sometimes be propagated in athymic nude mice (Klein *et al.*, 1974; Trumper *et al.*, 1977; Raab Traub *et al.*, 1983) and recently three such NPC-related tumours were established from North African patients (Busson *et al.*, 1987 and 1988). One of these, C15, was extensively analysed in this present study. Sources of fresh NPC biopsies (together with the means of preserving and transporting them) have also become available. Analyses of EBV "latent" protein expression (see Section I.1.4) in primary and nude mouse propagated NPC material has revealed that in NPC cells only EBNA1, and, in approximately 50%, of cases LMP are expressed; none of the other EBNA species have been detected (Young *et al.*, 1988; Fåhraeus *et al.*, 1988 and this study). Based on the available evidence it is far from clear what contribution EBV makes to the aetiology of NPC.

2.8 Oral Hairy Leukoplakia

Oral hairy leukoplakia (HL) is a lesion that appears clinically as white, irregular surface patches on the lateral margins of the tongue (Greenspan *et al.*, 1984 and 1985). It is found in HIV-positive individuals and is associated with the development of established AIDS (U.S. Centre for Disease Control, 1985; Greenspan *et al.*, 1987). The lesions invariably contain multiple copies of EBV DNA and late viral antigens and sometimes other intra-epithelial viruses such as human papilloma virus (Greenspan *et al.*, 1984 and 1985; Löning *et al.*, 1987). Both oral and intravenous administration of acyclovir are effective in producing complete regression of HL; the lesions return (at the original sites) two to three weeks after acyclovir treatment is discontinued (Andersson and Ernberg, 1988; I. Weller, personal communication). These observations are consistent with HL being the result of uncontrolled replication of EBV in oral keratinocytes, with cytopathic effects (including nuclear inclusions and ballooning) leading to clinically apparent lesions which morphologically resemble oedematous disruption of the oral epithelium (Löning *et al.*, 1987). HL most commonly develops in the latter stages of AIDS (Walter-Reed staging: 4, 5 and 6) when T cell mediated immunity is severely compromised and multiple opportunistic infections are becoming apparent (Greenspan *et al.*, 1987; Redfield and Burke 1988). A recent report has shown that epithelial Langerhan's cells are completely absent or reduced at the sites of HL

lesions (Daniels *et al.*, 1987). This specific defect in mucosal immunity may be a critical event in the pathogenesis of HL.

2.9 Other Diseases and Syndromes Associated with EBV

In addition to the well established links between EBV and the diseases considered above, there have been a number of (generally isolated) reports suggesting associations between EBV and various B cell, epithelial cell and even T cell related conditions. These include: carcinomas of tonsillar, supraglottal laryngeal, parotid and thymic epithelium (Brichacek *et al.*, 1984; Brichacek *et al.*, 1983; Saemundsen *et al.*, 1982; Leyvraz *et al.*, 1985). EBV has been demonstrated in the B cell proliferations in lymphocytic infiltration pneumonitis (LIP) of childhood AIDS (Andiman *et al.*, 1985) and in Castleman's syndrome (Hanson *et al.*, 1988). A recent report of a case of Kawasaki-like disease suggests that EBV may infect T cells (Kikuta *et al.*, 1988).

3 EBV-ASSOCIATED TUMOURS IN NON-HUMAN PRIMATES

Two species of non-human primates develop lesions after experimental infection with EBV, that is the owl monkey (*Aotus* sp., Epstein *et al.*, 1973 a, b) and the cotton-top tamarin (*Sanguinus oedipus oedipus*, Shope *et al.*, 1973). The latter has been chosen for experimental studies with EBV *in vivo* (Epstein and Morgan, 1986). Intraperitoneal and intramuscular EBV inoculations produce tumours in cotton top tamarins within 2-3 weeks. Histological analysis has shown that these lesions are large cell malignant lymphomas; they contain EBV DNA (2-25 copies/cell), express EBNA (as judged by ACIF) and are mono- or oligo-clonal (as determined by hybridization for Ig-gene rearrangements), (Cleary *et al.*, 1986). In all these respects the tamarin lymphomas resemble the tumours which develop in the iatrogenically immunosuppressed transplant recipients and the large cell lymphomas in severely immunocompromised AIDS patients (see Sections I.2.5 and 6).

4 LATENT PROTEINS

Latent EBV proteins are defined as those synthesised in B cells (for example in the LCL, IB4) which carry the EBV genome, generally as multiple copies of an

episome, but do not produce any early antigens, late antigens or virus. The latent proteins include one clearly defined and three putative membrane proteins and a complex of six nuclear antigens (see Figure 1.3). The available evidence suggests that all these proteins have functions associated with the processes of cellular immortalization and/or maintenance of latency.

4.1 Latent Membrane Protein

Latent membrane protein (LMP; also known as p63 or BNLFI), because of viral strain variation is a polypeptide of variable molecular weight (58-63 kD), encoded by abundant mRNA species (50-60 copies/cells which are transcribed right to left from the *Bam*HI N_{het} region of the EBV genome (Fennewald *et al.*, 1984; Hennessy *et al.*, 1984; Mann *et al.*, 1985). From the DNA sequence LMP is predicted to consist of a short hydrophilic amino terminus, six hydrophobic transmembrane domains and a long hydrophilic acidic carboxy terminus. It has been shown that both amino- and carboxy- terminal sequences are on the cytoplasmic surface of the plasma membrane connected *via* the six transmembrane domains. This results in the exposure of three short reverse turn loop sequences on the external surface of the cell (Liebowitz *et al.*, 1986; Thorley-Lawson and Isrealson, 1987). The protein is phosphorylated, predominantly on serine residues (Baichwal and Sugden, 1987; Mann and Thorley-Lawson, 1987) and is linked to the cytoskeletal matrix by association with vimentin (Liebowitz *et al.*, 1987; Mann and Thorley-Lawson 1987). Approximately 30-50% of cells in LCLs express LMP (Mann *et al.*, 1985; Rowe *et al.*, 1987) which has led to the suggestion that its expression may be cell-cycle related (Boos *et al.*, 1988).

The expression of LMP can transform immortal, but non-tumourigenic rodent fibroblasts (Rat 1 and Balbc 3T3, but not NIH3T3) into cells which are tumourigenic in nude mice. They also show loss of contact inhibition, anchorage independence and the ability to grow in soft agar (Wang *et al.*, 1985; Baichwal and Sugden, 1988). Recently it was found that expression of LMP, under the influence of the metallothionein promoter, in EBV-negative BL cells (Loukes) after DNA mediated gene transfer, results in profound alterations in the cell surface phenotype. LMP-Loukes cells exhibit increased homotypic adhesion ("clumping") due to increased

expression of the adhesion molecules LFA1 and ICAM1. The induction of LFA3 (together with ICAM1) also results in increased adhesion to T cells. There was an increase in acid production, cell size and plasma membrane ruffling. Expression of CD23 and the transferrin receptor were increased, as was the intracellular level of free calcium (Wang *et al.*, 1988). These data are all consistent with the hypothesis that LMP plays a key role in EBV-induced lymphocyte proliferation. Furthermore the influence which LMP has on adhesion molecule expression shows that it contributes to the LYDMA phenotype (see I.16) in two ways. It acts as a target for HLA Class 1 restricted CTLs (Murray *et al.*, 1988a) and it also induces expression of LFA3 and ICAM1, both of which participate in CTL killing (Gregory *et al.*, 1988a). This provides further clarification of the reports that Group I BL cells (which are LMP-negative) are resistant to CTL killing *in vitro* and avoid immunosurveillance *in vivo*.

A truncated LMP (trLMP), which lacks the N-terminus and first four transmembrane domains, has been described (Mann and Thorley Lawson, 1987; Rowe *et al.*, 1987). This protein is probably encoded by mRNAs transcribed from a second promoter located 3' to the promoter used for full length LMP transcription (Hudson *et al.*, 1985). Tr LMP is synthesised in B cells during productive EBV infection; it is carried in the virion and apparently lacks all the growth transforming properties and the LYDMA phenotype attributed to the full length molecule (Liebowitz *et al.*, 1988; Murray *et al.*, 1988a). The role of trLMP in the lytic cycle is unknown.

4.2 Terminal Protein

Recently a 2.0 kb cDNA clone was recovered from a B95-8 cDNA library which consisted of exons spliced from either side of the terminal repeats (*Bam*HI N_{het} and *Bam*HI C; Figure I.1). That is, the corresponding primary transcript ran from left to right through the linked ends of a circular genome (Laux *et al.*, 1988). Expression of this transcript was demonstrated in a wide variety of EBV-positive cell lines, including some considered "tightly latent". The putative product of the "terminal gene" was designated terminal protein (TP) and the predicted structure of TP suggested it may be an integral membrane protein (Laux *et al.*, 1988). Further

cDNA clones corresponding to the same transcription unit have since been recovered from an IB4 cDNA library (Sample *et al.*, 1989). These show that at least two similar transcripts are generated by differential splicing and they encode two related proteins. *In vitro* transcription/translation and membrane assembly confirmed that both proteins are membrane associated (Sample *et al.*, 1989).

4.3 BHRF1 - An EBV Encoded Homologue of *bcl2*

Analysis of cDNA clones from both human LCLs and the marmoset cell line B95-8 revealed transcripts which were spliced in a similar complex manner to those encoding EBNA species but which include the *Bam*HI H rightward frame 1 - BHRF1 as the 3' coding exon (see Figures I.1 and 3) (Bodescot and Perricaudet, 1986; Pfitzner *et al.*, 1987; Pearson *et al.*, 1987). Because at least two transcripts hybridizing to BHRF1 can be detected on northern blots of RNA extracted from the tightly latent IB4 cell line it has been suggested that BHRF1 encodes a latent protein (Pfitzner *et al.*, 1987). The protein product predicted from the DNA sequence is probably membrane-associated and shares homology with the 26 kD product of the *bcl2* protooncogene which is activated by t(14:18) translocations in human follicular lymphomas (Pfitzner *et al.*, 1987; Cleary *et al.*, 1986; Chen-Levy *et al.*, 1989). The claim that it is a latent protein is, however, controversial since using specific antibodies raised against a BHRF1-fusion protein, it has been shown that this protein is a component of the early antigen (EA) complex and cannot be detected in IB4 or another latent line, Namalwa (Pearson *et al.*, 1987).

4.4 Epstein Barr Virus Nuclear Antigen (EBNA)

All cells latently infected by EBV (whether LCLs or tumour cells) express a nuclear antigen, originally demonstrated by complement enhanced immunofluorescence (ACIF) using serum from EBV seropositive individuals (Reedman and Klein, 1973). Epstein Barr nuclear antigen (EBNA) appears 12-24 hours after infection of B-lymphocytes, before cellular DNA synthesis and mitosis occur (Einhorn and Ernberg. 1978; Robinson and Smith 1981; Moss *et al.*, 1981). The antigen was shown to include heat stable, DNA-binding components (Baron and Strominger, 1978) and to associate tightly with metaphase chromosomes (Reedman

and Klein, 1973) and chromatin (Spelsberg *et al.*, 1982; Sculley *et al.*, 1983). EBNA expression appears to be suppressed when cells enter the lytic cycle, differentiate and secrete immunoglobulin (Crawford and Ando, 1986; Wendel-Hanson *et al.*, 1987). It has always been assumed that EBNA plays an important role in both EBV-induced immortalization and tumourigenesis. Recent studies have shown that EBNA is a complex of at least six proteins, all of which contribute to EBNA ACIF reactivity.

4.5 EBNA1

EBNA1 was identified, by immunoblotting, as a protein of 65 kD - 73 kD in extracts from certain EBV-positive, but not EBV-negative cells. These antigens were only detected with EBV-positive sera (Strnad *et al.*,1981). The gene encoding EBNA1 was subsequently mapped to the *Bam*HI K region (the BKRF1 open reading frame; Figures I.1 and 3) by the stable transfection of mouse fibroblasts with recombinant plasmids including the *Bam*HI K fragment from B95-8. Nuclear fluorescence was demonstrated in transfectants by ACIF (Summers *et al.*, 1982) and a 78 kD polypeptide (which comigrated with the EBNA1 from B95-8 cells) was demonstrated by immunoblotting (Fischer *et al.*, 1984). Antibodies were produced against an EBNA1-β-galactosidase fusion protein (Hennessy and Kieff, 1983) and to synthetic peptides corresponding to repetitive sequences within EBNA1 (Dillner *et al.*, 1984) and these were used to confirm the initial findings. Comparisons of LCLs, eBL cell lines and EBV negative-BL cells converted with different strains of virus showed that the size variation of EBNA1 (69-94 kD) is dictated by individual viral genomes. Specifically a DNA sequence (sometimes called internal repeat 3, IR3) which is made up of the triplets GGG, GCA and GGA (Heller *et al.*, 1982) encodes a region of EBNA1 consisting entirely of a variable number of glycine and alanine residues (Hennessy and Kieff, 1983; Hennessy *et al.*, 1983; Sculley *et al.*, 1984a; Allday and MacGillivray, 1985a). This unusual domain can account for up to 50% of the native molecule and, presumably because of its repetitive nature and resistance to proteolysis, represents the major immunogenic and antigenic component of EBNA ACIF reactivity (Dillner *et al.*, 1984; Allday and MacGillivray, 1985b; Dillner *et al.*, 1987).

EBNA1 consists of a short N-terminal region, followed by the 20-40 kD glycine-alanine copolymer which is flanked by arginine rich sequences and a highly charged acidic carboxy-terminal sequence (Hennessy and Kieff, 1983; Baer *et al.*, 1984). It can exist in a phosphorylated form (Hearing and Levine, 1985). A bacterially synthesised 28 kD fusion-protein corresponding to the charged, acidic terminal region (Milman *et al.*, 1985) was used to show that this represents a DNA-binding domain which exhibits specificity for DNA sequences within the *Bam*HI C and Q regions of the EBV genome (Rawlins *et al.*, 1985). Recent studies have shown that the EBNA1 protein extracted from virus infected cells shows similar affinity and specificity of binding (Jones et al., 1989). In a parallel study Yates and Sugden demonstrated the functional significance of this (sequence) specific DNA binding. They showed that the EBNA1 binding sites within the BamHI C are components of a sequence necessary for the extrachromosomal maintenance of EBV episomes (plasmids). They designated the region oriP and showed that EBNA1 acting in trans is an absolute requirement for long-term plasmid maintenance (Yates et al., 1984 and 1985). Structural and functional analyses have since identified two components, separated by approximately 1000 bp, which are both necessary for plasmid replication, that is, a 5' region containing 20 copies of a 30 bp direct repeat and a 3' region containing a 65 bp region of dyad symmetry (Reisman et al., 1985). EBNA1 binds at high affinity within each of the 30 bp repeat elements and at 4 sites in and around the palindrome (Jones et al., 1989). Furthermore it has been shown that in addition to mediating plasmid replication, EBNA1 activates a transcriptional enhancer present in the oriP region. The functional significance of this in the intact viral genome is still, however, unclear (Lupton and Levine, 1985; Reisman and Sugden, 1986). The significance of the EBNA1 binding site in BamHI Q also remains to be determined (Jones et al., 1989).

4.6 EBNA2

When Strnad and colleagues (Strnad *et al.*, 1981) first demonstrated EBNA1 by immunoblotting, they noted that two of their EBV-positive sera detected an additional 81 kD band, which they showed was biochemically distinct from EBNA1. Subsequently a similar 82 kD nuclear antigen was demonstrated in a number of EBV-positive cell lines (Hennessy and Kieff, 1983). This polypeptide was shown to

be absent in cell lines, such as P3HR1, which contain viral genomes with a deletion in the BamHI WYH region (Sculley *et al.*, 1984b; Dambaugh *et al.*, 1984; see Figures I.1 and 3). Confirmation that the BamHI Y reading frame 1 (BYRF1) encoded this second nuclear protein, designated EBNA2, was obtained using antibodies raised against fusion proteins encoded by BYRF1 DNA sequences (Hennessy and Kieff, 1985) and synthetic peptides corresponding to the predicted amino acid sequence (Dillner *et al.*, 1985). DNA transfections with plasmids containing BamHI WYH sequences were used to generate cell lines stably expressing EBNA2 (Rymo *et al.*, 1985; Mueller-Lantzsch *et al.*, 1985).

The molecular weight of EBNA2, like EBNA1 and LMP, varies slightly in cells infected by different viral strains (Hennessy and Kieff, 1983); it is generally about 80 kD. The polypeptide includes a polyproline region, a glycine-arginine repeat and a highly charged and acidic carboxyl terminus (Dambaugh *et al.*, 1984). Using an anti-peptide monoclonal antibody in immunoprecipitations it has recently been shown that EBNA2 is phosphorylated and coprecipitates with a 32 kD cellular protein (Dillner *et al.*, 1988). It also binds non-specifically to single- and double-stranded DNA (Kallin *et al.*, 1986).

Antigenic differences between the EBNA2 protein from different EBV isolates have revealed two EBV strains designated 2A (represented by B95-8) and 2B (represented by AG876 and Jijoye) (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985; Rowe *et al.*, 1985; Zimber *et al.*, 1986). World wide, type 2A viruses appeared to be more common; however, it now appears that 2B virus is widely distributed (T. Sculley and J. Sixbey, personal communications) and not restricted to the tropics as was originally thought (Zimber *et al.*, 1986; Young *et al.*, 1987). There are reports that dual infection of individuals with 2A and 2B viruses may occur (Arrand *et al.*, 1989; J. Sixbey and T. Sculley, personal communications).

The *Bam*HI WYH region, which includes the EBNA2 encoding exon, appears to be essential (but not sufficient) for the immortalization of B cells. Superinfection of the Raji cell line (which does not produce virus) with non-immortalizing P3HR1 virus results in the production of recombinant, immortalizing virus (Fresen *et al.*, 1978). All the immortalizing recombinants analysed acquire the *Bam*HI WYH region

from Raji and the capacity to synthesise EBNA2 (Skare *et al.*, 1985). Early gene transfer experiments suggested EBNA2 could transiently stimulate cellular DNA synthesis (Volsky *et al.*, 1984) and recently it has been shown that expression of EBNA2 in rodent cells reduces their dependence on serum factors (Dambaugh *et al.*, 1986b). Infection of the EBV-negative BL, Loukes, with a recombinant retrovirus encoding EBNA2, induced the expression of the activation antigen CD23 (Wang *et al.*, 1987; see Section I.1.4). This result is consistent with the induction of CD23 reported after conversion of EBV-negative BL lines with B95-8 but not with P3HR1 (Calender *et al.*, 1988). A comparison of cells immortalized with either 2A or 2B viruses showed differences in their growth properties. The 2B virus containing lines grow slower, exhibit a greater sensitivity to seeding at limiting dilution and have a lower saturation density (Rickinson *et al.*, 1987). Of course the possibility that other regions of the 2A and B genomes may contribute to these phenotypic differences cannot be excluded.

EBNA2 thus appears to possess a number of properties consistent with it having an important role in the induction and maintenance of cell proliferation. The absence of EBNA2 expression from group I eBL cells suggests, however, that it is unnecessary in cells which are already proliferating (and prevented from leaving the cell cycle to enter G_o by a deregulated *cmyc* gene; see Section I.2.4). Furthermore its role as a component of LYDMA (see Section I.1.6) could explain why tumour cells failing to express it are selected for, *in vivo*. EBNA2 expression *in vitro*, on the other hand, may be of selective advantage because it can induce an autocrine growth factor, sCD23 (see Section I.1.4).

4.7 EBNA5 (EBNA-Leader Protein)

DNA sequence analysis of a cDNA clone, designated T1, from the latently infected Raji cell line showed it contained two exons from the *Bam*HI W internal repeat, three exons from *Bam*HI Y and an exon from *Bam*HI H (Bodescot *et al.*, 1984; Figures I.1 and 3). A long open reading frame through the W and Y exons (encoding 261 amino acids) was predicted which would contain a repetitive element of 66 amino acids from each internal repeat. Dillner and colleagues (Dillner *et al.*, 1986) synthesised peptides corresponding to the deduced amino acid sequence, used

them to raise rabbit antisera and showed that these antipeptide antibodies recognised a 46 kD nuclear polypeptide (which they called EBNA5) in the tightly latent cell line, IB4 (Dillner *et al.*, 1986). They also showed EBNA5 species in various LCLs varying in molecular weight between 22 kD and 70 kD and a ladder of multiple polypeptides (41 kD-70 kD) induced 1-2 days after infection of tonsillar B cells with the B95-8 strain of virus. These data were confirmed and extended to demonstrate expression in a number of well established lines from eBL using mouse monoclonal or immunoaffinity purified human antibodies (Finke *et al.*, 1987). Since EBNA5 is predominantly encoded by the (large) internal repeats, it is assumed that multiple molecular weight forms are induced because of heterogeneity in the number of repeats per genome (see Heller *et al.*, 1981), the use of multiple promoters (one from each repeat), or differential splicing (see Sample *et al.*, 1986; Finke *et al.*, 1987).

Further cDNA analysis has revealed that EBNA5 is encoded by bicistronic messages which also code for other EBNA species (see Figure I.3). The start of the E5 open reading frame is created by the first splice junction, - AT in exon 1, - G in exon 2 (Sample *et al.*, 1986; Wang *et al.*, 1987; Bodescot and Perricaudet, 1987). Because EBNA5 is encoded within the "leader" sequence of messages including other EBNAs it is also known as Leader Protein (LP). The function of EBNA5/LP has not yet been determined but it may be significant that the protein in the non-immortalizing P3HR1 virus is truncated because the exons encoding Y_1 and Y_2 are absent (see Figures I.1 and 3).

4.8 The High Molecular Weight Family of EBNAs

At the time when this project was started, only EBNAs 1 and 2 had been characterised and mapped on the EBV genome. There were, however, indications that other EBV encoded nuclear proteins might exist. Specifically, using sera from patients with rheumatoid arthritis, Sculley and colleagues (Sculley *et al.*, 1984b) had demonstrated two bands on immunoblots of nuclear extracts from latently infected EBV-positive cell lines. They had apparent molecular weights of over 100 kD and appeared unrelated to EBNAs 1 and 2. In addition, serum from a patient with chronic IM had been described (serum WC) which was negative for EBNA1 and had very weak reactivity with EBNA2, but still gave a strong ACIF reaction (Miller *et*

al., 1985; Dillner and Kallin, 1988). Combined, these data suggested the existence of at least two further EBNA species. Subsequent studies in different laboratories (including that presented in this thesis) have established the existence of a high molecular weight group of three EBNA polypeptides (approximately 140-180 kD). These EBNAs (3, 4 and 6) are encoded by tandemly arranged open reading frames in the *Bam*HI L-Z region of the EBV genome (see Figure I.3; results in Section II and discussion in Section III).

4.9 Nomenclature of EBV Latent Proteins

Because the EBNAs have been identified and characterised independently their nomenclature has not been systematised. The exons encoding EBNAs 1 (from *Bam*HI K), 2A and 2B (from *Bam*HI Y and H) have been identified and clearly defined and there is general agreement with respect to the designation of these (see Figure I.3). The high molecular weight EBNAs, which form part of the present study, have, however, been designated either EBNA 3, 4 and 6 (Ricksten *et al.*, 1988; Allday *et al.*, 1988; this study) or EBNAs 3a, 3b and 3c (Petti *et al.*, 1988a and b). They are encoded by the predicted open reading frames BLRF3/BERF1, BERF2a/2b and BERF3/4 respectively. The nuclear antigen encoded by the repeated *Bam*HI W exon (and exons from *Bam*HI Y) has variously been called EBNA5 (Dillner *et al.*, 1986), EBNA leader protein (EBNA-LP) (Sample *et al.*, 1986) and EBNA IV or 4 (Rowe *et al.*, 1987) (see Figure I.3). Fortunately, there is general agreement about the designation of latent membrane protein (LMP) for the protein encoded by *Bam*HI N_{het}. However, the products of the gene formed when the genome circularizes have been called terminal protein (TP) (Laux *et al.*, 1988) and more recently LMP2A and LMP2B (Sample *et al.*, 1989).

5 RATIONALE FOR THIS PROJECT

Preliminary studies in various laboratories (see Section I.4.9) suggested the existence of EBNA species in addition to EBNAs 1 and 2 (A + B). The aim of this project has been to identify, genetically map and investigate the expression of one or more of these.

The rationale for investigating in particular the BERF4 gene (exon) was threefold: Firstly, this was one of three uncharacterised predicted long open reading frames (o.r.f.), arranged in tandem and running from left to right on the EBV genome in a similar manner to EBNAs 1 and 2. Each o.r.f. has coding potential for proteins of over 100 kD (Baer *et al.*, 1984; Figures I.3 and II.26). Secondly, there was an unconfirmed report that this region was transcribed at low levels in latently infected cells (King *et al.*, 1980) which was confirmed in our laboratory (MJA unpublished). Finally, a comparison of the amino acid sequences of known viral nuclear proteins suggested the product of BERF4 may have a nuclear distribution. The peptide Lys-Arg-Pro-Arg (KRPR), thought to be part of a signal necessary for the rapid transport to the nucleus of polyoma large T (Richardson *et al.*, 1986) and adenovirus E1a proteins (Lyons *et al.*, 1987), is also present in EBNA1 and both variants of EBNA2. An analysis of other EBV open reading frames revealed KRPR also in BERF4. A further search of all the available computer data bases showed that this short sequence was not common and was almost entirely restricted in eukaryotes to proteins known (or suspected) to have a nuclear distribution (see Table I.2).

My working hypothesis was, therefore, that the open reading frames BERFs 1, 2b and 4 encode three high molecular weight nuclear proteins, that these are transcribed and regulated in a similar manner to EBNAs 1 and 2 and that they are involved in the process of EBV-induced immortalization and possibly tumourigenicity.

Table I.2: Nuclear Localisation Signals - confirmed or predicted

Protein*	Sequence	Nuclear	ANA†
Viral			
Polyoma Large T Ag	PVSRKRPRPAG	+	+
Adenovirus 5/2 E1a	NLSCKRPRP-COOH	+	-
Adenovirus 12 E1a	DLSVKRPRCN-COOH	+	-
Adenovirus 2 polypeptide VI precursor	GRGEKRPRPD	+	?
EBNA1	GRGEKRPRSPS	+	+
EBNA2A	QGPSKRPRPSIQ-COOH	+	+
EBNA2B	GGPSKRPRTSTQ-COOH	+	+
BERF4/EBNA6	ATTPKRPRVEE	+	+
HIV2 env polyprotein	QPINKRPRQAW	?	?
HSV1 DNA polymerase	PSPAKRPRETP	+	+
HSV1/2 exonuclease	DSPPKRPRPNS	+	+
VZV orf 59	KKTRKRPRGLP	?	?
Eukaryotic-Homeotic Proteins			
Drosophila engrailed	TNDEKRPRTAF	+	+
Drosophila inverted	SPEDKRPRTAF	+	+
Honeybee E60	RPEEKRPRTAF	+	+
Honeybee E30	GPEEKRPRTAF	+	+
Mouse En1	KEDKRPRTAF	+	+
Mouse En2	KEDKRPRTAF	+	+
Eukaryotic-Other Proteins			
30S Ribosomal protein S8	KNKQKRPRSRT	+	+
Prokaryotic			
Gene 278 protein filamentous phage Pf3	EPGAKRPRGDR	N/A‡	+
Plasmids R100 and R1 RepA4 protein	LITEKRPRPGP	N/A	+
Phage 434 Cro protein	AGVKRPRFLF	N/A	+

* For references see Allday *et al.*, 1988

† Associated with Nucleic Acids. ‡ Not applicable

II RESULTS

1 IDENTIFICATION OF HUMAN SERA CONTAINING ANTIBODIES TO THE PROTEIN ENCODED BY BERF4

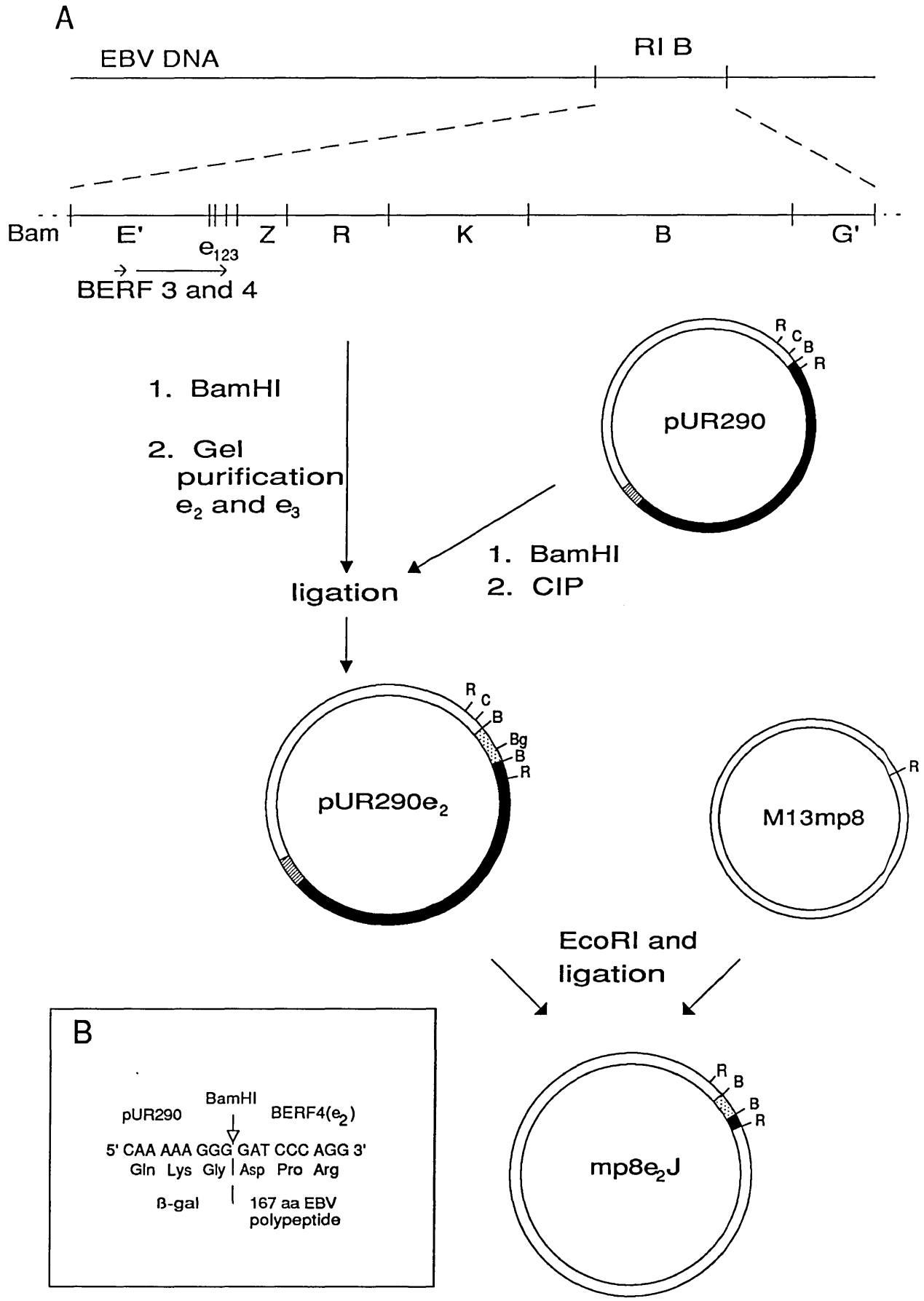
1.1 Construction of pUR290e_2

In order to determine whether the EBV BERF4 open reading frame is expressed *in vivo* and to identify any human sera which would be useful for studying the protein(s) it encodes, a recombinant plasmid, designated pUR290e_2, was constructed. This is an expression vector derived from one of the pUR series of β-galactosidase-fusion plasmids constructed by Rüther and Müller-Hill (1983). pUR290e_2 encodes a fusion protein consisting of β-galactosidase and 167 amino acids corresponding to the C-terminal end of BERF4. This chimeric gene is under the transcriptional control of the lacZ promoter/operator and is thus inducible by lactose analogues such as isopropyl thio-galactoside (IPTG). The fusion protein produced in this way is ideal for screening sera by immunoblotting.

An analysis of the EBV DNA sequence (Baer *et al.*, 1984) and comparison with the sequence of pUR290 (Rüther and Müller-Hill, 1983) revealed that this particular pUR vector would yield an in-frame fusion protein if ligated in the correct orientation to the EBV *Bam*HIe_2 fragment. The scheme for the construction of pUR290e_2 is summarised in Figure II.1 and details are given in the Materials and Methods section.

The resulting recombinant plasmids were screened by cleavage with *Bam*HI and *Bgl*II in order to distinguish between clones containing e_2 and those containing e_3. (The latter does not contain a *Bgl*II site). Twelve pUR290e_2 clones were selected for further analysis. Cleavage with *Bgl*II and *Cla*I revealed that 5/12 were in the correct orientation with the continuous open reading frame from β-galactosidase maintained through BERF4. Definitive confirmation was obtained by cloning the 686 bp *Eco*RI fragment from pUR290e_2 (which contains the *Bam*HI junction between e_2 and pUR290) into the M13 vector mp8 (Figure II.1). DNA sequence analysis by the Sanger dideoxy method was performed on two clones (by T. Hara).

Figure II.1: Construction of Recombinant Plasmid pUR290e_2. **A.** Plasmid DNA including the *Eco*RI fragment of EBV (Arrand *et al.*, 1981) was digested with *Bam*HI and after agarose gel electrophoresis the bands corresponding to e_2 and e_3 (507 and 521 bp, respectively) were copurified using NA45 paper. These were then ligated to *Bam*HI cleaved and phosphatased (CIPed) pUR290 and the resulting recombinants were screened as described in the text for those with e_2 in the correct orientation. Confirmation that the open reading frame between lac Z and EBV BERF4 was maintained was obtained by DNA sequence analysis after subcloning the *Eco*RI fragment (containing the *Bam*HI junction) into M13mp8 to produce mp8e_2J as shown. The location of *Bam*HI sites and the BERF3 and 4 open reading frames in the *Eco*RI B fragment of EBV DNA are indicated. ▨ represents the lacZ promoter/operator region; ■ , the lacZ coding sequence; ▩ the e_2 fragment of EBV. Restriction enzyme sites are: R(*Eco*RI), B(*Bam*HI), C(*Cla*I) and Bg(*Bgl*II). **B.** DNA and protein sequence through the junction between pUR290 and BERF4.


A
EBV DNA
RI B
Bam
E'
e_{123}
Z
R
K
B
G'
BERF 3 and 4
1. BamHI
2. Gel purification e_2 and e_3
pUR290
1. BamHI
2. CIP
ligation
pUR290e_2
M13mp8
EcoRI and ligation
mp8e_2J
B
pUR290
BamHI
BERF4(e_2)
5' CAA AAA GGG GAT CCC AGG 3'
Gln Lys Gly Asp Pro Arg
ß-gal
167 aa EBV polypeptide

These clones were determined to be in-frame and thus were used in subsequent experiments.

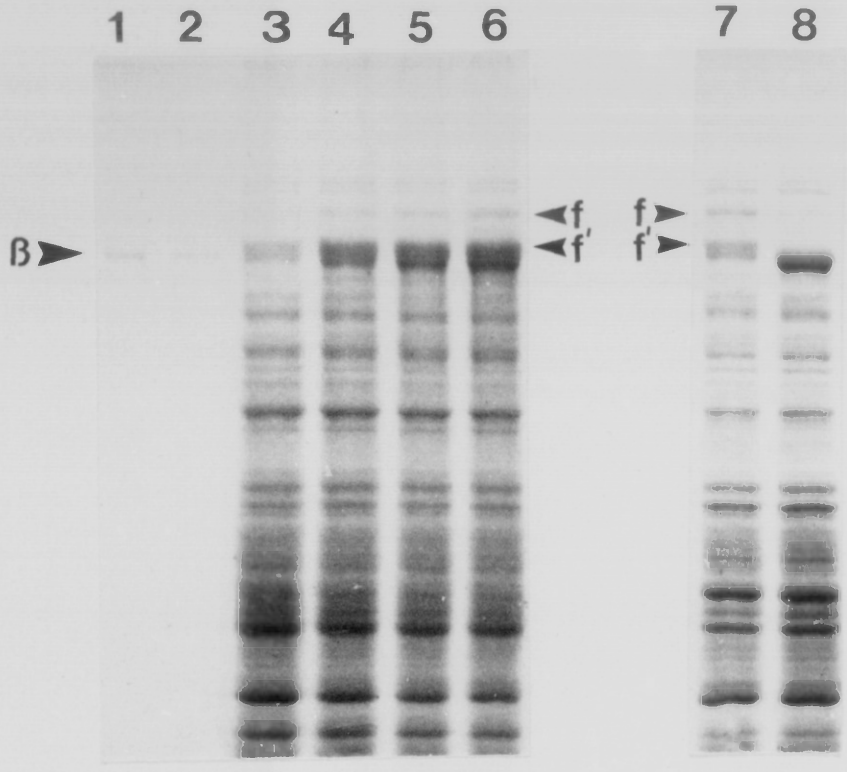
1.2 Expression and Partial Purification of the e_2-β-Galactosidase Fusion Protein

Overnight cultures of *E. coli* K12 strain TG2 carrying either pUR290e_2 or pUR290 were induced by the addition of IPTG. Total cellular proteins were analysed by SDS-PAGE after 1, 2, 3 and 4 hours of induction at both 37°C and room temperature. A series of such experiments were performed on different pUR290e_2 clones and they revealed that induction for 4 hours at room temperature was optimal for stable synthesis of the e_2-β-galactosidase fusion protein (e_2–β-gal). Coomassie blue stained 10% SDS-polyacrylamide gels show separation of proteins from two typical experiments (Figure II.2 A). A band corresponding to a fusion product (f) can be seen migrating at a higher molecular weight than the unmodified β-galactosidase encoded by pUR290 and marker β-galactosidase (β). (Compare tracks 1, 7 and 8). In tracks 3 and 7 an additional band (f') only slightly larger than β can also be seen .

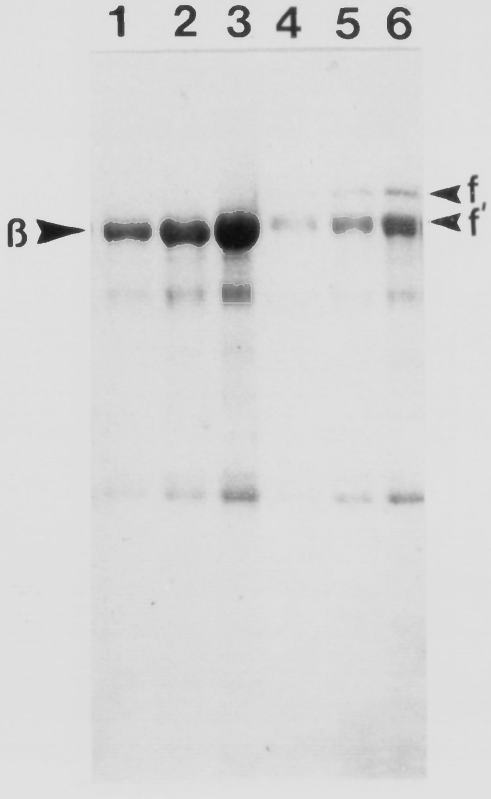
Substrate analogue affinity chromatography depends on the reversible binding of enzymes to an analogue of their normal substrate which in turn is bound to an insoluble matrix. It was judged to be a convenient and efficient method for the partial purification of fusions such as e_2–β-gal (Steers *et al.*, 1971; Koenen *et al.*, 1985). Total cytosolic proteins of TG2 were extracted and clarified by ultracentrifugation (Koenen *et al.* 1985). The supernatants were applied to p-amino-phenyl-β-D-thiogalactoside (APTG) agarose columns and after extensive washing β-galactosidase species were eluted and dialysed against phosphate buffered saline. Figure II.2.B shows the partially purified proteins derived from pUR290e_2 (tracks 4, 5 and 6) and pUR290 with no insert (tracks 1, 2 and 3) separated on a 10% SDS-polyacrylamide gel and stained with Coomassie blue. These results also show that in addition to a fusion product (f) of a size predicted from the complete amino acid sequence, the second species (f') which is only slightly larger than the unmodified β-galactosidase is also purified. This species presumably results from an inherent instability of the fusion in *E. coli* as has been reported for other hybrid polypeptides

Figure II.2: Expression of e_2-β-Galactosidase. **A.** Coomassie blue dye stained 10% SDS-polyacrylamide gels showing induction of β-galactosidase fusion products f and f' : 1 hour (track 3); 2 hours (track 4); 3 hours (track 5) and 4 hours (track 6) after induction at room temperature with IPTG. Track 7 shows the e_2-β-galactosidase fusion products after 1 hour of induction. Bacteria containing pUR290 with no insert were induced in the same way (track 8). Unmodified β-galactosidase protein (Sigma) is shown in tracks 1 and 2. **B.** Coomassie blue dye stained 10% SDS-polyacrylamide gel showing e_2-β-galactosidase polypeptides partially purified by substrate affinity chromatography (tracks 4, 5 and 6) and the product of pUR290 without an insert prepared in a similar manner (tracks 1, 2 and 3). Fusion products (f and f') are indicated as is the unmodified β-galactosidase (β). (tracks 1 and 4, 2 and 5 and 3 and 6 were loaded with 5μl, 10μl and 25μl of eluate from a 1.5ml APTG-agarose column respectively).

A



B



of this type (Rüther and Müller-Hill, 1983). An increase in the induction period or temperature results in a concomitant decrease in f and an increase in f' (not shown).

1.3 Human Antibodies to e_2-β-Galactosidase

The partially purified fusion products (shown in Figure II.2.B) were used to screen, by immunoblotting, more than 100 human sera. These were chosen from a variety of disease groups and also normal EBV seropositive individuals. Only a small number (average less than 20%) clearly recognised the protein on such blots. The results which are summarised in Table II.1 suggest seropositivity is not associated with any particular group of individuals. Figure II.3 shows a representative immunoblot (from a 7.5% SDS-polyacrylamide gel) probed with a high titre positive serum, RT, (track 1). It can be seen that the antibodies in the positive serum which are directed against the fusion protein are specific for the EBV component (that is the C-terminus of BERF4). Also it is the full length fusion product (f) which is predominantly recognised suggesting that the product of proteolytic cleavage loses major EBV specific epitopes. There is no binding to the unmodified β-galactosidase derived from pUR290 (track 2). Probing a similar blot with a murine monoclonal anti-β-galactosidase antibody, on the other hand, produces binding to both unmodified and modified proteins (tracks 7 and 8). An EBV negative serum recognises neither protein (Tracks 4 and 5). Fifteen human sera which clearly recognise the EBV encoded component of e_2-β-gal were identified in this way. Two of these, RT (from a normal healthy donor) and 062 (from a patient in Hong Kong with nasopharyngeal carcinoma, NPC) had particularly high titres and produced very "clean" immunoblots. These were used in subsequent investigations on EBNA expression.

1.4 Monoclonal Antibodies to e_2-β-Galactosidase

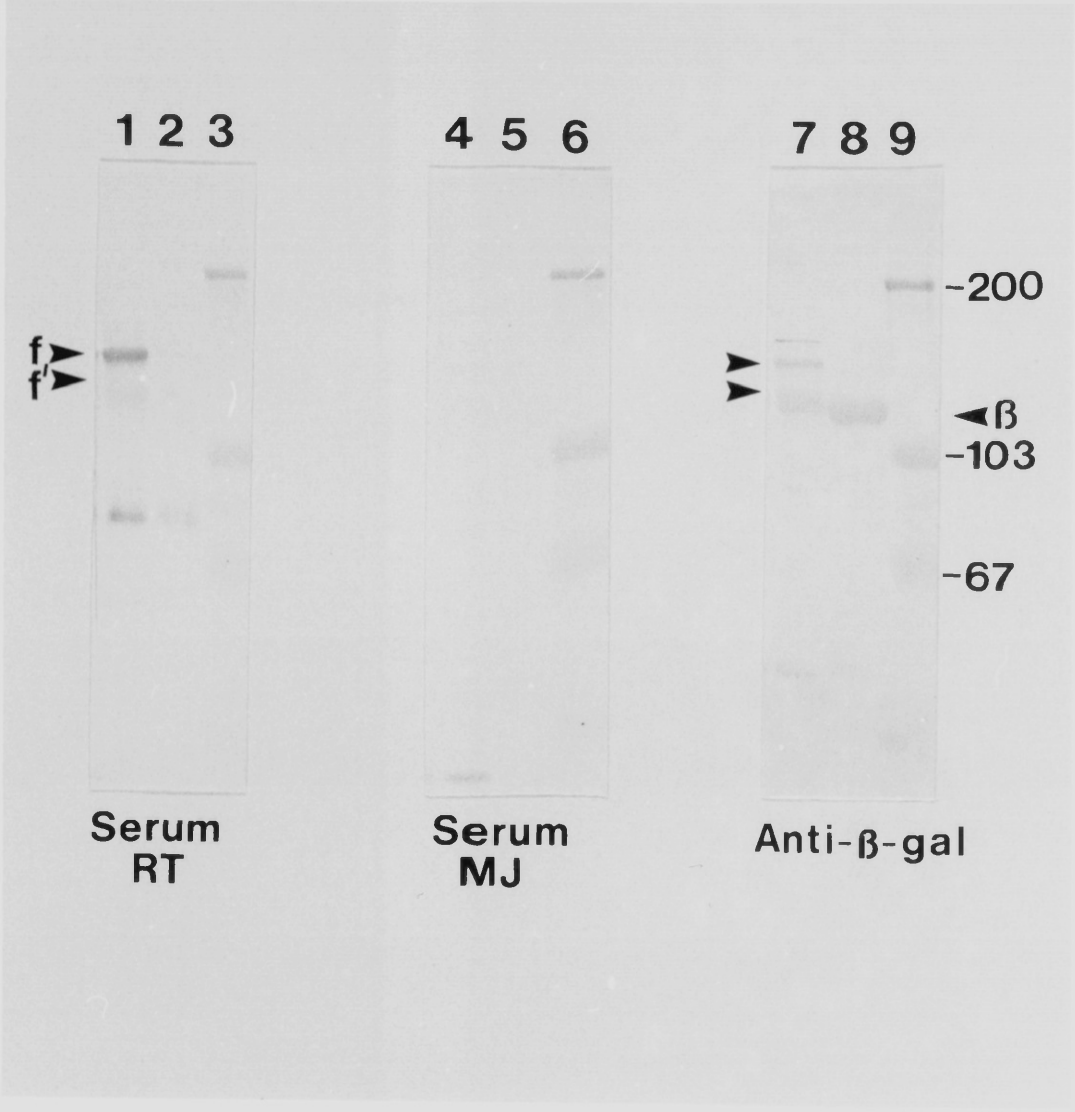
Mice have been immunised with the e_2-β-gal fusion products with the aim of producing monoclonal antibodies. These experiments (carried out in collaboration with D.H. Crawford and M. Rowe, Birmingham) are currently in progress.

Table II.1: Human Serum containing Antibodies which Recognise e_2-β-Gal on Immunoblots.

Normals	Rheumatoid Arthritis	Infectious Mononucleosis	Nasopharyngeal Carcinoma[+]	African Burkitt's lymphoma	Total
3/10	3/30	0/5	7/50	2/20	15/115
(33%)	(10%)	(0%)	(14%)	(10%)	(13%)

[+] 40 North African, 5 European, 5 from Hong Kong

Figure II.3: Immunoblots of e_2-β-Galactosidase. The partially purified protein products of pUR290e_2 (tracks 1, 4 and 7) and pUR290 (tracks 2, 5 and 8) were separated on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose. Blots were probed with EBNA-positive serum, RT (1/100); EBNA-negative serum MJ (1/50); and an anti-β-galactosidase monoclonal antobody (1/10) as indicated. f (tracks 1 and 7) indicates the position of the largest species of e_2-fusion and f' (tracks 1 and 7) the more weakly staining product probably resulting from proteolytic degradation (see text): β shows the position of unmodified β-galactosidase and tracks 3, 6 and 9 show prestained high molecular weight standards (BRL) of indicated MW (kD).


1 2 3
4 5 6
7 8 9
f
f'
-200
β
-103
-67
Serum RT
Serum MJ
Anti-β-gal

2 DEMONSTRATION THAT BERF4 ENCODES A LATENT NUCLEAR PROTEIN (EBNA6)

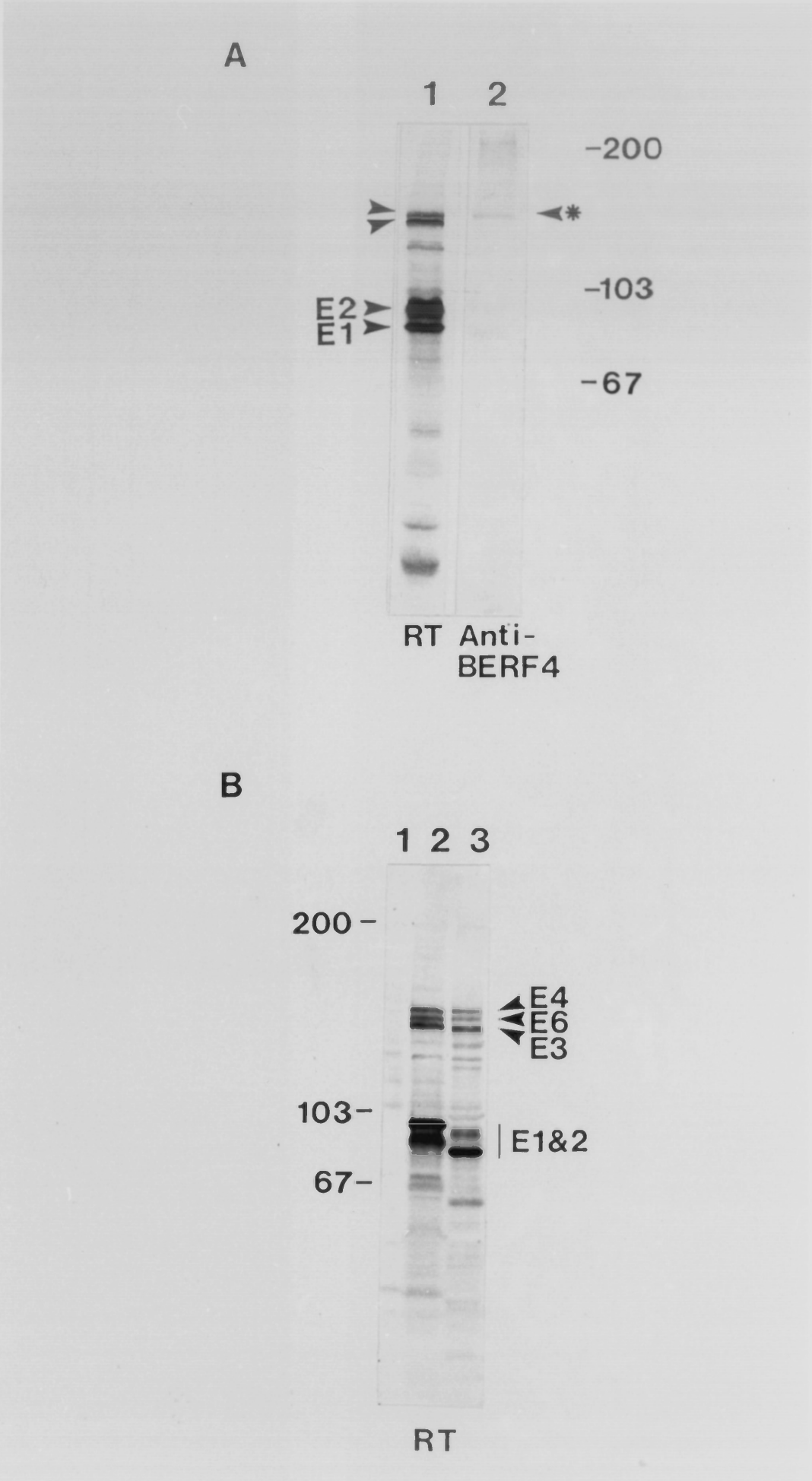
2.1 The Protein Encoded by BERF4 is one of a Family of Three High Molecular Weight Species

The studies of Sculley *et al.* (1984b) showed that certain human sera recognised on immunoblots, in addition to EBNAs 1 and 2, a group of EBV associated proteins with molecular weights over 100 kD. In order adequately to resolve these species by SDS-PAGE and ensure efficient transfer to nitrocellulose either low percentage (7.5%) or gradient (5-15%) gels are required. The protein encoded by BERF4 was suspected of being part of this high molecular weight complex, thus immunoblots from 7.5% SDS-polyacrylamide gels were probed with sera 062 and RT. The results of such a blot can be seen in Figure II.4. Serum RT identifies two proteins of approximately 140-160 kD in the tightly latent lymphoblastoid cell line, IB4 (A, track 1). These are in addition to EBNAs 1 and 2 which have molecular weights of approximately 80 and 90 kD, respectively (see Introduction, Section I.4). A further band of about 170 kD is detected in both the marmoset B cell line, B95-8 and a spontaneous LCL from an African child, 2C9 (obtained from K. Lam), (B, tracks 3 and 2, respectively). None of these protein bands are detected by serum RT in the EBV negative Burkitt's lymphoma cell line, Ramos (B, track 1). Antibodies specific for BERF4 were immunoaffinity purified from serum RT by elution off e_2-β-gal which was immobilised on nitrocellulose (see Allday *et al.*, 1988 and Materials and Methods). These monospecific antibodies recognise a single protein band of approximately 160 kD (A, track 2). This corresponds to the larger of the two protein bands detected by serum RT in the latent IB4 line and the middle band in B95-8 (compare tracks A1, A2 and B3; also compare tracks 2 and 3 in Figure II.6.A).

2.2 Nuclear Location

In order to determine the cellular location of the proteins recognised by serum RT, cells of the LCL, 2C9 (see above), were fractionated into nuclei and cytoplasm. The nuclear fraction was further extracted with 0.4M NaCl and finally

Figure II.4: Expression of BERF4 (EBNA6) in B cells. **A.** SDS-solubilized proteins from the EBV-positive LCL, IB4, separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with serum RT (1/100) in track 1 and immunoaffinity purified anti-BERF4 specific antibodies in track 2. EBNAs 1 and 2 and novel high molecular weight species are indicated in track 1. The putative EBNA6 polypeptide is also indicated (*). **B.** Similar immunoblot of EBV-negative BL line Ramos (track 1); LCL, 2C9 (track 2) and marmoset B cell line, B95-8 (track 3) probed with serum RT (1/100). The positions of EBNA species and high molecular weight standards (kD) are indicated.


A
1 2
-200
-103
-67
E2
E1
RT Anti-
BERF4
B
1 2 3
200-
103-
67-
E4
E6
E3
E1&2
RT

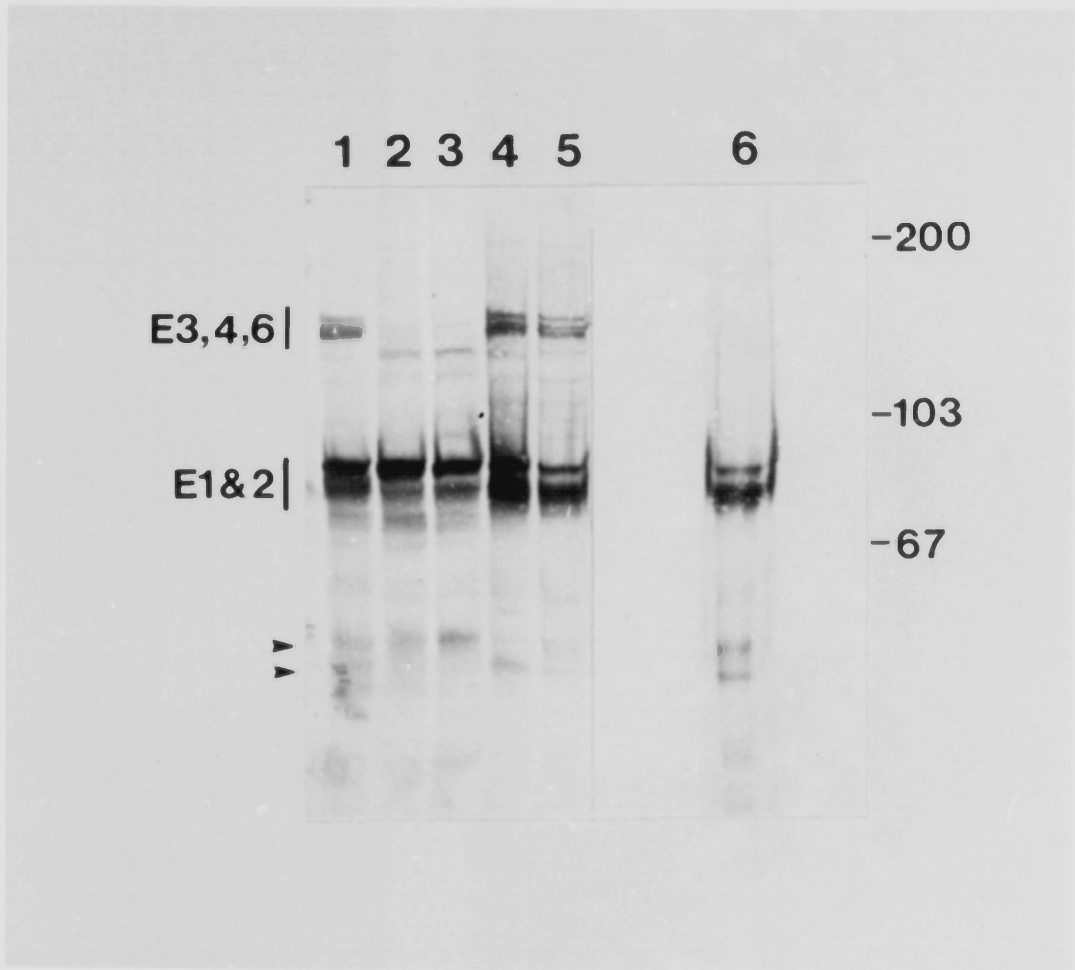
the insoluble nuclear chromatin pellet was solubilised in 5M urea. The proteins associated with each fraction were completely solubilised and denatured in SDS-sample buffer and, after separation on a 7.5% gel, analysed by immunoblotting with serum RT. The doublet of proteins migrating at approximately 80-90 kD and previously identified as EBNAs 1 and 2 is found in the cytoplasmic as well as the nuclear fractions (Figure II.5. Compare tracks 2 and 3 with track 4). This observation is consistent with previous reports of their leakage from the nucleus (Sculley *et al.*, 1983; Allday *et al.*, 1985c). The 140-170 kD species, on the other hand, appear to be entirely associated with the nuclear fractions (track 5). However, these three proteins are all completely released from nuclei by the 0.4M NaCl extraction. This is unlike EBNAs 1 and 2, a small percentage of which remains associated with the insoluble chromatin pellet, and suggests the high molecular weight species are not tightly associated with either chromatin or the nuclear matrix (Sculley *et al.*, 1983).

Thus the antigen encoded by BERF4 appears to have a predominantly nuclear distribution. It was designated EBNA6 to conform with designations presented elsewhere (Ricksten *et al.*, 1988). Further studies with different sera, other cell lines (see below) and data from a number of different groups (Kallin *et al.*,1986; Ricksten *et al.*, 1988) have shown that the additional bands recognised by serum RT correspond to EBNAs 3 and 4 (see Figures II.4 and 6).

2.3 EBNA Expression in Various Cell Lines

Serum RT recognises five main EBV-associated nuclear proteins in the marmoset B cell line, B95-8 and human LCL, 2C9, and only four in the human LCL IB4. It has been shown previously that IB4 expresses EBNAs 1, 2 and 3 (Hennessy *et al.*, 1985), also EBNA5 (Dillner *et al.*, 1986; Finke *et al.*, 1987), and this study shows that it synthesises EBNA6. Thus by a process of elimination the antigen missing was identified as EBNA4. In order to establish whether its absence is a common feature of LCLs, immunoblot analysis with RT sera was performed on more than 20 LCLs from a variety of sources. Lines spontaneously derived from peripheral blood and lines established by *in vitro* B95-8 virus infection have been studied. All the lines analysed expressed EBNAs 1,2,3 and 6 and most expressed EBNA 4 (see Figure II.6

Figure II.5: Nuclear Distribution of EBNA Species. The LCL, 2C9, was fractionated as described in the Materials and Methods and fractions were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with serum RT (1/100). Proteins from whole cells (track 1); cytoplasmic fractions (tracks 2 and 3); intact nuclei (track 4); 0.4 M NaCl nuclear extract (track 5); 5 M Urea-solubilized chromatin (track 6) are shown. EBNAs and molecular weight standards (kD) are indicated. Also, two polypeptides (40-50 kD) which appear to be EBV-related are shown (arrows). These species, which are predominantly associated with nuclear chromatin, have not yet been identified.


1 2 3 4 5 6
E3,4,6
E1&2
-200
-103
-67

for examples). However, two of the LCLs, which were established using B95-8 virus (by N. Sullivan, ICRF, London) are similar to IB4 and do not express detectable levels of EBNA4 (Figure II.6.B, tracks 2 and 3).

A similar analysis was performed on the Burkitt's lymphoma cell lines Raji, P3HR1 and Daudi, each of which have been shown to contain defective viral genomes with well characterised deletions (see Section I.3 and Figure I.1). Immunoblots probed with serum RT suggest that neither Raji, P3HR1 nor Daudi express EBNA6 (Figure II.7.A): Raji appears to express EBNAs 1, 2, 3 and 4 (track 1); Daudi only 1, 3 and 4 (track 2) and P3HR1 only EBNAs 1 and 3 (track 4). The absence of EBNA6 from these lines was confirmed by probing a similar immunoblot with serum MS which contains antibodies specific for EBNAs 1, 2 and 6 only (M. Rowe, personal communication). A band of 160 kD (or thereabouts) was not detected in any of these BL lines (Figure II.7.B, tracks 1, 2 and 4) although it is present in B95-8 (track 5). The identity of the band thought to correspond to EBNA3 in P3HR1 has been confirmed by others (M. Rowe and T. Sculley, personal communications). Fusion of P3HR1 cells with cells of the myeloid leukaemia line MLK562 to produce Putko surprisingly results in a line with an even more restricted pattern of expression than the parent. Here immunoblotting with sera RT and MS detects only EBNA1 (Figure II.7, tracks A3 and B3).

3 DNA MEDIATED GENE TRANSFER OF BERF 4 AND EXPRESSION OF EBNA 6

3.1 Construction of pSVE3/4 and pMMTVE3/4

Analysis of the DNA sequence of the EBV genome reveals that the open reading frames BERF3 and 4, which together are thought to encode the protein designated here as EBNA6, do not have an obvious transcriptional start site within 10 kb 5' of their predicted translational initiation sites. In order to express these exons, isolated from the remainder of the EBV genome, two recombinant expression vectors were constructed in which BERF3/4 is located downstream of promoters derived from other mammalian viruses. pSVE3/4 encodes BERF3/4 under the transcriptional control of the simian virus (SV) 40 early region promoter and in

Figure II.6: Expression of EBNAs in LCLs. A. Immunoblot, from a 7.5% SDS-polyacrylamide gel, of proteins from B95-8 (track 2); IB4 (track 3); six spontaneous LCLs established from African children (tracks 4-9) probed with serum RT (1/100). Track 1 shows prestained high molecular weight standards (kD). **B.** A similar immunoblot showing B95-8 (track 1) and two B95-8 derived LCLs (tracks 2 and 3) which fail to synthesise detectable levels of EBNA4.

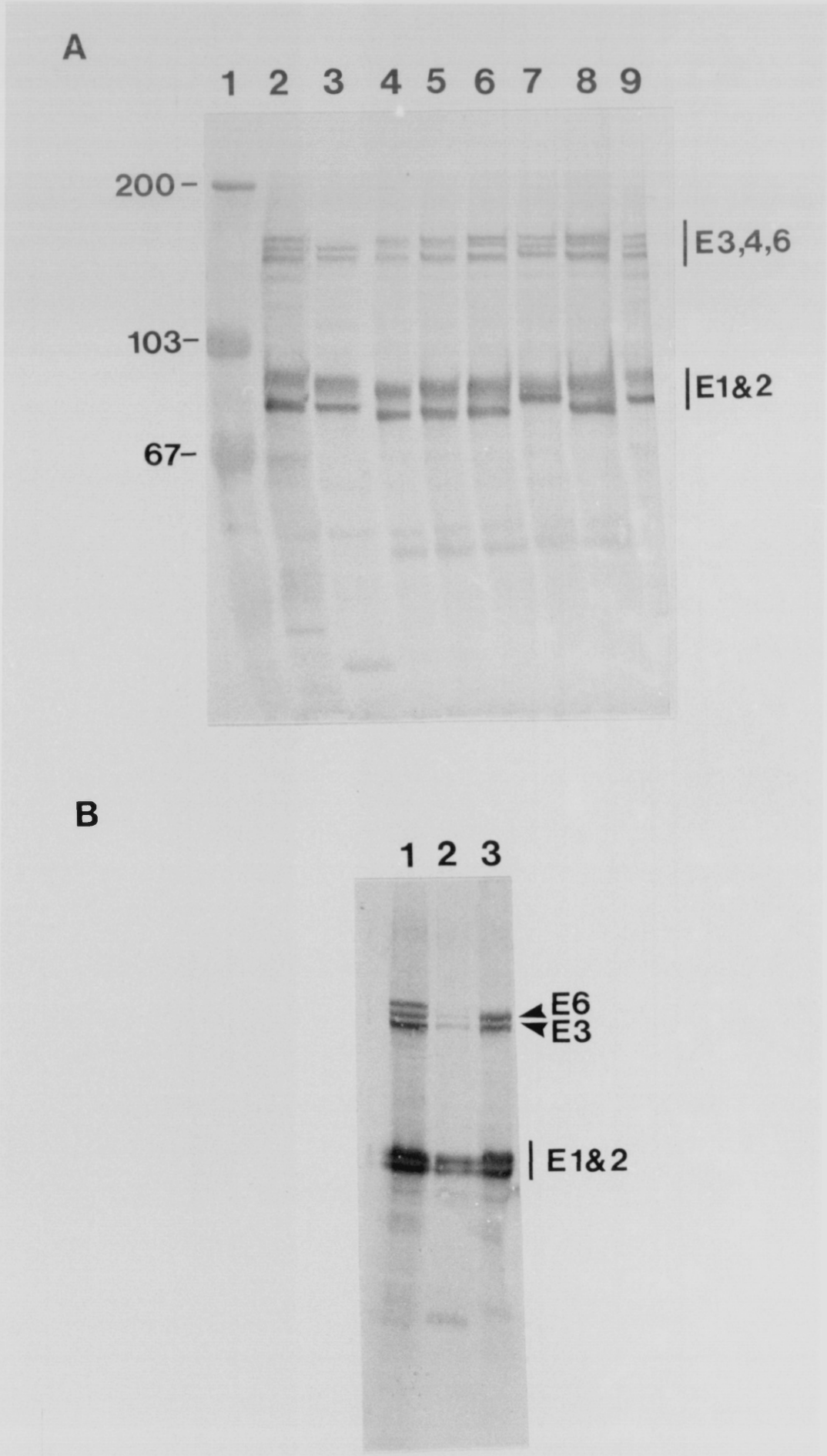
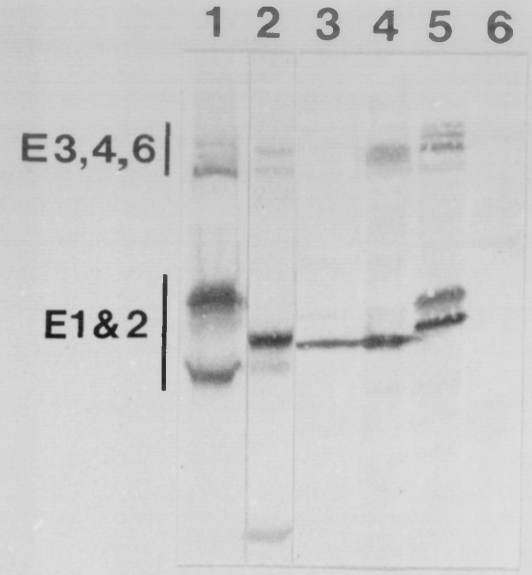

A
1 2 3 4 5 6 7 8 9
200–
103–
67–
E3,4,6
E1&2
B
1 2 3
E6
E3
E1&2

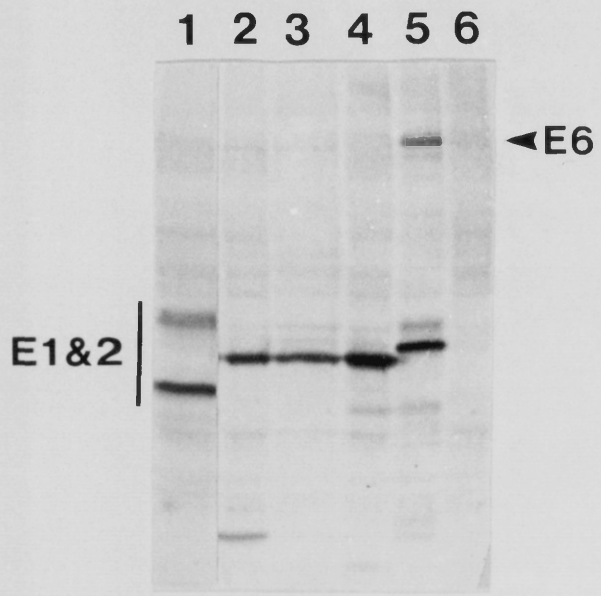
Figure II.7: Expression of EBNA Species in Established BL-lines. Immunoblots from 7.5% SDS-polyacrylamide gels probed with: **A**, serum RT (1/100) and **B**, serum MS (1/100). Raji (track 1); Daudi (track 2); Putko (track 3); P3HR1 (track 4); B95-8 (track 5) and Ramos (track 6). The position of EBNA (E) species is indicated.

A



Serum
RT

B



Serum
MS

pMMTVE3/4 it is under the control of a murine mammary tumour virus (MMTV) long terminal repeat (LTR). The scheme used for the construction of these recombinant plasmids is summarised in Figure II.8 and described in detail in the Materials and Methods section. The orientation of the EBV gene in the final plasmids was determined by digestion with *Bam*HI and *Hin*dIII. Two clones of each vector were then grown in large scale preparations and DNA purified by caesium chloride gradient centrifugation for transfection studies.

3.2 Transient Expression of EBNA6

Forty eight hours after calcium phosphate transfection of pSVE3/4 DNA or salmon sperm carrier DNA (control) onto Cos 7 cells they were fixed and stained by indirect immunofluorescence (IF). The results show that in the cells transfected with pSVE3/4, IF using serum 062 produced nuclear staining (Figure II.9,a). The fluorescent staining had a distinctly punctate appearance and nucleoli were generally unstained (see also Allday *et al.*, 1988). Cos 7 cells transfected with salmon sperm DNA gave no fluorescent staining above background levels (not shown) and an EBV negative serum elicited no fluorescence response (Figure II.9,b). The frequency of stained cells (approximately 1%) was of the order expected from the transfection protocol used (MJA unpublished observations; van der Eb and Graham, 1980). Transient expression of EBNA6 in Cos 7 cells did not produce sufficient antigen for Western immunoblot analysis.

The EBV negative Burkitt's lymphoma line, EB4, is of B-cell origin, grows in suspension culture and therefore proved difficult to transfect by the calcium phosphate technique. This line was thus transfected with pSVE3/4 DNA by electroporation (Potter *et al.*, 1984). Cells were "pulsed" with a "Gene Pulser^{TM}" (Biorad) as described in the Materials and Methods. After 72 hours the samples were harvested by cytospin, fixed and stained by anticomplement immunofluorescence (ACIF) with serum RT. Cells pulsed at only one setting (500 V, 400 μF, time constant 11) produced nuclear fluorescence in approximately 1% of cells (Figure II.10,a). This was not detected in cells transfected at the same setting with pSV2Neo DNA or cells transfected at any other setting (not shown) and an EBV-negative serum produced no fluorescence staining above background levels on successfully

Figure II.8: Construction of Recombinant Plasmids pSVE3/4 and pMMTVE3/4. The 7652 bp *Hind*III/*Eco*RI cleavage product from the *Eco*RI B clone of EBV DNA (Arrand *et al.*, 1981) was purified by dialysis elution. This fragment was then redigested with *Sma*I and the 5729 bp fragment was ligated to *Sma*I cleaved Bluescribe. This produced two plasmids with the EBV insert in opposite orientations [pBSE3/4(1) and pBSE3/4(2)]. pSVE3/4 was generated by ligating the purified 5780 bp *Hind*III/*Eco*RI fragment from BSE3/4(1) to a 2133 bp *Hind*III/*Eco*RI subfragment of pSV2CAT containing the SV40 early region promoter/enhancer (Gorman *et al.*, 1982). pMMTVE3/4 was generated by ligating the purified 5780 bp fragment from BSE3/4(2) to a 4000 bp subfragment of pMLE containing an MMTV LTR (F. Farzaneh, personal communication). ▒ represents the EBV insert containing BERF3/4; ▥ represents the SV40 promoter/enhancer and MMTV LTR in the respective recombinant plasmids. Restriction enzyme sites are: R(*Eco*RI), B(*Bam*HI), H(*Hin*dIII) and S(*Sma*I). The location of EBV open reading frames BERF3 and 4 and BZLF2, restriction sites within the *Eco*RI B fragment and the region deleted from the viral DNA in the Raji Burkitt's lymphoma cell line are all shown.

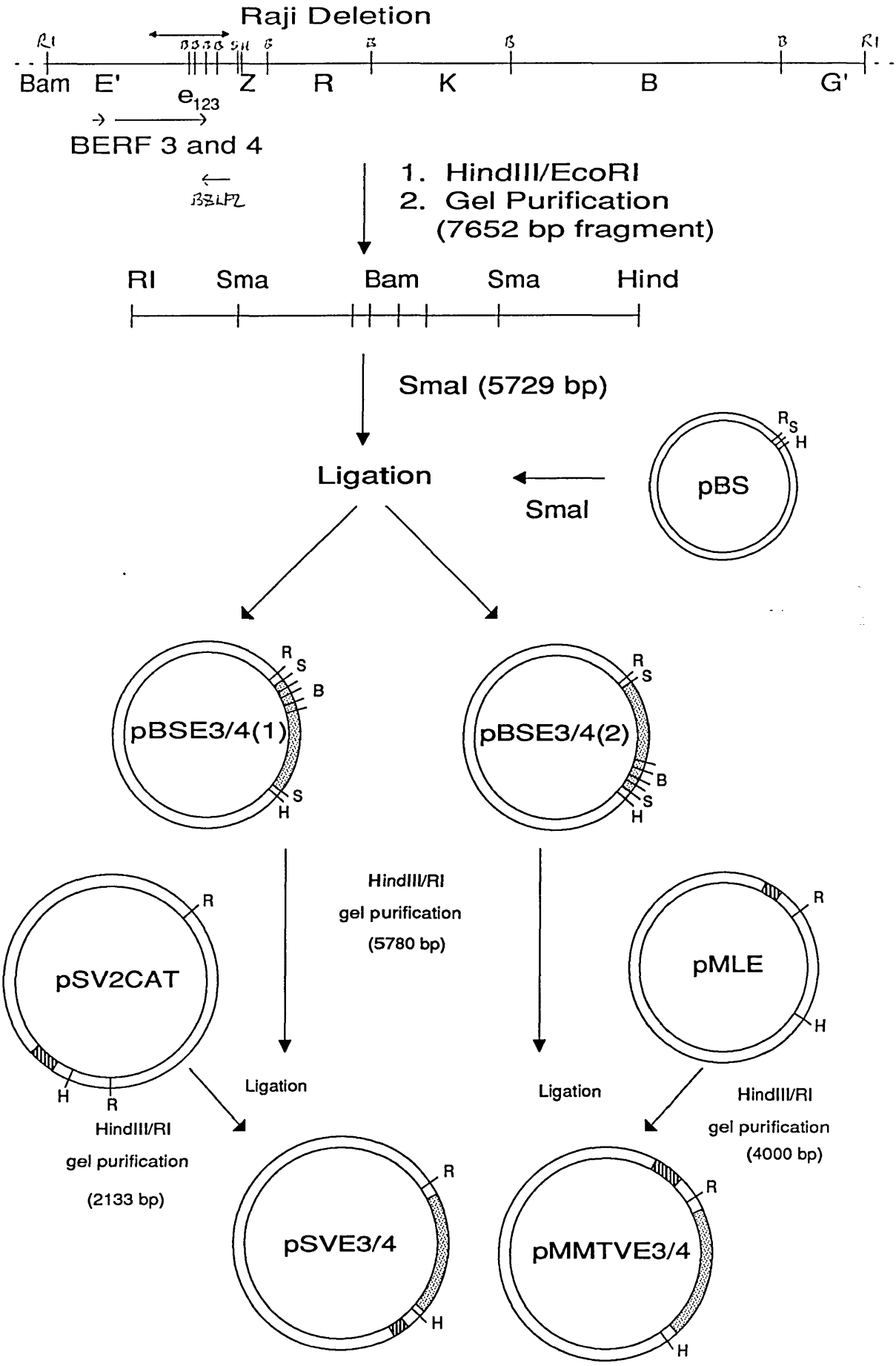
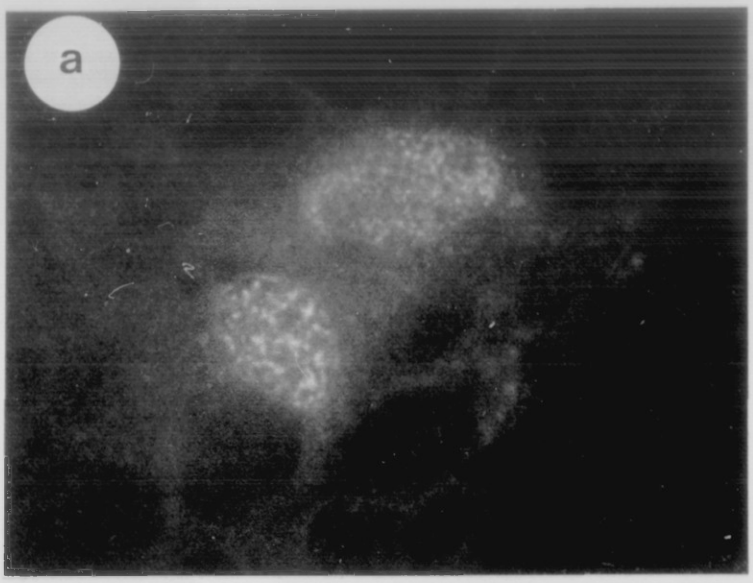
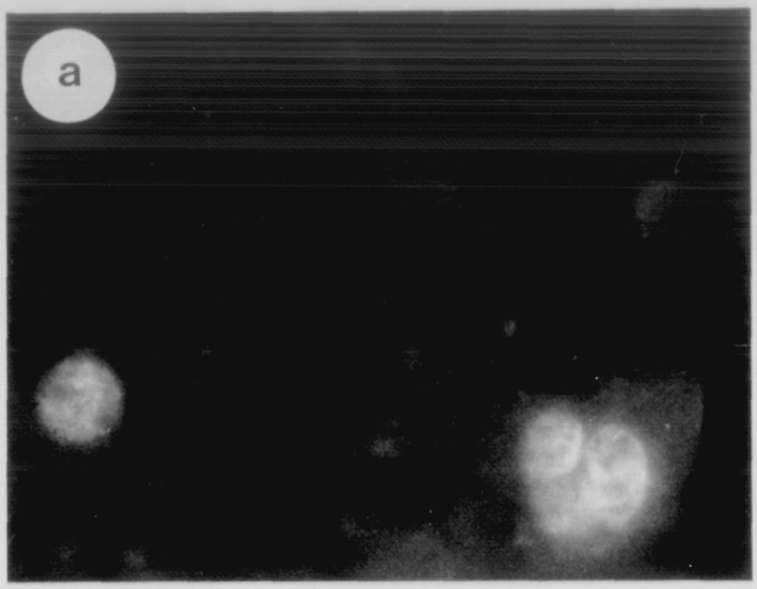

Raji Deletion
Bam
E'
e123
Z
R
K
B
G'
BERF 3 and 4
1. HindIII/EcoRI
2. Gel Purification
(7652 bp fragment)
RI
Sma
Bam
Sma
Hind
SmaI (5729 bp)
Ligation
SmaI
pBS
pBSE3/4(1)
pBSE3/4(2)
HindIII/RI
gel purification
(5780 bp)
pSV2CAT
pMLE
Ligation
Ligation
HindIII/RI
gel purification
(2133 bp)
HindIII/RI
gel purification
(4000 bp)
pSVE3/4
pMMTVE3/4

Figure II.9: Transient expression of EBNA6 in Cos 7 cells. Plate (a). After transfection with pSVE3/4 DNA and staining by indirect immunofluorescence using serum 062. **Plate (b).** Similarly transfected cells stained with EBV-negative serum, MJ. (Magnification x 1500).


a


b

Figure II.10: Transient expression of EBNA6 in EB4 B lymphocytes. Plate (a). After electroporation with pSVE3/4 DNA and staining by ACIF with serum RT. **Plate (b).** Similarly transfected cells stained with EBV-negative serum, MJ. (Magnification x 1000).


a


b

transfected cells (Figure II.10,b). As in the Cos7 cells the level of transient expression did not produce sufficient antigen for immunoblot analysis. The strictly nuclear distribution of EBNA6 in the transient assays in both mouse and human cells is consistent with the results of the cell fractionation studies performed on the LCL, 2C9 (section 2.2).

3.3 Attempts to Establish EBNA6 Expressing Cell Lines

pSVE3/4 does not include a dominant selectable marker, such as a gene conferring drug-resistance. Therefore in attempts to establish cell lines stably expressing EBNA6, either pSV2Neo (which confers resistance to Geneticin, G418) or pSV2Hyg (which confers resistance to Hygromycin-B) were used in cotransfections. Six different cell types were cotransfected with pSVE3/4 DNA and either pSV2Neo DNA or (in one case) pSV2Hyg DNA; the results of these experiments are given in Table II.2. No experiment successfully produced drug-resistant clones stably expressing EBNA6. The results of an experiment in which DNA from two different clones of pSVE3/4 were independently cotransfected along with pSV2Neo DNA onto Balbc 3T3 fibroblasts are shown in Figure II.11. In the control dish, cells were cotransfected with pSV2Neo and salmon sperm DNAs and approximately 60 resistant colonies/dish had grown 21 days after applying G418 selection (panel c). However, similar experiments with the pSVE3/4 DNAs produced only one resistant colony (a and b). To determine if the failure of these cells to proliferate was due specifically to expression of BERF3/4, pSVE3/4 DNA was cleaved with *HindIII* prior to transfection. This disengages the SV40 early promoter from the EBV encoded open reading frames (see Figure II.8). It can be seen that after cleavage, cotransfection of this DNA with SV2Neo DNA produced almost as many G418 resistant colonies (30-40/dish) as on the control dish (compare panel e and f with c). It appears that constitutive expression of EBNA6 from a "strong" viral promoter in transfected recombinants is not compatible with long term proliferation of Balbc 3T3 (or a number of other) cells.

The vector pMMTVE3/4 (Figure II.8) utilises the promoter/enhancer elements located in the long terminal repeat (LTR) of the murine mammary tumour virus (MMTV). This promoter generally has a low level of constitutive activity

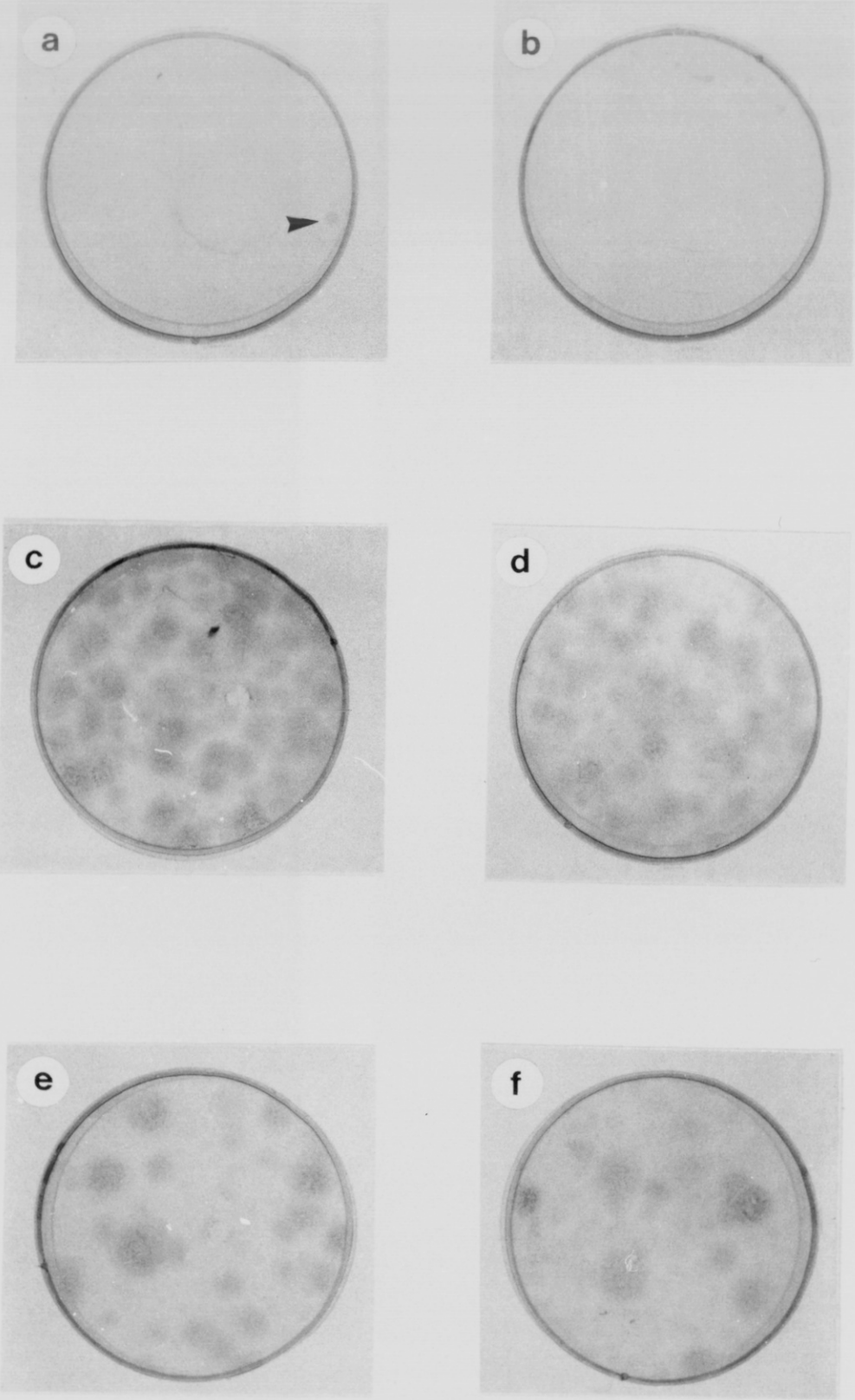
Table II.2: Summary of SVE3/4 Transfections

Cells	Species	Cell Type	Transfection Technique	Transient EBNA6 Expression+	Selection	Stable EBNA6 Expression
Permanent Line						
Cos 7	Monkey	Fibroblast	Calcium Phosphate	+	G418	-
CV1	Monkey	"	"	+	"	-
Balbc 3T3	Mouse	"	"	+	"	-
EB4	Human	B lymphocyte	Electroporation	+	Hygromycin	-
Tera 2*	Human	Undifferentiated teratocarcinoma	Calcium phosphate	N/D	G418	-
Primary cells						
REF	Rat	Embryonic	Calcium phosphate	N/D	G418	-

* Transfected by J. Sinclair

+ Demonstrated by Immunofluorescence

Figure II.11: Cotransfections of Balbc 3T3 Fibroblasts. Cells were transfected with the combinations of DNA indicated below and 21 days after applying G418 selection they were washed, fixed and stained (0.15% Leishman's stain in methanol). (a) and (b) pSVE3/4 + pSV2Neo; (c) Salmon Sperm DNA + pSV2Neo; (d) pMMTVE3/4 + pSV2Neo; (e) and (f) *Hind*III digested pSVE3/4 + pSV2Neo. The arrow in (a) denotes a single G418 resistant colony.


a
b
c
d
e
f

which is enhanced by the addition of steroid hormone analogues such as dexamethasone (M. Strauss, personal communication). Cotransfection of pMMTVE3/4 with pSV2Neo onto Balbc 3T3 cells produced G418 resistant colonies which were stained 21 days after applying the drug selection (see Figure II.11, panel d). A similar experiment was set up and 10 days after applying selection six colonies were picked and subcultured using cloning rings into a 24 well (flat bottom) plate. When they reached confluence the cells were passaged to a six well plate. A sample of cells from each well was transferred to a multichamber microscope slide and at confluence were fixed and stained by using IF. Five of the six clearly showed nuclear fluorescence on the majority of the cells with serum 062 (Figure II.12, a, b, c, e and f) even in the absence of dexamethasone. However, when the clones were subcultured and regrown to confluence in 5 cm dishes, analysed by IF (by D.H. Crawford) and also immunoblotted (Figure II.13, tracks 2-7) none appeared to be expressing EBNA6, as judged by either technique. Similar results were obtained when the experiment was repeated and extracts were made from uncloned cells at 3 and 4 weeks after applying drug selection and immunoblotted; that is, no EBNA6 was detected (not shown). These results are consistent with those obtained from the transfections with pSVE3/4 and the results of another group who, using similar expression vectors, have also consistently failed to establish stable EBNA6 expressing lines (L. Rymo, personal communication).

4 LATENT GENE EXPRESSION DURING THE INITIATION OF IMMORTALIZATION

4.1 Introduction - The Use of PLL Cells

It has been reported that EBNA expression, as determined by anti-complement immunofluorescence (ACIF) is detectable in a subpopulation of B-cells approximately 12-24 hours after they are infected with EBV (Moss *et al.*, 1981). It has also been suggested that EBNA2 expression may precede that of EBNA1 (Kallin *et al.*, 1986; Moss *et al.*, 1986). However, there has been no detailed study of the temporal sequence of expression from all the identified latent EBV genes immediately after viral infection. Immunological reagents and techniques for the visualisation in fine detail of the EBNA complex have been developed (see sections

Figure II.12: Expression of EBNA6 in Balbc 3T3 Fibroblasts after Transfection with pMMTVE3/4 DNA. Cells were transfected, selected for G418 resistance and cloned as described in the text and Materials and Methods. They were then fixed and stained by indirect immunofluorescence with serum 062. **Plates a-f** represent 6 independent G418 resistant clones.

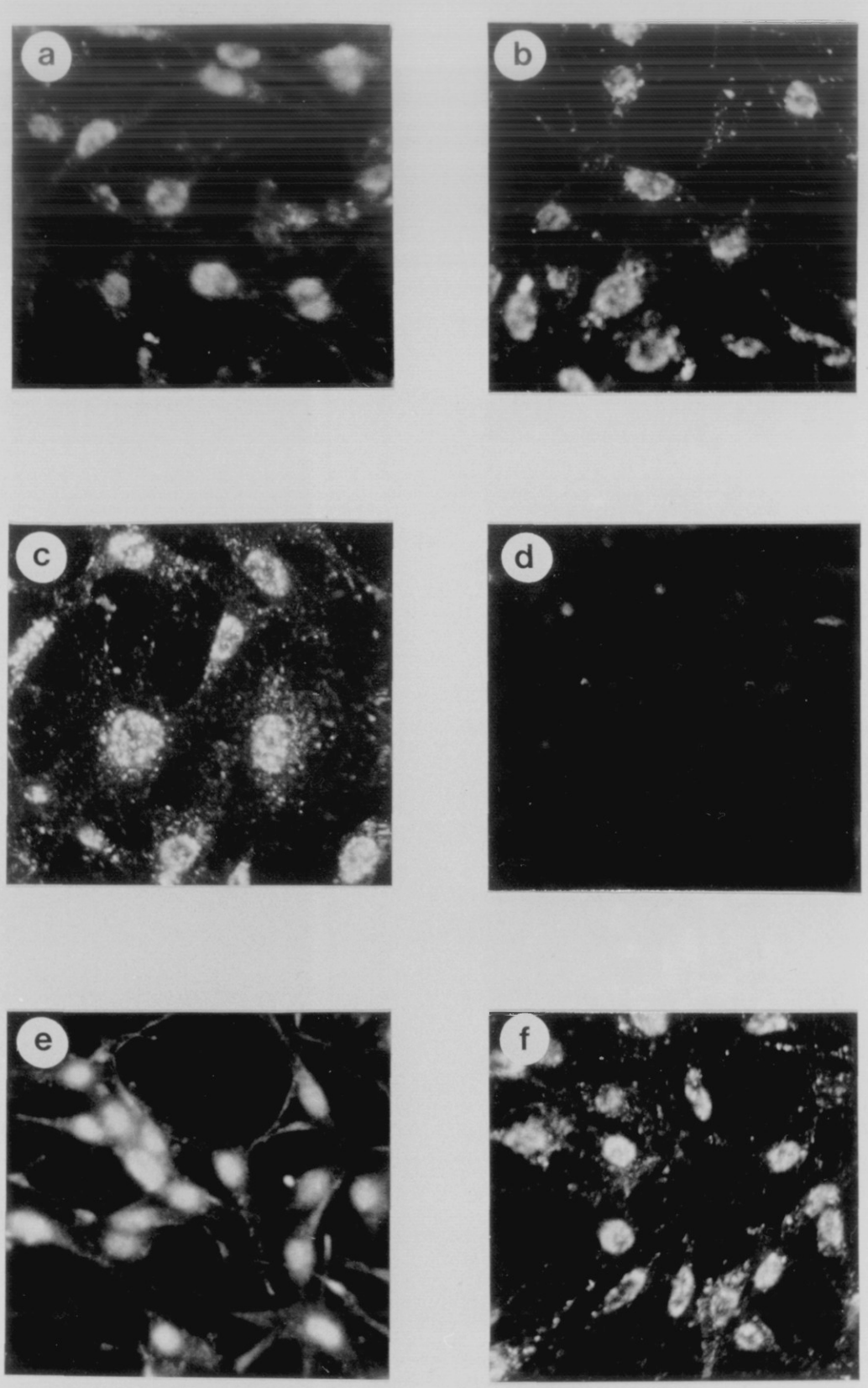
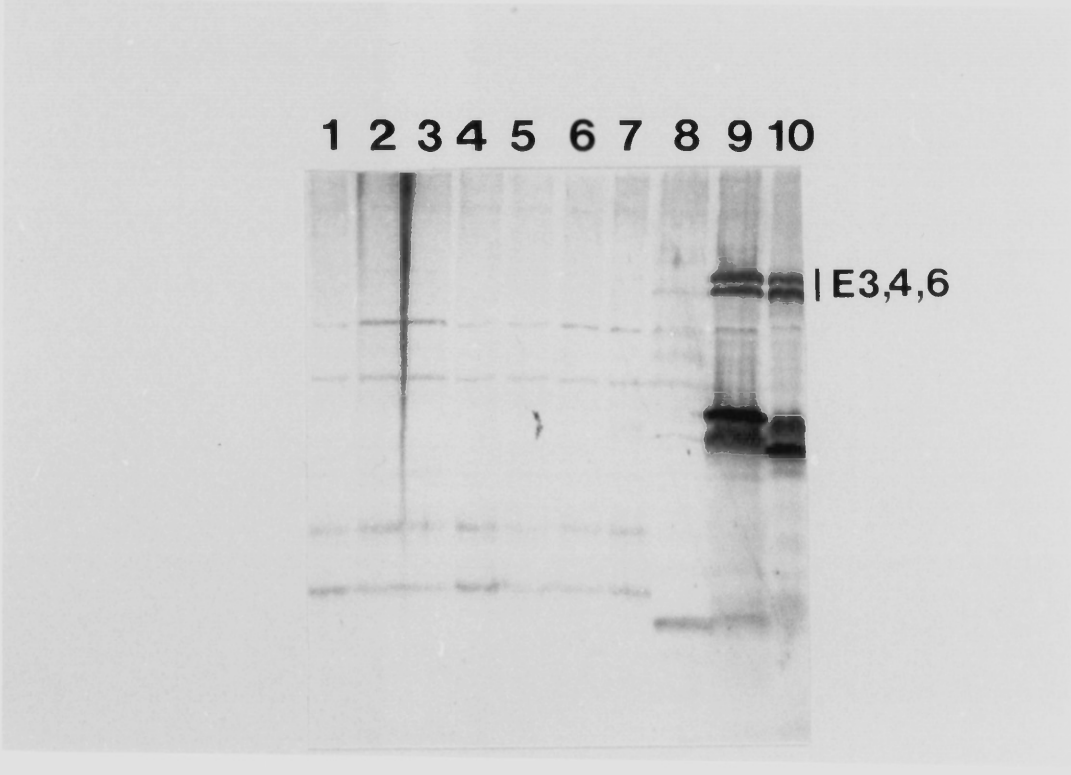

a
b
c
d
e
f

Figure II.13: Immunoblot Analyses of pMMTVE3/4 Transfectants. The six G418 resistant clones shown in Figure II.12. were expanded and total cellular proteins extracted, separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with serum RT (1/100). Immunoreactive proteins from clones a-f are represented in tracks 2-7, untransfected Balbc 3T3 cells in track 1; EBV-negative Ramos in track 8 and positive controls, 2C9 and IB4 in tracks 9 and 10, respectively. The position of the EBNA (E) 3, 4, 6 cluster is indicated. the uppermost band in track 10 corresponds to EBNA 6.


1 2 3 4 5 6 7 8 9 10
E3,4,6

II.1 and 2). An analysis of EBNA (and latent membrane protein, LMP) was therefore carried out during the initiation of immortalization when resting B-cells are stimulated to enter and traverse the cell cycle. Preliminary studies suggested that EBNA fluorescence staining could be detected in both cord blood lymphocytes (CBL) and adult peripheral blood lymphocytes (PBL) as early as 8 hours after infection with the B95-8 strain of virus (D.H. Crawford, personal communication). However, it is not possible to identify the component EBNA proteins in such cells by immunoblotting until at least 24 hours after infection (Kallin *et al.*, 1986, and M.J.A. unpublished observations). This is consistent with the small percentage (10%-20%) of infected CBL and PBL which actually express EBNA. Prolymphocytic leukaemia (PLL), cells on the other hand, have been identified as a much more sensitive target population for analysing the early events after EBV infection (Walls *et al.*, 1989; D.H. Crawford personal communication). These are leukaemic cells "frozen" at an early lymphoblastic stage of differentiation. They form a monoclonal, relatively homogenous population of non-proliferating B cells up to 60% of which are activated and immortalized by EBV (Walls *et al.*, 1989). In this respect, and also with respect to other B-cell activators, they appear to behave in a very similar manner to the subpopulation of normal resting B cells which are immortalized by EBV. Because PLL cells are available in large numbers from a single individual it has been possible to carry out many experiments on identical populations. In addition to determining the temporal sequence of EBNA synthesis, LMP expression was also investigated.

4.2 EBNA Expression

Eight hours after infection of PLL cells with B95-8 EBV, anticomplement immunofluorescence, using a human serum (RT) which recognises EBNAs 1, 2, 3, 4 and 6, produces a faint but characteristic EBNA fluorescence pattern in approximately 50% of cells (Figure II.14 panel a). Prior to this time no staining was detectable. (Data obtained were indistinguishable from those with uninfected cells stained with RT, Figure II.15a). From 8-48 hours the intensity of staining increased and the pattern altered slightly with some cells exhibiting pronounced nucleolar staining (Figure II.14 panel d, arrowed). The number of clearly positive cells rose slightly as staining became more intense. To establish which EBNA species contribute to this fluorescence pattern, samples of cells were taken at various time

Figure II.14: Photomicrographs showing Anti-EBNA ACIF. Staining (with serum RT) of B95-8 virus infected PLL cells at (a) 8 hours; (b) 12 hours; (c) 24 hours; (d) 48 hours post infection, (Magnification x 500).

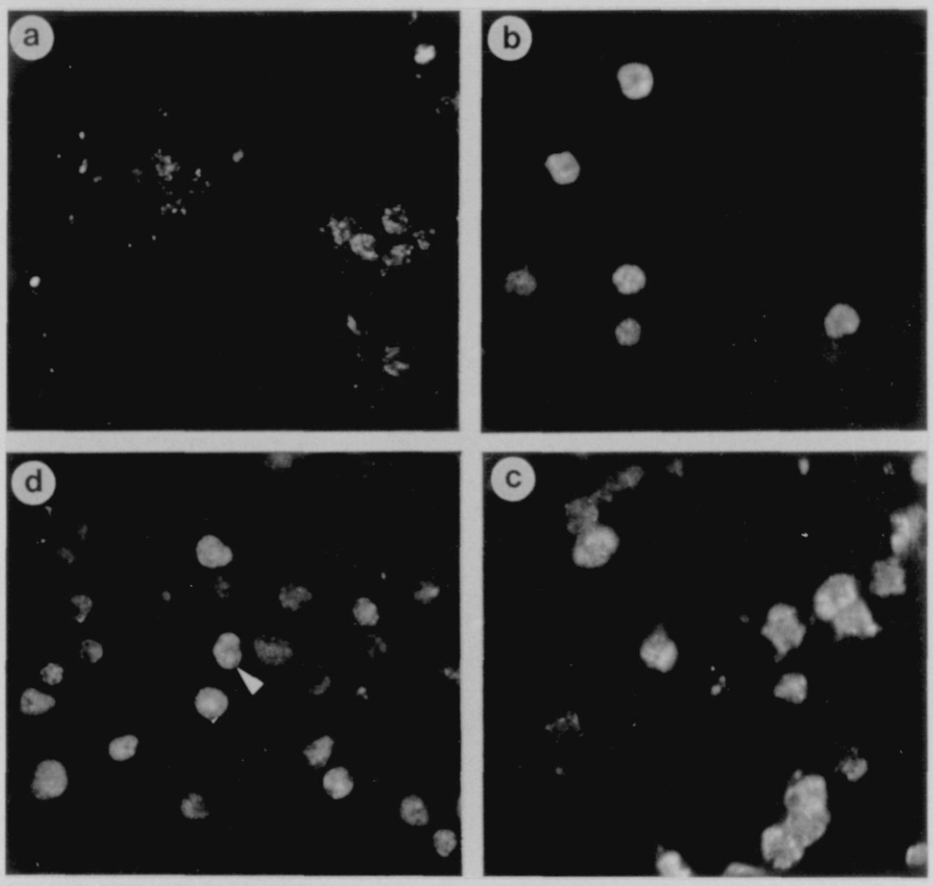
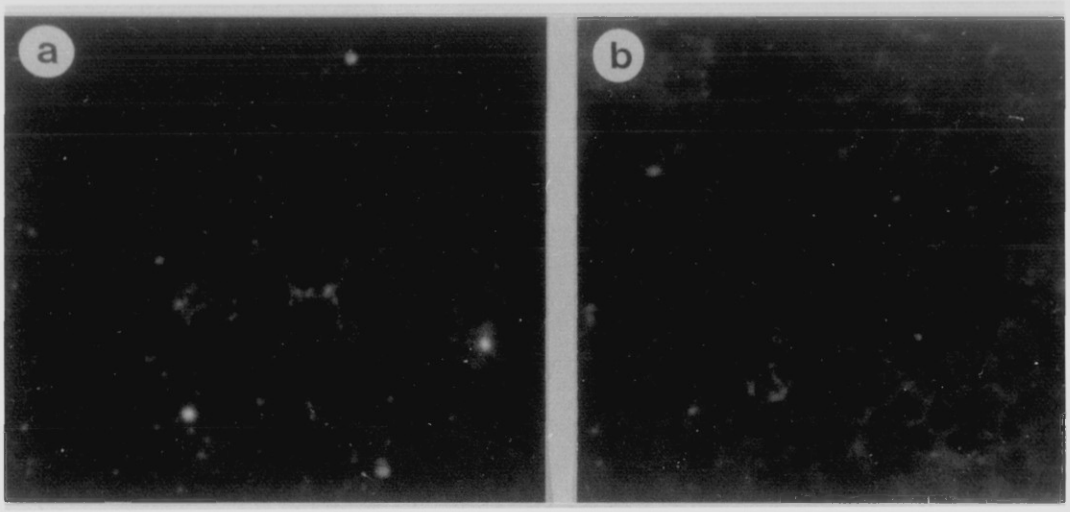

a
b
d
c

Figure II.15: Photomicrographs showing Anti-EBNA ACIF (Negative Controls). Staining of (**a**) uninfected PLL cells with serum RT or (**b**) B95-8 infected PLL cells 48 hours post infection with EBV-negative serum MJ (Magnification x 600) (compare with Figure 14 II.14, panel d).


a
b

points after infection and protein extracts were immunoblotted with serum RT. At 10 hours post-infection EBNA2 was just detectable (Figure II.16.A, track 6). By 12 hours the level of this protein had increased and by 18 hours (Figure II.16.B, track 2) EBNAs 1, 3, 4 and 6 can also be clearly seen. Between 18 and 24 hours the levels of EBNAs 1 and 2 reach that found in established LCLs (compare Figure II.16.A, track 8 with tracks 10 and 11). Although during this period EBNAs 3, 4 and 6 were weakly detectable it was not until the 40 hour time point that they were present at levels equivalent to those as in established cell lines (compare Figure II.16.B, track 3 with track 6). Immunoblotting with a serum (RM), which contains high titres of antibody to EBNA5, shows a characteristic ladder of EBNA5 species (see Finke, *et al.*, 1987) appearing between 12 and 24 hours (Figure II.17, tracks 2 and 3), at about the same time as EBNAs 1, 3, 4 and 6. The apparently low levels of EBNA2 in this blot merely reflect the low titre of anti-EBNA2 antibodies in serum RM with respect to RT. Compare Figure II.16,A (tracks 8 and 10) with Figure II.17 (tracks 1 and 3).

4.3 LMP Expression

Indirect immunofluorescence staining with a cocktail of four anti-LMP monoclonal antibodies produced the cytoplasmic staining pattern (in 5-10% of PLL cells) typical of LMP (Rowe *et al.*, 1987), 48 hours after infection with B95-8 virus (Figure II.18, panel b). Prior to this cells were negative apart from low level non-specific staining (see Figure II.18, panel a). The number of positive cells then increased over the next 48 hours to approximately 30% (c and d). This figure, which is based on a comparison with cells viewed under phase contrast (not shown), is consistent with previous reports (Mann *et al.*, 1985; Rowe *et al.*, 1987). To confirm these data, cell extracts were immunoblotted with the same cocktail of monoclonal antibodies. The results (Figure II.19,A) show that LMP is only detectable after 72 hours (track 2). The failure to detect LMP at 48 hours by immunoblotting is consistent with the relatively small percentage of cells observed to be positive by IF.

4.4 Infection with P3HR1 Strain of EBV

Expression of the latent genes after P3HR1 infection of PLL cells, in similar studies to those carried out with B95-8, were analysed. EBNAs 1 and 3 (Figure

Figure II.16: Immunoblots of EBNAs 1, 2, 3, 4 and 6. **A.** SDS-solubilized proteins from 10^6 PLL cells were taken at different time points (0-24 hours) post-infection with B95-8 virus, as indicated below each track (1-8); after separation on a 7.5% SDS polyacrylamide gel and transfer to nitrocellulose they were probed with serum RT (1/100). Track 9, contains proteins from uninfected PLL cells at 24 hours, tracks 10 and 11 from B95-8 and IB4 (EBV positive LCLs), respectively, and track 12 from Ramos (an EBV negative BL line). **B.** Similar immunoblot carried out on proteins from B95-8 infected PLL 18-90 hours post-infection (tracks 2-5). Track 1 contains uninfected PLL cells at 18 hours and track 6, IB4. In each blot the positions of MW standards (kD) is shown.

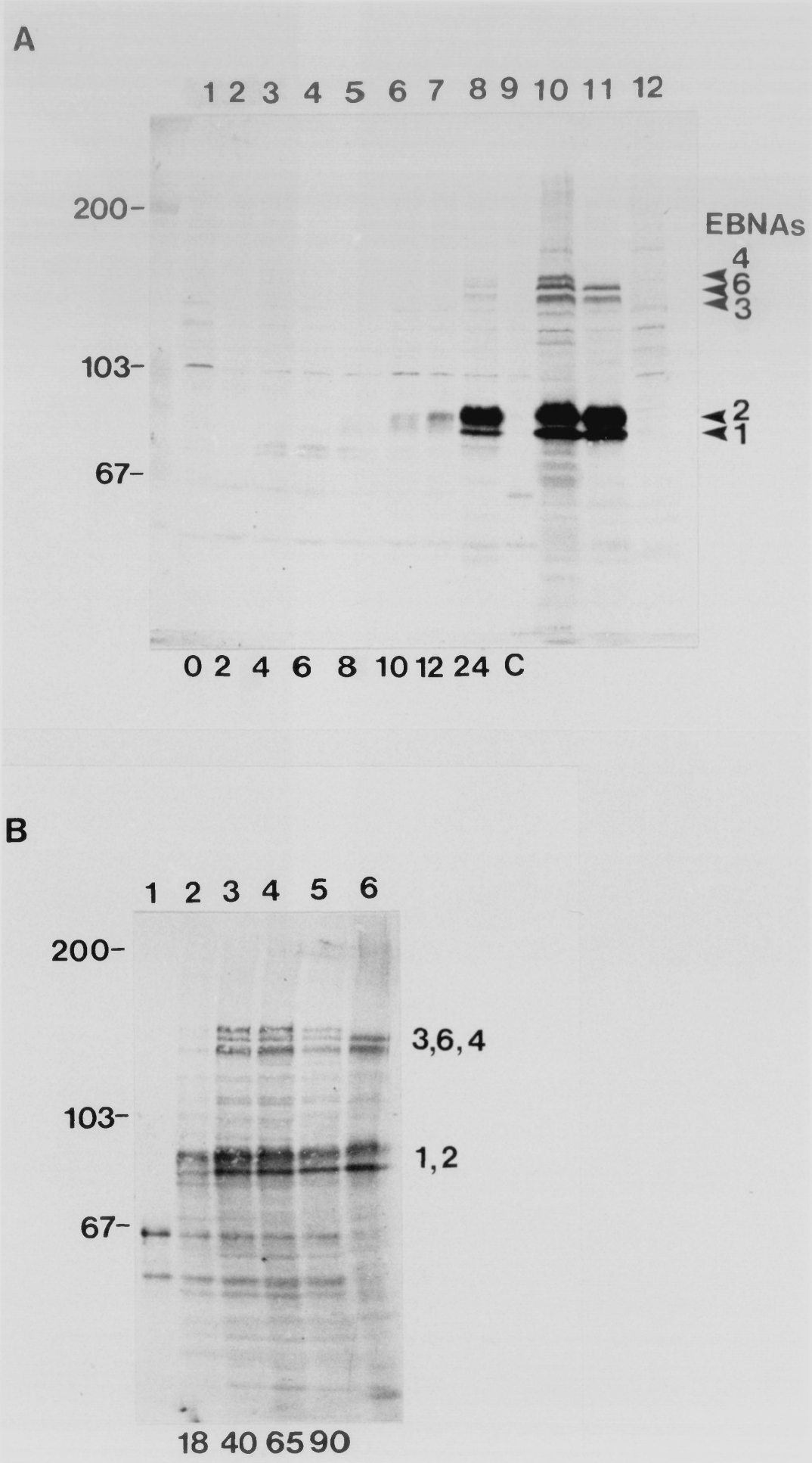

A
1 2 3 4 5 6 7 8 9 10 11 12
200-
103-
67-
EBNAs
4
6
3
2
1
0 2 4 6 8 10 12 24 C
B
1 2 3 4 5 6
200-
103-
67-
3,6,4
1,2
18 40 65 90

Figure II.17: EBNA5 Expression after EBV Infection of PLL cells. Immunoblot probed with serum RM (1/500) showing EBNA5 expression in B95-8 virus infected PLL cells (tracks 2 and 3) and P3HR1 virus infected PLL cells (tracks 4 and 5). B95-8 (track 1) and P3HR1 (track 6) were similarly blotted and probed. The hours post-infection of PLL cells are shown below each track, and the approximate position of MW standards (kD) are indicated. (See Figure II.16 for location of other EBNA species).

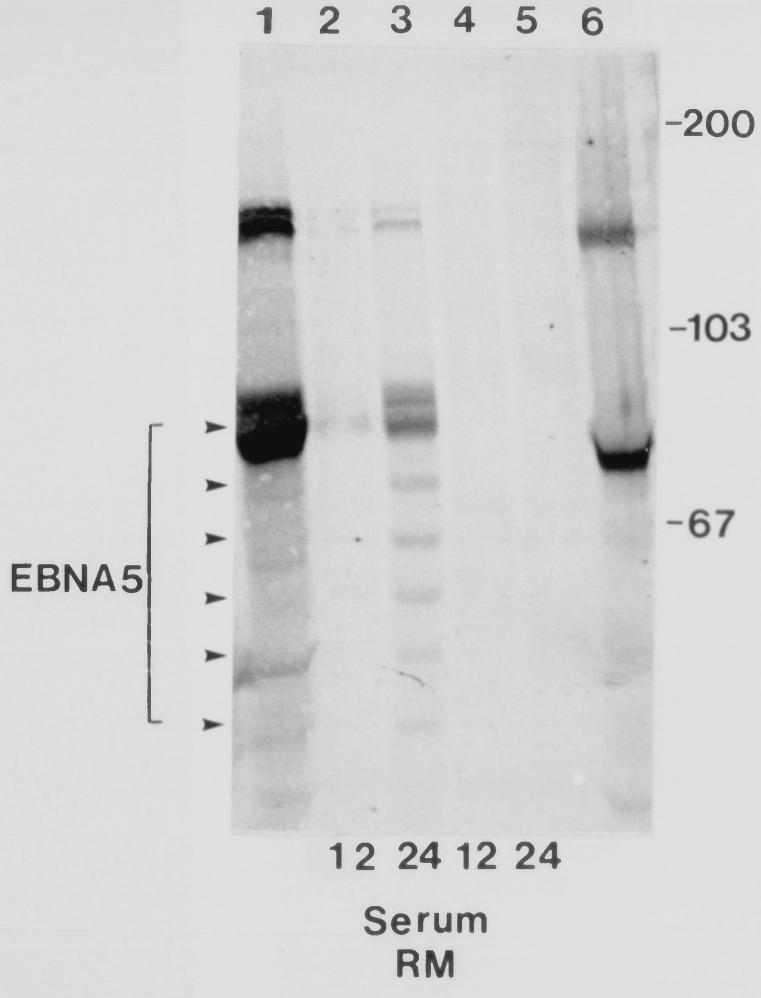

1 2 3 4 5 6
-200
-103
-67
EBNA5
1 2 24 1 2 24
Serum
RM

Figure II.18: Photomicrographs showing Anti-LMP Indirect Immunofluorescence. Staining of B95-8 virus infected PLL cells at (a) 24 hours, (b) 48 hours, (c) 72 hours and (d) 96 hours post infection (Magnification x 500).

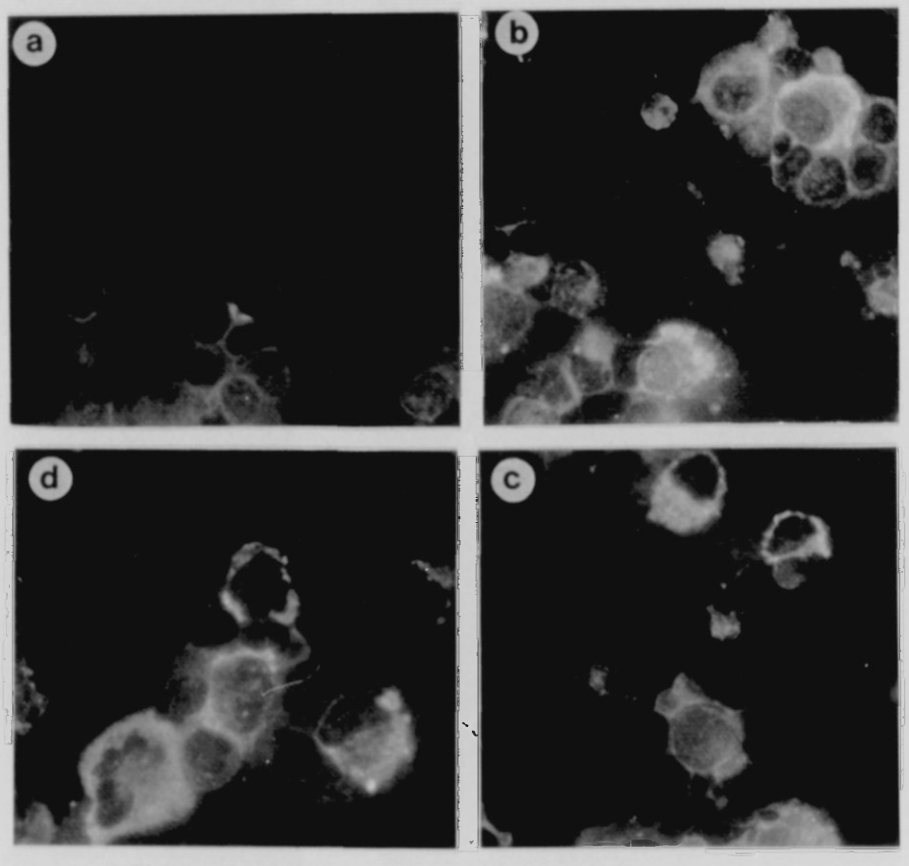
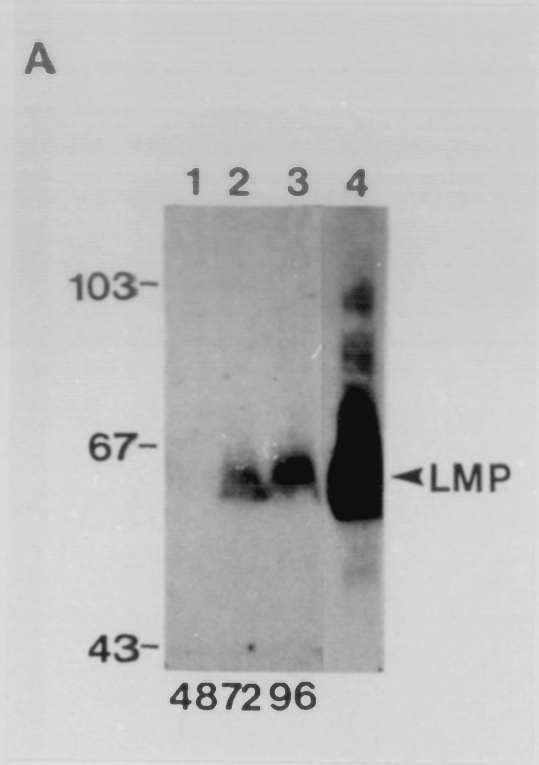

a
b
d
c

Figure II.19: Immunoblots of LMP probed with CS1-4. **A.** 2 x 10^6 PLL cells were taken at various time points (48-96 hours) after infection with B95-8 virus as indicated below tracks (1-3). IB4 cells were similarly blotted (track 4). **B.** P3HR1 virus infected PLL cells (tracks 1-3) and P3HR1 cells (track 4) were immunoblotted in a similar manner. MW standards (kD) are indicated.


A
1 2 3 4
103-
67-
43-
LMP
48 72 96
B
1 2 3 4
103-
67-
43-
LMP
48 72 96

II.20, track 13) and EBNA5 (Finke *et al.*, 1987), are expressed in the parent P3HR1 cell line. However, in the PLL cells during the first 24 hours post infection, when B95-8 infected cells have synthesized EBNAs 1-6, there appears to be no expression of the P3HR1 EBNA genes as determined by fluorescence (not shown) or immunoblotting with either serum RT (Figure II.20, tracks 1-8) or RM (Figure II.17, tracks 4 and 5). 48 hours after infection a small number of cells (approximately 10%) showed a punctate nuclear staining characteristic of EBNA1 (see Figure II.21,A) with various sera, including RT and RM. There is no evidence to suggest that these cells proliferate and by 96 hours fluorescent staining was no longer detectable (not shown). Attempts to analyse this transient expression are hampered by the small number of EBNA expressing cells and the low levels of antigen. A representative immunoblot of extracts made at 48 hours post-infection (Figure II.21,B) (probed with serum ST) shows a low level of EBNA1. Both fluorescence (not shown) and immunoblotting (Figure II.19,B, tracks 1-3) fail to detect LMP expression in the PLL cells up to four days post-infection although the parent P3HR1 cell line synthesizes readily detectable levels of LMP (Figure II.19,B, track 4).

4.5 Immortalization

It was established on several occasions that PLL cells could be immortalized with B95-8 virus; cells from various experiments were allowed to proliferate and were passaged for several months. On the other hand, P3HR1 infected cells were consistently dead after five days in culture, as were uninfected PLL cells.

4.6 Latent Gene Expression in Immortalizable and Non-Immortalizable B Cell Subsets

All mature B lymphocytes will bind and internalise EBV. However, limiting dilution analyses suggest that only a small percentage of these cells become immortalized after infection (see Section I.1.3). Recent studies undertaken to identify the population which immortalizes have used the CD23 (Blast-2) B cell activation antigen and have revealed that immortalization is restricted to the CD23-positive cells (Thorley-Lawson and Mann, 1985; Azim and Crawford, 1988).

Figure II.20: Immunoblot after P3HR1 Infection. SDS-solubilized proteins from 10^6 PLL cells were taken at different time points (0-24 hours) after infection with P3HR1 virus as indicated below tracks (1-8); after separation on a 7.5% SDS-polyacrylamide gel and transfer to nitrocellulose they were probed with serum RT (1/100). Track 9 contains proteins from uninfected PLL cells at 24 hours. Proteins from B95-8 (track 10); IB4 (track 11); Ramos (track 12) and the parent P3HR1 cell line (track 13) are shown. The positions of EBNAs 1 and 3 in P3HR1 are indicated and the approximate position of MW standards (kD) is shown.

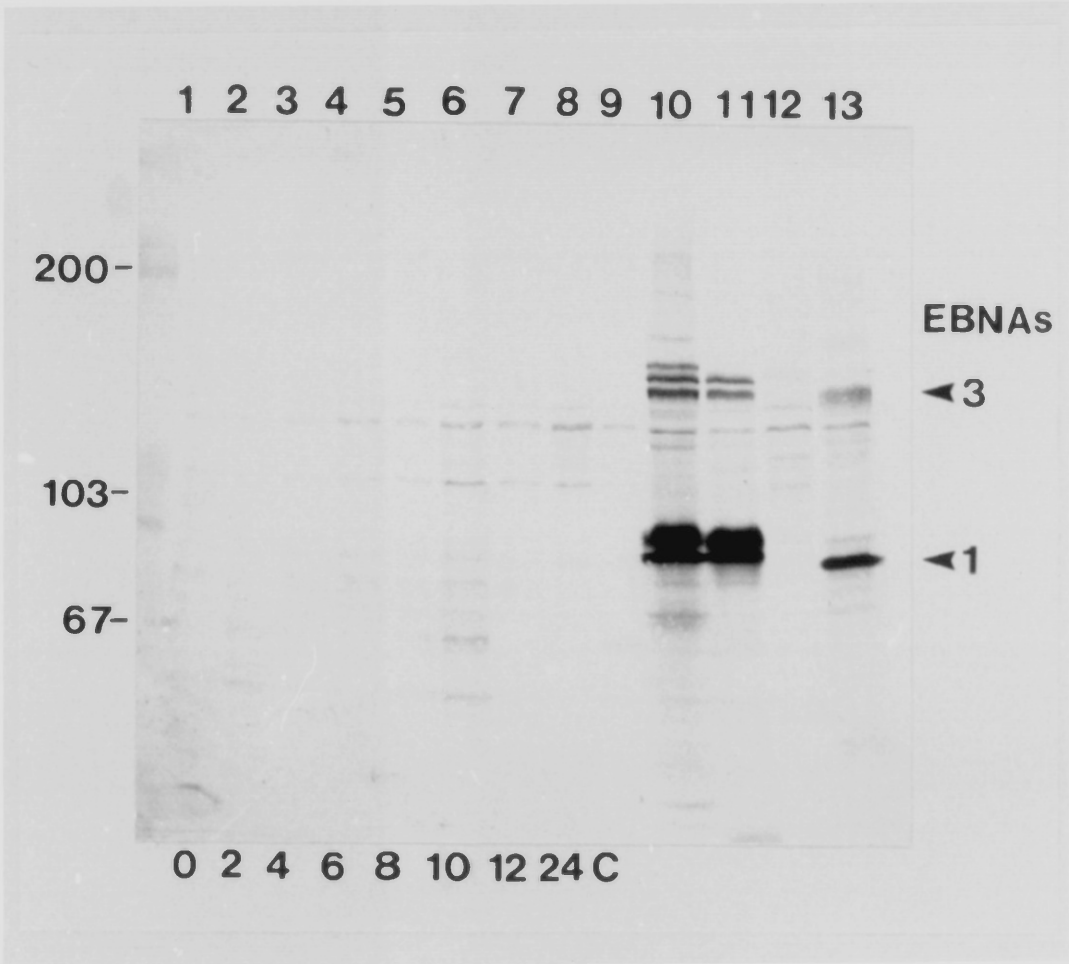
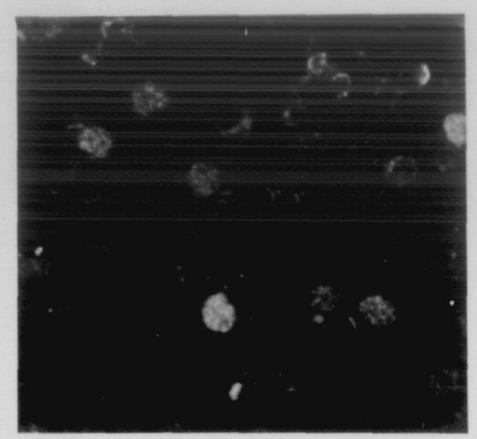
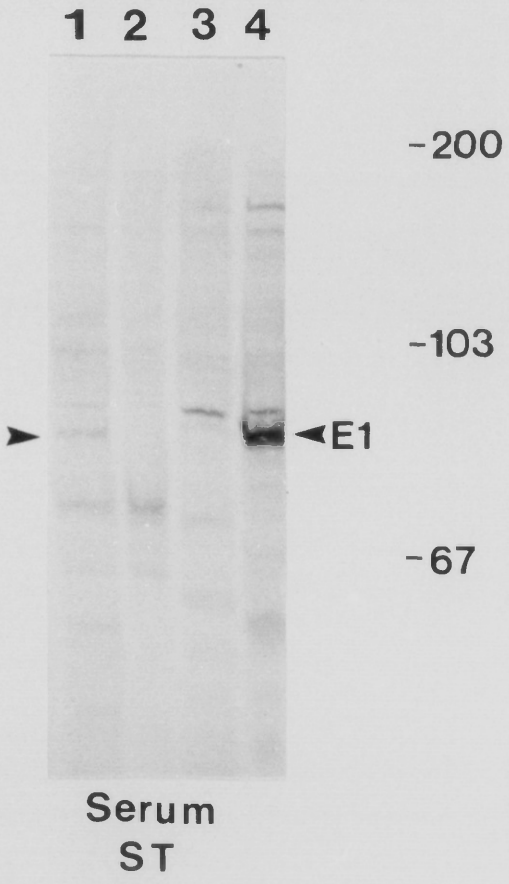

1 2 3 4 5 6 7 8 9 10 11 12 13
200-
103-
67-
EBNAs
3
1
0 2 4 6 8 10 12 24 C

Figure II.21: EBNA Expression 48 hours after P3HR1 Virus Infection. **A.** Photomicrograph showing anti-EBNA ACIF staining, with serum RT, of P3HR1 infected PLL cells harvested at 48 hours post infection (Compare with Figure II.15 panel a) (Magnification x 500). **B.** Immunoblot, probed with serum ST (1/500) showing EBNA1 expression in PLL cells 48 hours post-infection with P3HR1 virus (track 1). Immunoreactive proteins from uninfected PLL cells (track 2); Ramos (track 3) and P3HR1 (track 4) are shown. The position of EBNA 1 in P3HR1 (◄) and MW standards (kD) are indicated.

A



B



However, cells expressing EBNA (as determined by ACIF using polyclonal serum) are found in both fractions. The pattern of latent EBV gene expression in these subsets was therefore determined.

Three days after infection with B95-8 virus, E-rossette negative peripheral blood lymphocytes (PBL) were separated into CD23-positive and negative fractions using antibody-coated magnetic microbeads. (This method routinely produces a CD23-negative fraction with less than 1% contamination with CD23 expressing cells (T. Azim, personal communication). The cells were washed with PBS and solubilised in SDS-sample buffer. After separation by SDS-PAGE, the proteins were analysed by immunoblotting with serum RT and the anti-LMP monoclonal antibodies CS1-4. The results show that EBNAs 1 and 2 are expressed to about the same degree in both fractions (Figure II.22,A. Compare tracks 1 and 2). This was unexpected as it has been shown in another study that expression of CD23 is specifically stimulated by EBNA 2 (Wang et al., 1987). On the other hand, expression of EBNAs 3, 4, and 6 does not appear equivalent in both populations. Despite there being protein from a greater number of cells in track 2 (which corresponds to the CD23-negative population) EBNAs 3, 4, 6 cannot be detected, whereas in the CD23-positive cells (track 1) they are readily detectable. The immunoblot probed with CS1-4 revealed a similar restriction of LMP to the CD23-positive fraction (Figure II.22,B, track 1).

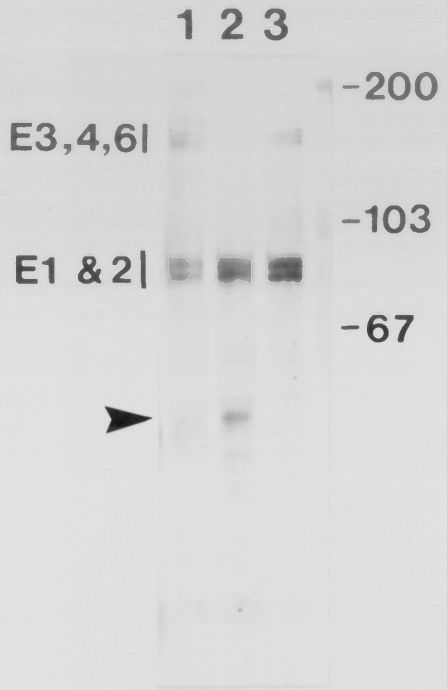
5 LATENT GENE EXPRESSION IN A NASOPHARYNGEAL CARCINOMA EXAMINED BY IMMUNOBLOTTING AND cDNA ANALYSIS

5.1 C15 Tumour

The C15 tumour was originally obtained from a 13-year-old girl with a locally advanced tumour and early metastatic spread. After histological diagnosis it was classified as a poorly differentiated nasopharyngeal carcinoma and biopsy fragments were established subcutaneously in Swiss nude mice (Busson *et al.*, 1988). The tumour has been regularly passaged in nude mice over 30 times in several different laboratories, including our own. The main genomic and phenotypic characteristics have been regularly checked and remain stable with time (Busson *et al.*, 1988). In particular the number of EBV viral genomes has remained constant

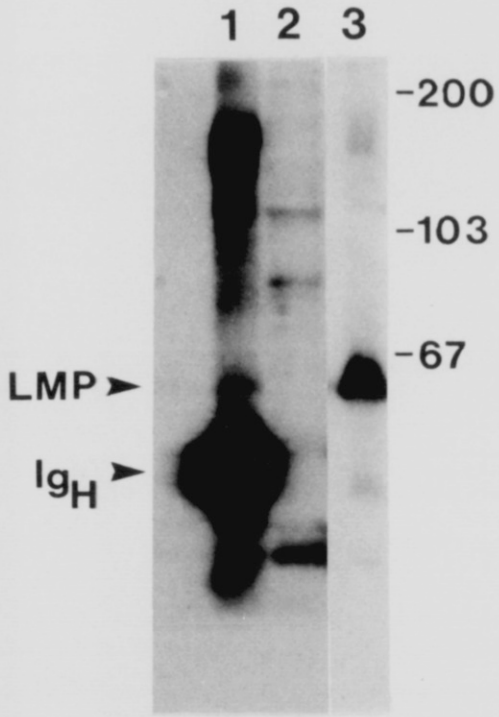
Figure II.22: Latent gene Expression in Immortalizable and Non-immortalizable B cell Subsets. **A.** 72 hours after infection with B95-8 virus, E-rossette negative PBLs were separated into CD23-positive and negative fractions. Protein solubilized from approximately 10^6 CD23-positive (track 1) and 2 x 10^6 CD23-negative (track 2) cells, were separated on a 7.5% SDS-polyacrylamide gel and immunoblotted with serum RT (1/100). Track 3 shows proteins from a B95-8-derived LCL similarly immunoblotted. EBNAs (E) 1, 2, 3, 4 and 6 and the positions of MW standards (kD) are indicated. Human Ig heavy chain in the CD23-negative fraction is also shown (➤). **B.** Similar immunoblot to A, probed with monoclonal antibodies CS1-4. CD23-positive fraction (track 1); CD23-negative fraction (track 2); and LCL (track 3). The position of LMP, Ig heavy chain (which was bound to the magnetic beads in the CD23-positive fraction and also binds the ^{125}I-protein A probe) and MW standards (kD) are indicated. (Tracks 1 and 2 were exposed to X-ray film for 7 days; track 3 is an overnight exposure).

A



Serum
RT

B



CS1-4

(about 30 copies/cell), no VCA components have ever been detected and there has been no evidence of virus production *in vivo*.

5.2 Antigen Expression in the C15 Tumour

Protein extracts were made from the C15 tumour at various times between passage 18 and passage 25 and examined by immunoblotting for the presence of functions associated with latent infection in B cells, namely EBNAs 1, 2 and the recently characterised EBNAs 3, 4 and 6, as well as LMP. The pattern of antigen expression in the tumour (at passage 18) is highly restricted. Only EBNA 1 (Figure II.23,A, track 3 and B, track2) and LMP (Figure II.24,B, track 2) are detected. This pattern has remained unchanged during continuous passage. C15 virus has been rescued into continuously propagated lymphoblastoid cell lines by cocultivation of tumour-derived cells either with adult lymphocytes from an EBV-seronegative individual or with cord blood lymphocytes (I. Ernberg, personal communication; Fåhraeus *et al.*, 1988). It can be seen that whereas in C15 only EBNA1 is detected, one of the C15 virus-derived LCLs (NAD-C15-STO-B) expresses EBNAs 1, 2, 3, 4 and 6 (Figure II.23,A track 4). Because EBNAs 1 and 2 in the LCL comigrate, the presence of EBNA2 was revealed by immunoblotting with serum WC which (in addition to high antibody titres against lytic antigens) contains antibodies specific for EBNAs 2 and 3 but not EBNA1 (Dillner *et al.*, 1986) (See Figure II.24,A tracks 5 and 6). This blot also confirms that C15 does not express EBNAs 2 and 3 (track 7) or any of the lytic cycle antigens (LA) seen in 2C9 (track 3), B95-8 (track 4) and NAD-C15-STO-B (track 6). (the two bands seen below 67 kD and between 67 and 103 kD are also in CV1 cells and therefore not EBV-specific). Thus, in the C15 tumour, as has recently been found in many NPC biopsies (Young et al,. 1988), only a subset of the B cell associated EBV latent genes appear to be expressed. However, no gross defects exist in the genome that prevent expression, as shown here in NAD-C15-STO-B and by Southern blot analysis of the C15 EBV DNA (Hitt *et al.*, submitted for publication).

Figure II.23: Expression of Latent Proteins in the C15 Tumour. A. Proteins were extracted from: EBV negative Ramos B-cells (track 1); 2C9 LCL (track 2); C15 tumour (track 3); and NAD-C15-STO-B (track 4). They were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with serum RT (1/100). The positions of the EBNA (E) species 1, 2, 3, 4 and 6 are indicated. In 2C9 cells EBNA1 has a MW greater than EBNA2. **B.** Proteins extracted from B95-8 (track 1) and C15 (track 2), separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with monospecific rabbit antibodies reactive with a glycine/alanine repeat peptide in EBNA1. MW standards (kD) are indicated.

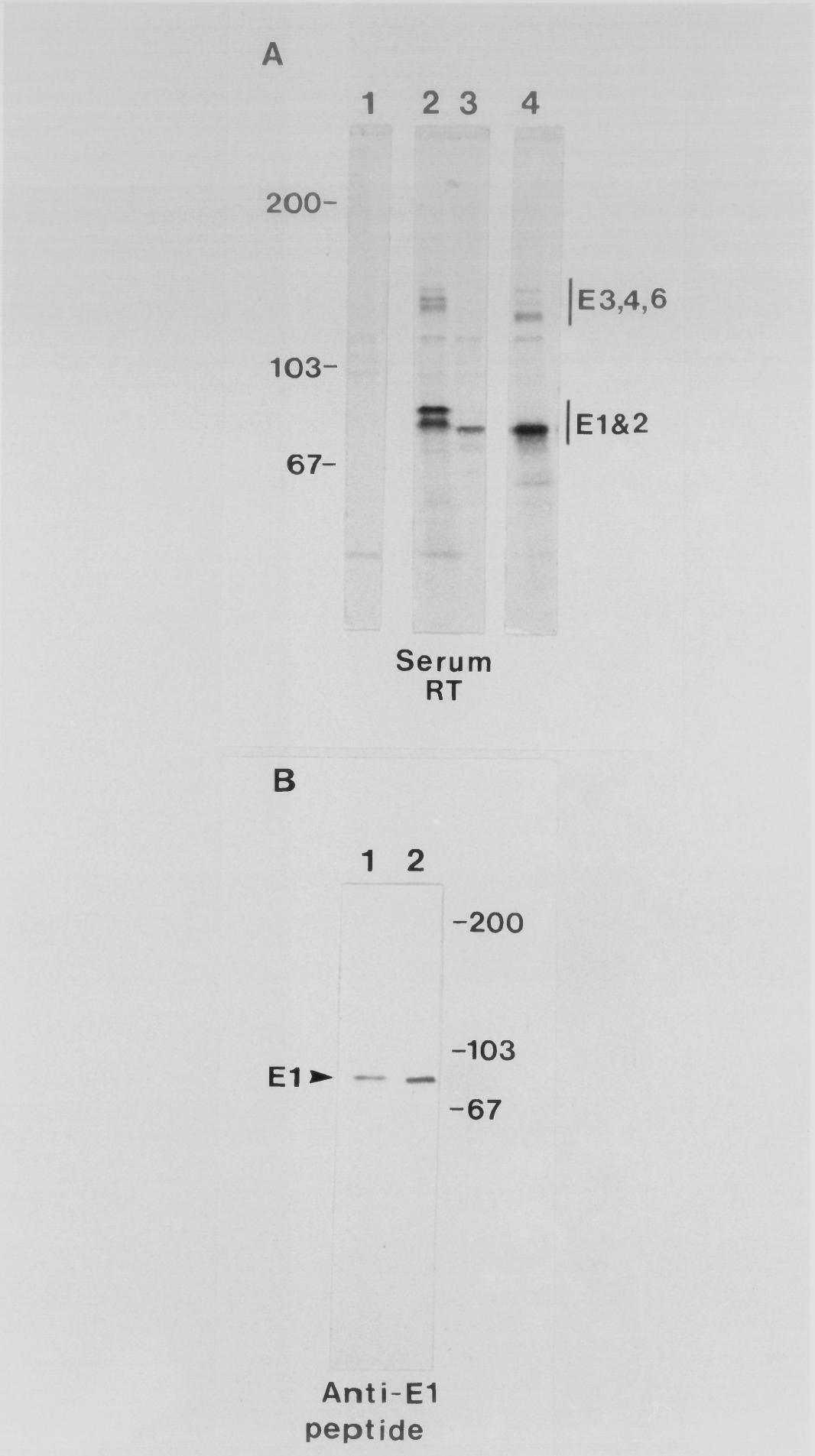
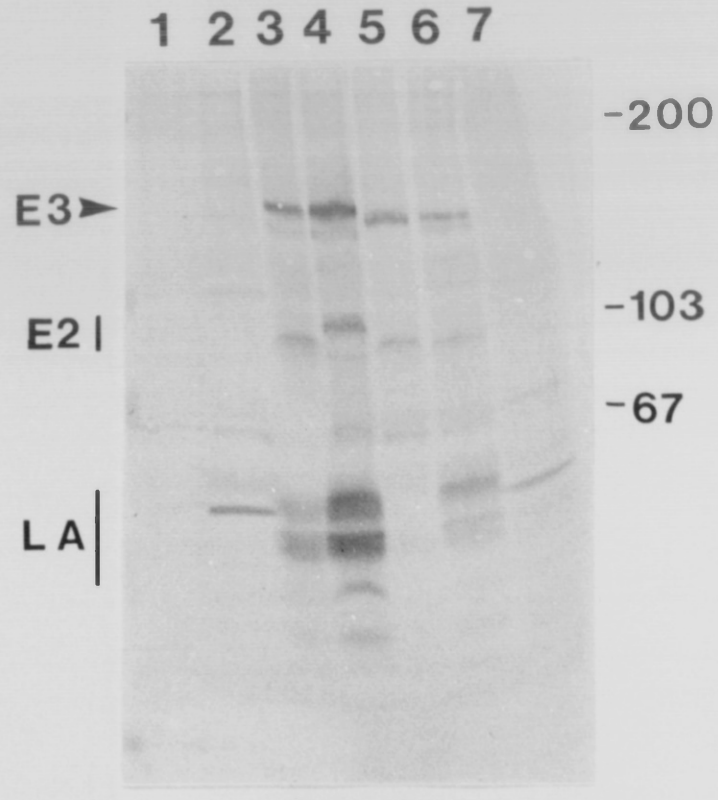

A
1 2 3 4
200-
103-
67-
E3,4,6
E1&2
Serum
RT
B
1 2
-200
-103
-67
E1
Anti-E1
peptide

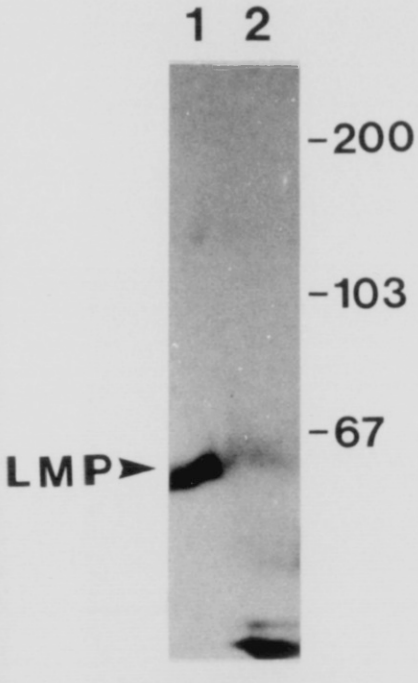
Figure II.24: Expression of Latent Proteins in the C15 Tumour. **A.** Protein extracts were made from: EBV-negative Ramos (track 1); EBV-negative CV1 cells (track 2); 2C9 (track 3); B95-8 (track 4); CBL-C15-STO-B (track 5); NAD-C15-STO-B (track 6) and C15 tumour (track 7). Proteins were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with serum WC (1/100). The positions of EBNAs 2 and 3 are indicated, as are proteins thought to correspond to lytic-cycle antigens (LA). The approximate positions of MW standards (kD) are shown. **B.** Protein extracts from NAD-C15-STO-B (track 1) and C15 tumour (track 2) separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with CS1-4. Locations of LMP and MW standards (kD) are indicated.

A



Serum
WC

B



CS1-4

5.3 Construction of cDNA Libraries

In order to determine the EBV transcription pattern in the C15 tumour a large cDNA library was produced and comprehensively analysed. However, prior to this, the methodology for construction of such a library and the means of assessing its quality were extensively studied.

The frequency at which cDNA clones of a particular mRNA species appear in a cDNA library is generally proportional to the abundance of that species in the mRNA population (Huynh *et al.*, 1985). In order to isolate clones corresponding to rare mRNAs it is necessary to be able to construct a very large library ($>10^5$ recombinants) representative of the complex poly(A)$^+$ RNA population. Phage lambda (λ) vectors take advantage of the high efficiency and reproducibility of *in vitro* packaging of λ DNA for introducing recombinant DNA into *E. coli* and make the generation of a library consisting of $>10^6$ recombinants feasible.

A cDNA library was therefore constructed in λgt10 starting from 5μg of poly(A)$^+$ selected RNA extracted from the EBV-producing marmoset B-cell line, B95-8. This established a protocol for preparing, screening and analysing cDNAs. Details of this protocol are given in the Material and Methods section and are summarised in a flow diagram (Figure II.25). The library of 1.2 x 10^6 individual recombinants obtained was amplified as described (Materials and Methods) and approximately 6 x 10^5 independent clones were then screened with gel purified DNA corresponding to the *Bam*HI E and e_2 fragments of the EBV genome. These should hybridise to cDNAs spanning BERFs 1, 2a and b, 3 and 4, that is the exons probably encoding EBNAs 3, 4 and 6 (see Introduction). 14 positive λgt10 clones were obtained, subcloned into the plasmid vector Bluescribe (Stratagene) and characterised by restriction enzyme cleavage and (after further subcloning into M13mp8) DNA sequence analysis. Although at least 3 productive and one latent transcript hybridize to *Bam*HI e_2 (King *et al.*,1980; P. Farrell, personal communication; MJA, unpublished data) surprisingly no clones mapping to the right hand end of *Bam*HIE or e_2 were identified. This probably reflects interference with cDNA synthesis by the repetitive sequences located in this region (see Figure II.26). Other groups have encountered similar problems with EBV repetitive DNA sequences (Speck and

Figure II.25: Strategies used to Generate cDNA Libraries representing Gene Expression in the B95-8 Marmoset B Cell Line and the C15 Tumour (see text and Materials and Methods for details)

cDNA Cloning Strategies

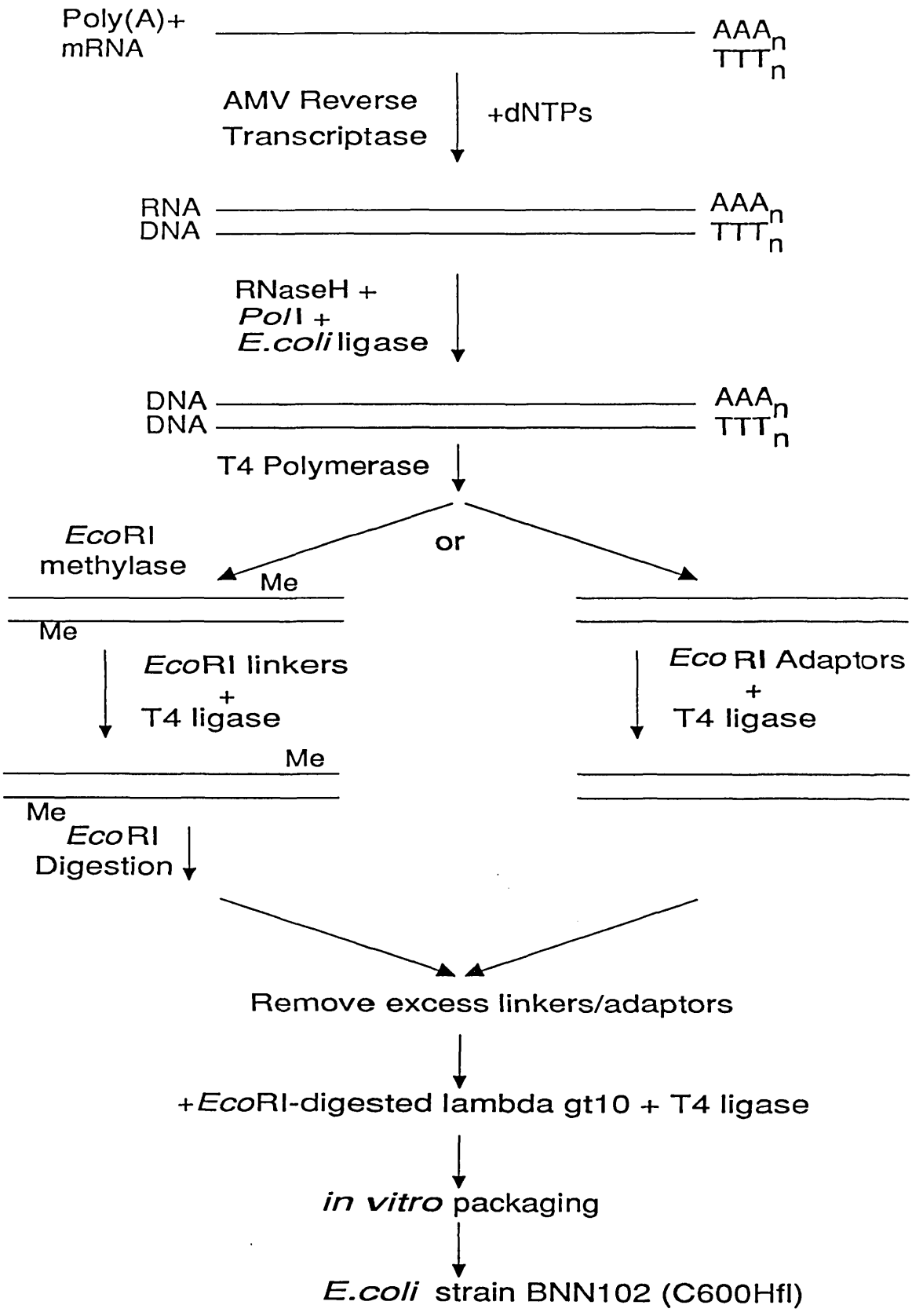
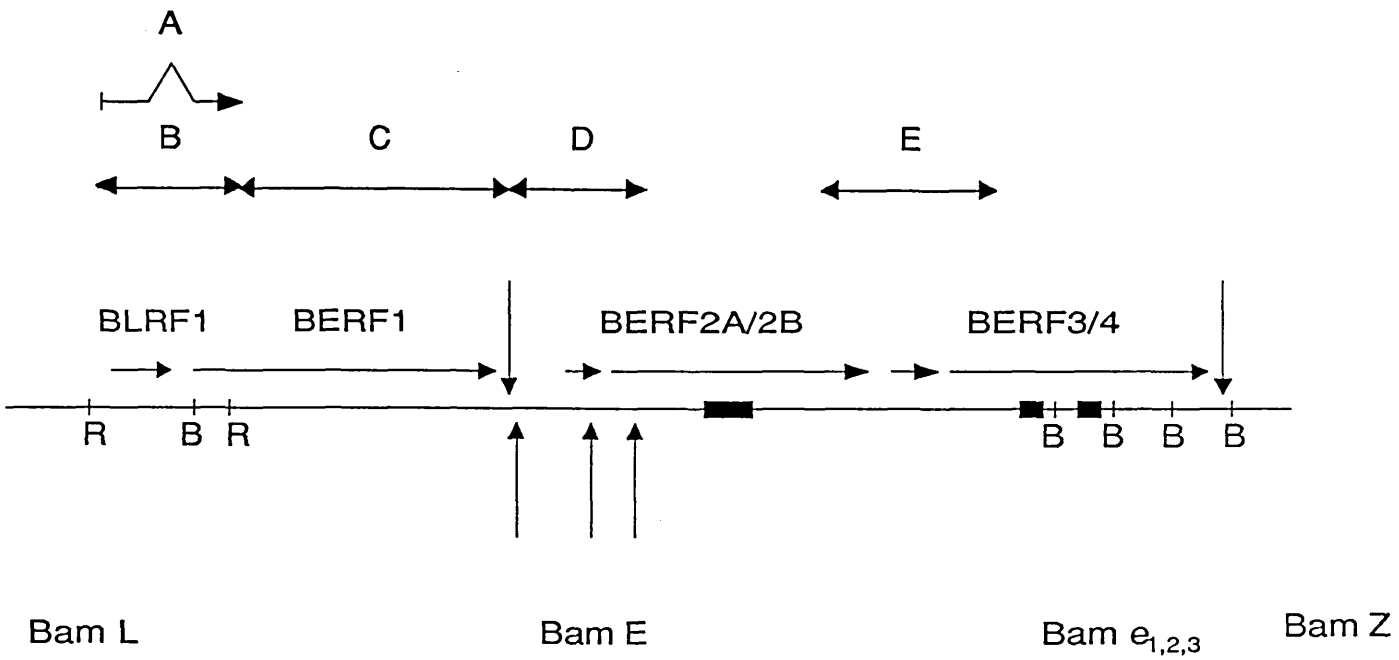


Figure II.26: BamHI E-positive Clones from a B95-8 cDNA Library. Physical map showing the BamHI L-Z region of the EBV genome and the regions corresponding to the 5 different types of clone (A-E) which were recovered from the B95-8 cDNA library. Vertical arrows indicate the position of predicted poly(A) addition sites; ▬ indicates the location of repetitive DNA sequences. The six predicted open reading frames encoding EBNA3, EBNA4 and EBNA6 (that is, BLRF3 and BERF1, 2a, 2b, 3 and 4; Baer *et al.*, 1984) are shown. Clone A includes an in-frame splice from BLRF to BERF1 and probably represents a truncated cDNA from an EBNA3 transcript (see text and Figure II.27).


A
B
C
D
E
BLRF1
BERF1
BERF2A/2B
BERF3/4
R
B
R
B
B
B
B
Bam L
Bam E
Bam e1,2,3
Bam Z

Strominger, 1985; J. Sample and M. Perricaudet, personal communications). The positive clones identified were mapped on the EBV genome by determining the DNA sequence of both their 5' and 3' ends. The clones were found to fall into 5 groups (see Figure II.26) and after restriction enzyme mapping, 13 of them (in 4 groups) were shown to represent cDNA fragments colinear with genomic DNA (that is, they were unspliced). One clone (of 1020 bp) was, however, spliced and identified as corresponding to a putative EBNA3 transcript (Bodescot *et al.*, 1986; Joab *et al.*, 1987 and Figure II.27). This clone does not, however, represent a full length mRNA; during the cloning *Eco*RI cleavage of the cDNA appears to have removed the 5' end of the transcript, despite *Eco*RI specific methylation. It is interesting that the clone has the same splice junction linking BLRF3 to BERF1 as the cDNA clone (T2) reported by Bodescot *et al.* (1986) but a different splice pattern 5' to BLRF3. The T2 clone splices from the *Bam*HI L region to *Bam*HI W using a splice acceptor site 5 bases upstream from the BLRF3 ATG initiation codon. However, in the clone reported here such a splice must occur 5' to the *Eco*RI site at which the cDNA was truncated. This indicates that alternative splice patterns can generate messages which have the coding potential for EBNA3.

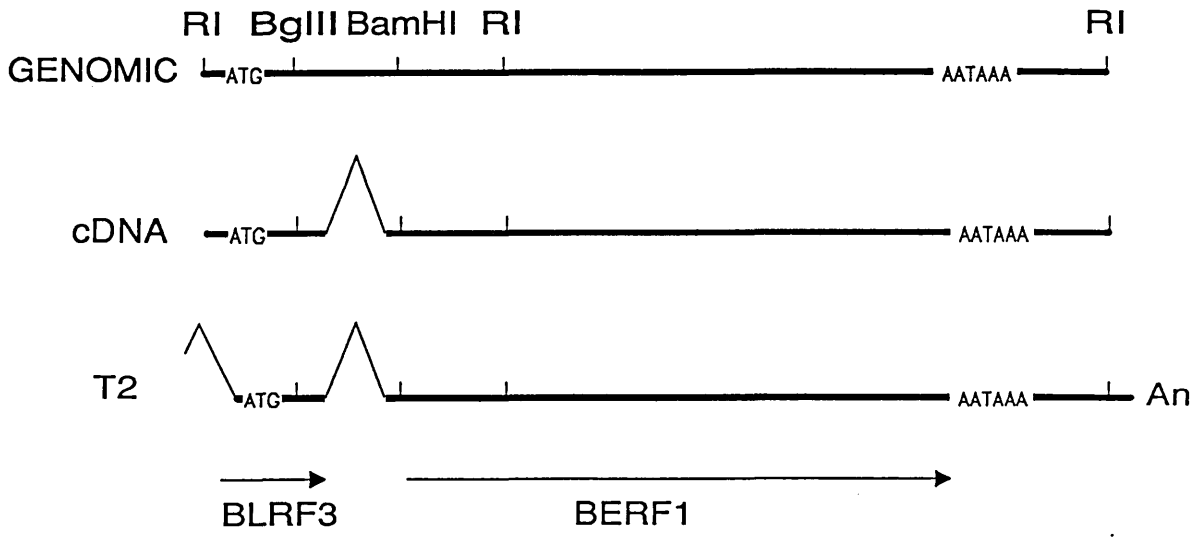
5.4 Preparation and Analysis of the C15 cDNA Library

The basic protocol for cDNA cloning, established in the preparation of the B95-8 library, was modified for the production of a library from the C15 tumour RNA. Notably *Eco*RI "linkers" (which necessitate methylation of the cDNA and *Eco*RI digestion prior to ligation to λgt10) were replaced by *Eco*RI "adaptors" (see Figure II.28). This removed the risk of truncating cDNAs by cleaving them at internal *Eco*RI sites.

A cDNA library consisting of 3-4 x 10^6 individual recombinant clones was constructed from approximately 5 μg of total poly(A)$^+$ RNA extracted from C15 (Hitt *et al.*, submitted for publication). Analysis of the B95-8 library had shown that screening 6 x 10^5 clones detected a clone corresponding to a low abundance latent transcript (<5 copies/cell) which was consistent with the experiences of others (Speck and Strominger, 1985; Bodescot *et al.*, 1986; Sample *et al.*, 1986) who have reported similar very rare EBNA-associated clones obtained from libraries of about

Figure II.27: Putative EBNA3 cDNA Clone. **A.** The structure of a clone recovered from the B95-8 cDNA library which, by restriction enzyme mapping and DNA sequence analysis, was shown to include an in-frame splice from BLRF3 to BERF1 is compared with the region of EBV genomic DNA to which it corresponds and the T2 cDNA clone of Bodescot *et al.*, (1986). **B.** DNA sequence showing the splice junction (▼) and predicted protein sequence. These data were generated by subcloning into M13 and sequencing the *Bam*HI/*Eco*RI subfragment containing the splice junction.

A


RI
BglII
BamHI
RI
RI
GENOMIC
ATG
AATAAA
cDNA
ATG
AATAAA
T2
ATG
AATAAA
An
BLRF3
BERF1

B

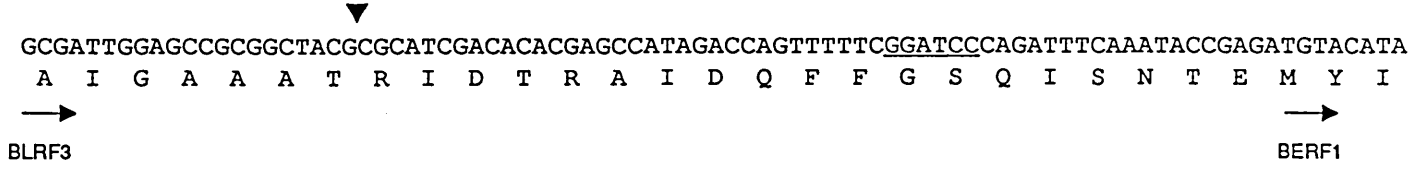

GCGATTGGAGCCGCGGCTACGCGCATCGACACACGAGCCATAGACCAGTTTTTCGGATCCCAGATTTCAAATACCGAGATGTACATA
A I G A A A T R I D T R A I D Q F F G S Q I S N T E M Y I
BLRF3
BERF1

Figure II.28: ***Eco*RI Oligodeoxynucleotide Adaptors used in C15 cDNA Cloning.**

17-mer 5′-AATTCCTCGAGAGTTGC-3′

13-mer 5′-GCAACTCTCGAGG-3′

13-mer kinased and oligodeoxynucleotides annealed:

```
5′ HO-     AATTCCTCGAGAGTTGC-OH 3′
3′ HO-         GGAGCTCTCAACG-P  5′
```

*Eco*RI *Xho*I site
cohesive
end

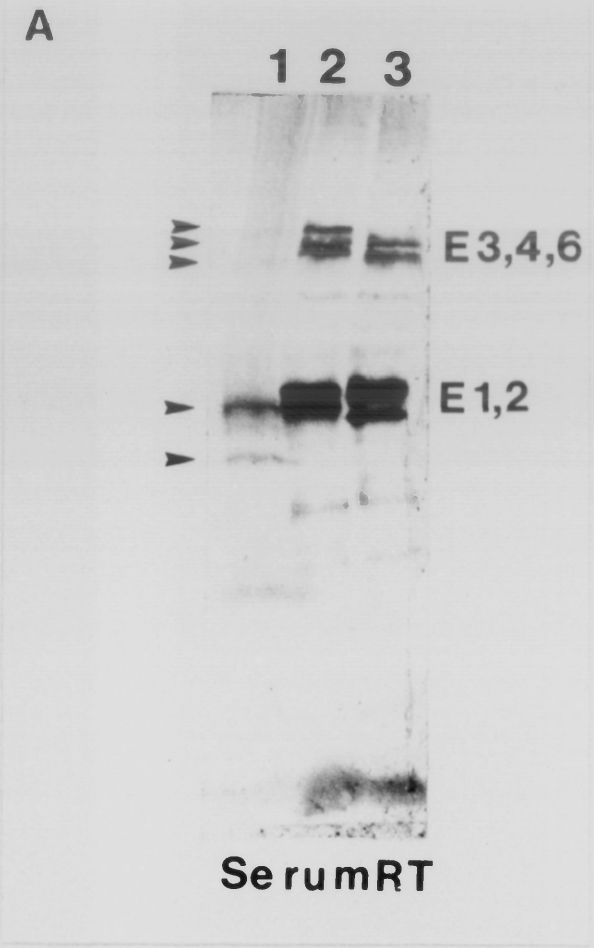
10^6 recombinants. By this criterion the C15 library was judged large enough to be totally representative of the RNA population from which it was derived. It was probed in detail by screening a minimum of 10^6 independent clones from the primary, as well as the amplified library (see Materials and Methods) with individual EBV DNA fragments. The results, summarised in the discussion section (Figure III.3) and presented in detail elsewhere (Hitt *et al.*, submitted for publication) reflect a highly restricted pattern of transcription in the C15 tumour. With respect to EBNA expression, screening 10^6 clones from the unamplified library and a further 10^6 after amplification with *Bam*HI fragments C, W, E, $e_{1,2,3}$ and K yielded several recombinants hybridizing to *Bam*HI K DNA, that is, corresponding to EBNA1 transcripts, but none to the other EBNA-encoding regions. These data are completely consistent with the immunoblot analysis of the latent antigens in C15 which revealed only EBNA1 (and LMP) expression.

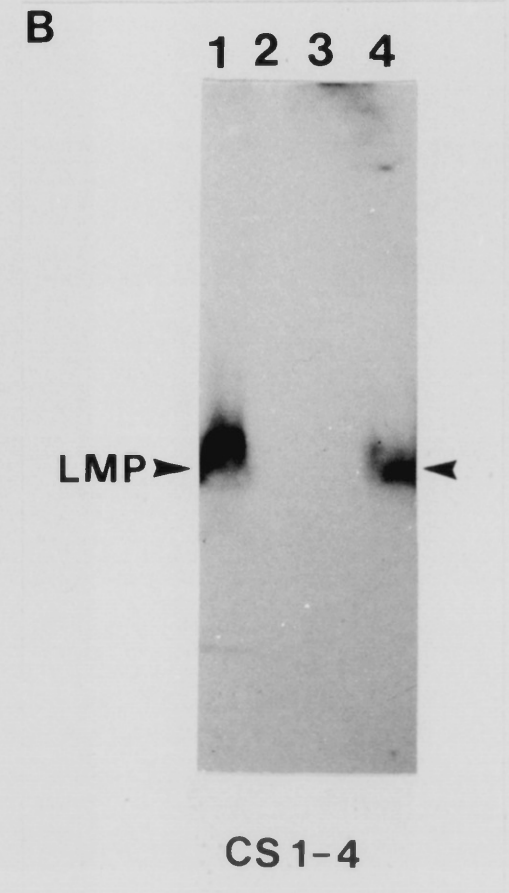
6 LATENT GENE EXPRESSION IN OTHER EBV ASSOCIATED TUMOURS

6.1 Transplantation lymphomas

Lymphoproliferative lesions from 5 patients, 3 of whom had received renal and 2 who had received heart/lung transplants were examined for EBV latent gene expression. The clinical data from these patients, their EBV serology and the results of a detailed immunocytochemical study on the lesions are presented elsewhere (Thomas *et al.*, 1989). All the specimens were also examined by immunoblotting with EBV specific sera and some with anti-LMP monoclonal antibodies. However, due to the age and/or size of the specimens only one biopsy (from a diffuse undifferentiated large cell lymphoma in a renal transplant recipient receiving cyclosporin A and azathioprin; Thomas *et al.*, 1989) produced blots which were sufficiently distinct for meaningful interpretation (Figure II.29). In confirmation of the immunocytochemical demonstration of multiple EBNA components, bands corresponding to EBNAs 1 and 2 were revealed by serum RT (A, track 1). Faint but detectable bands consistent with high molecular weight EBNAs 3, 4 and 6 were additionally observed (compare tracks 1, 2 and 3). A similar immunoblot probed

Figure II.29: Expression of Latent Proteins in a Transplant-associated Tumour (Diffuse Undifferentiated Large Cell Lymphoma). **A.** Protein extracts were made from: tumour tissue (track 1); 2C9 LCL (track 2); IB4 LCL (track 3), separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with serum RT (1/100). The positions of the putative EBNA (E) species in the tumour are indicated (➤). **B.** Protein extracts from P3HR1 (track 1), LMP-negative BL cell lines AW Ramos (track 2), BL41 (track 3) and tumour tissue (track 4), separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with CS1-4. The position of LMP is indicated.


A
1 2 3
E3,4,6
E1,2
SerumRT


B
1 2 3 4
LMP
CS 1-4

with monoclonal antibodies CS1-4 also revealed LMP expression in this lesion (B, track 4). The pattern of latent expression is therefore reminiscent of that seen in LCLs.

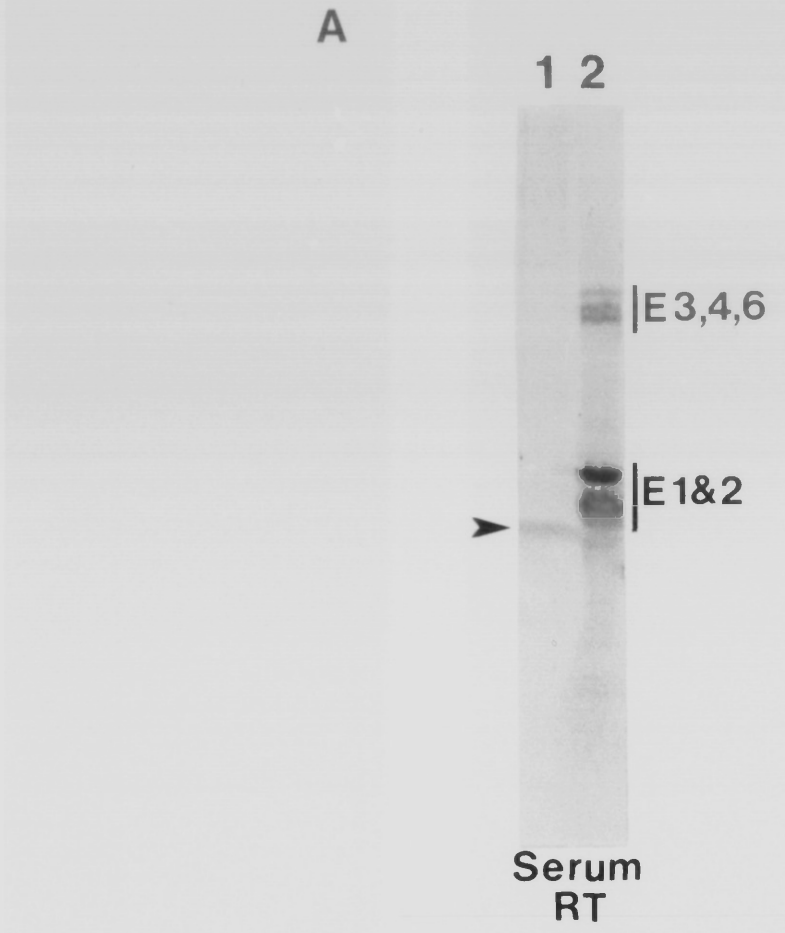
6.2 EBNA 1 Expression in an Early-Passage Endemic BL Cell Line

A cell line (BLMH) was established from a Burkitt's lymphoma needle-biopsy specimen (transported) from Malawi and found to be EBNA-positive by ACIF (D. Kundu, personal communication); histochemistry (S.Lucas, personal communication) confirmed the BL status of this biopsy. Prior to extensive passaging (<20) protein was extracted with SDS-sample buffer and analysed for EBNA and LMP expression by immunoblotting . The results revealed only a single EBV-associated protein band in BLMH; this probably corresponds to EBNA1 (Figure II.30A, track 1) and is consistent with previous reports that in eBL biopsies and early passage eBL cell lines only EBNA 1 is expressed (Rowe *et al.*, 1986; Rowe *et al.*, 1987). LMP was not detected (Figure II.30B, track 1).

6.3 EBNA 1 Expression in an AIDS-Associated Non-Hodgkin's Lymphoma

An autopsy specimen of a non-Hodgkin's large cell lymphoma from a 60-year-old haemophiliac suffering from AIDS (G. Bird. personal communication) was examined for EBNA and LMP expression. The tumour was shown to be EBNA positive by immunocytochemistry (J. A. Thomas, personal communication). Proteins were extracted with RIPA buffer, totally solubilised in SDS-sample buffer and separated on a 7.5% SDS-polyacrylamide gel. After transfer to nitrocellulose the blot was probed with serum RT and anti-LMP monoclonal antibodies CS1-4. Only a single band, which probably corresponds to EBNA 1 was detected with RT (Figure II.31,A, track 2). No bands were revealed with CS1-4 (Figure II.31,B, track 2). A similar blot probed with an EBNA2B virus-specific serum also produced only a single (EBNA1) band (not shown). This pattern of latent expression is similar to that found in endemic Burkitt's lymphoma biopsies (Rowe *et al.*, 1986; Rowe *et al.*, 1987 and previous section). (In this respect, it should be noted that the HIV positive patient in this study was not grossly immunocompromised; his peripheral blood $CD4^+$ T cell count, 0.4 x $10^3/mm^3$ [normal range: 0.5-1.2 x $10^3/mm^3$], was not particularly

Figure II.30: Expression of Latent Proteins in BLMH, an Early Passage Burkitt's Lymphoma Cell Line. Protein extracts from 10^6 BLMH cells (track 1) and the LCL 2C9 cells (track 2) were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with **A.** Serum RT (1/100) and **B.** CS1-4. The apparent EBNA1 protein in BLMH is indicated [A, track 1 (➤)]. After probing, blot **B** was exposed to X-ray film for 4 days; an even longer (10 day) exposure (not shown) also failed to reveal an LMP band in BLMH.


A
1 2
E3,4,6
E1&2
Serum
RT

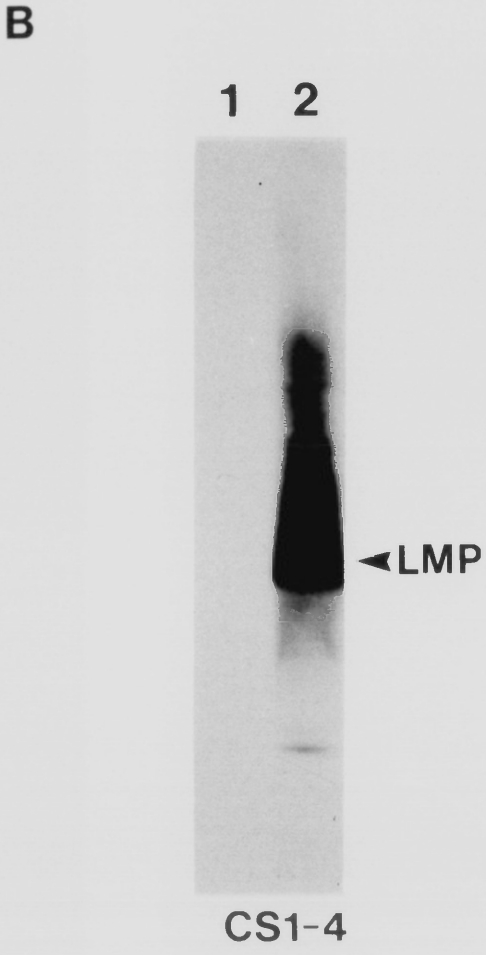
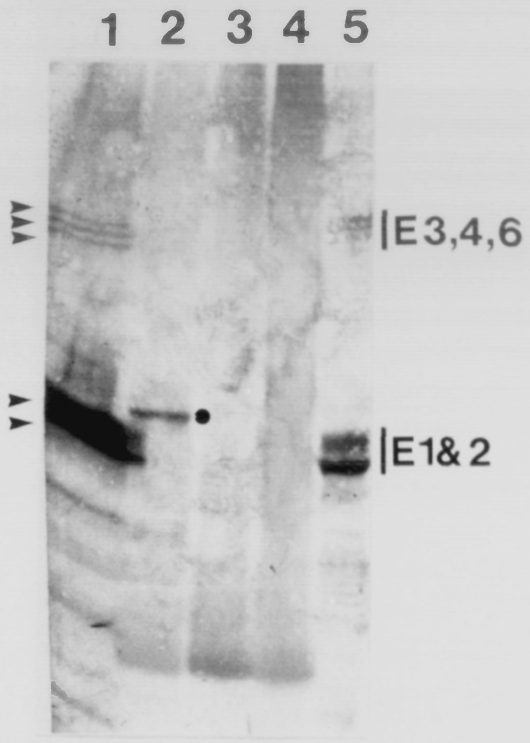

B
1 2
LMP
CS1-4

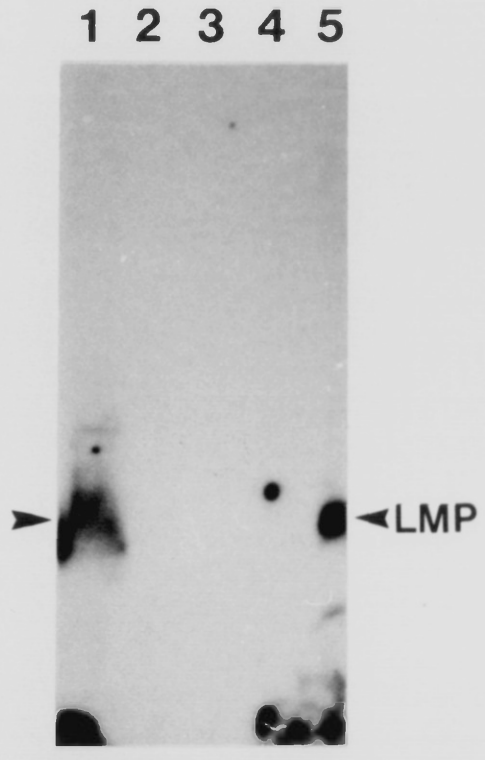
Figure II.31: Expression of Latent Proteins in an AIDS-Associated Non-Hodgkin's Lymphoma and a Tamarin Lymphoma (B124) Protein extracts were made from: Tamarin tumour (track 1); AIDS-lymphoma (track 2); non-tumourous tissue from the AIDS patient (tracks 3 and 4) and B95-8 (track 5). They were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with **A**, serum RT (1/100) and **B**, CS1-4. Latent proteins [EBNAs (E) 1, 2, 3, 4 and 6 and LMP] expressed in the Tamarin tumour are indicated (➤) as is the putative EBNA1 protein in the AIDS-lymphoma (•). After probing, blot **B** was exposed to X-ray film for 2 days. A longer exposure (7 days) also failed to reveal any LMP protein in the AIDS tumour (not shown).

A



Serum RT

B



CS 1-4

low for someone with established AIDS. Also, the very rapid growth rate of the tumour was typical of a Burkitt's lymphoma; G. Bird, personal communication.) The absence of the cellular antigen CD23 from these tumour cells (J. A. Thomas, personal communication) is consistent with a highly restricted, BL-like, pattern of EBV latent gene expression (Rowe *et al.*, 1987; Calender *et al.*, 1987; Wang *et al.*, 1988). No EBV related antigens were detected in protein extracts made from a lymph node and other non-tumourous tissue from the same patient (Figure II.31,A and B, tracks 3 and 4).

6.4 LCL-Like Expression in a Lymphoma from a Cotton-Top Tamarin

Tumours were induced in a young adult cotton top tamarin (*Sanguinus oedipus oedipus*) by intraperitoneal inoculation with a standard 100% tumourigenic dose of B95-8 virus (S. Finerty, personal communication).

A sample of a mesenteric lymph node tumour (B124) was teased into a cell suspension and immediately frozen (with 10% DMSO) (S. Finerty, personal communication). After thawing the cells and washing twice with PBS the proteins were solubilised with SDS-sample buffer, separated by SDS-PAGE and immunoblotted with serum RT and CS1-4. This revealed a pattern of expression identical to the LCL-like pattern in the marmoset cell line, B95-8 (compare Figure II.31, A and B, tracks 1 and 4).

7 PATTERNS OF EBNA EXPRESSION AND CpG METHYLATION OF VIRAL DNA IN EBV-ASSOCIATED TUMOURS

The experiments reported in sections 4, 5 and 6 suggested that patterns of EBNA protein expression in cell lines and tumours fall into two major categories and that the differences probably reflect patterns of transcription. There are those cells in which EBNAs 1, 2 and all (or most) of the other EBNA species are synthesised and are readily detectable. This type is exemplified by the LCLs established from human cells with the B95-8 strain of virus; it is also found in Burkitt's lymphoma cell lines, such as Raji, which have been passaged in culture for many years and in B cell lymphomas from cotton top tamarins and certain immunocompromised humans

Table II.3 Inverse Correlation between EBNA Expression and CpG Methylation in Various Cell Lines and EBV Associated Tumours

Antigen*	Raji	LCLs[1,2] (C15 & Maja)	C15[1,2] NPC Tumour	Group 1 BL-lines (BLMH & Eli[3])	Cotton Top Tamarin lymphoma	Transplant[4] lymphoma	AIDS-related Burkitt's-like lymphoma
EBNA 1	+	+	+	+	+	+	+
EBNA 2	+	+	-	-	+	+	-
EBNA 3	+	+	-	-	+	+	-
EBNA 4	+	+	-	-	+	ND	-
EBNA 5	ND	+	-	ND	+	ND	-
EBNA 6	-	+	-	-	+	ND	-
LMP	+	+	+	-	+	ND	-
CD23#	+	+	ND	ND	ND	+	-[5]
CpG Methylation in BamHI W	-	-	+	+	-	-	+

* Data presented is from immunoblotting, immunohistochemical and immunofluorescence analyses from this and previous studies: [1] Hitt *et al.*, submitted; [2] Fahraeus *et al.*, 1988; [3] Rowe *et al.*, 1987; [4] Thomas *et al.*, 1989; [5] J.A. Thomas, personal communication.

CD23 is a B-cell activation antigen whose expression is associated with EBNA2 (Wang *et al.*, 1987).
ND - Not done

(see Table II.3). In Burkitt's lymphoma biopsies and early passage BL cell lines and many NPCs, on the other hand, only EBNA 1 can be detected. However, it is not clear from these experiments, or from published data, what restricts EBNA expression in the tumour cells. This problem has therefore been approached by considering whether the DNA sequences controlling EBNA expression are chemically modified. Specifically [as the transcriptional regulatory region(s) for EBNAs 3, 4 and 6 have not been definitively identified], the pattern of CpG methylation in the 5' regulatory region of the EBNA 2 gene (Ricksten *et al.*, 1988) was analysed. This, and the surrounding sequences in *Bam*HI W are particularly rich in CpG dinucleotides (Figure II.32). Therefore total DNAs extracted from various tumours and cell lines were digested with the methylation sensitive endonuclease *Hpa*II and, as a control, its isoschizomer *Msp*I, which cleaves methylated sites. Southern blotting and probing these DNAs with the *Bam*HI W fragment of EBV revealed a tight inverse correlation between methylation and EBNA(2-6) expression. Several such blots are shown in Figure II.33 and the data are summarised in Table II.3.

None of the DNAs from the NPC-derived tumour (C15) (Figure II.33,A, track 1 and B, track 2), a stable group 1 BL cell line, Eli (Rowe *et al.*, 1987) (A, track 3) nor the early passage BL, BLMH (B, track 5) were completely cleaved by *Hpa*II - indicating methylation of CpG sites within the W repeats. Analysis of DNA from the BL-like tumour from an AIDs patient revealed a similar pattern of methylation (C, track 5). On the other hand, DNAs from an LCL established with C15 virus (A track 2 and B, track 1), and LCL established with B95-8 virus (C, track 1), Raji (B track 6), the tamarin lymphoma, B124 (C track 3) and a transplant-associated lymphoma (C, track 4) - all of which express EBNA2 - were cleaved at most (or possibly all) the *Hpa*II sites within *Bam*HI W. These results are consistent with the hypothesis that methylation of certain sites in the regulatory region interferes with the expression of EBNA2. The viral DNAs were completely digested by *Msp*I (A tracks, 4, 5, 6; B tracks, 3, 4, 7, 8; C6). Furthermore, the data presented here are consistent with those from another group who also showed that demethylation induced by the drug 5' azacytidine activated expression of silent EBNA genes in a stable group 1 BL cell line (I. Ernberg, personal communication).

Figure II.32: Physical Map of the BamHI W - Large Internal Repeat Region of the B95-8 EBV Genome. A. Vertical lines indicate the distribution of all the CpG dinucleotides, as well as the more limited CpGs within *Hpa*II (and *Msp*I) recognition sites. The *Bam*HI fragment used as a probe in Fig. II.33 is indicated as well as the major (558 bp) fragment that could be obtained upon complete *Hpa*II/*Msp*I digestion. **B.** The putative 5' flanking regulatory region of the EBNA2 gene showing the transcription initiation site (↳), recognition signals and the location of the site at which a cellular protein binds ▦ (Ricksten *et al.*, 1988). CpG dinucleotides and *Hpa*II/*Msp*I recognition sites are indicated.

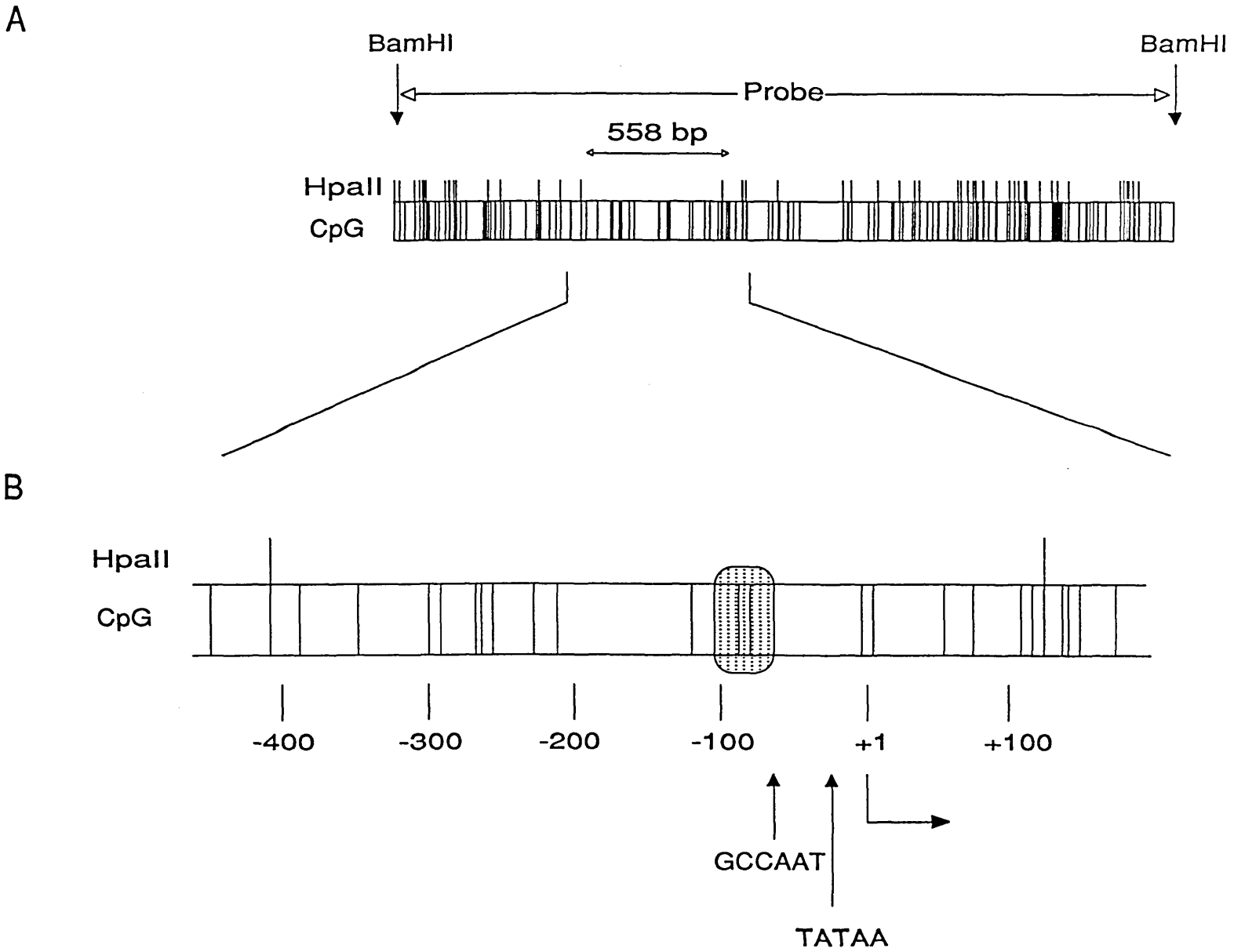
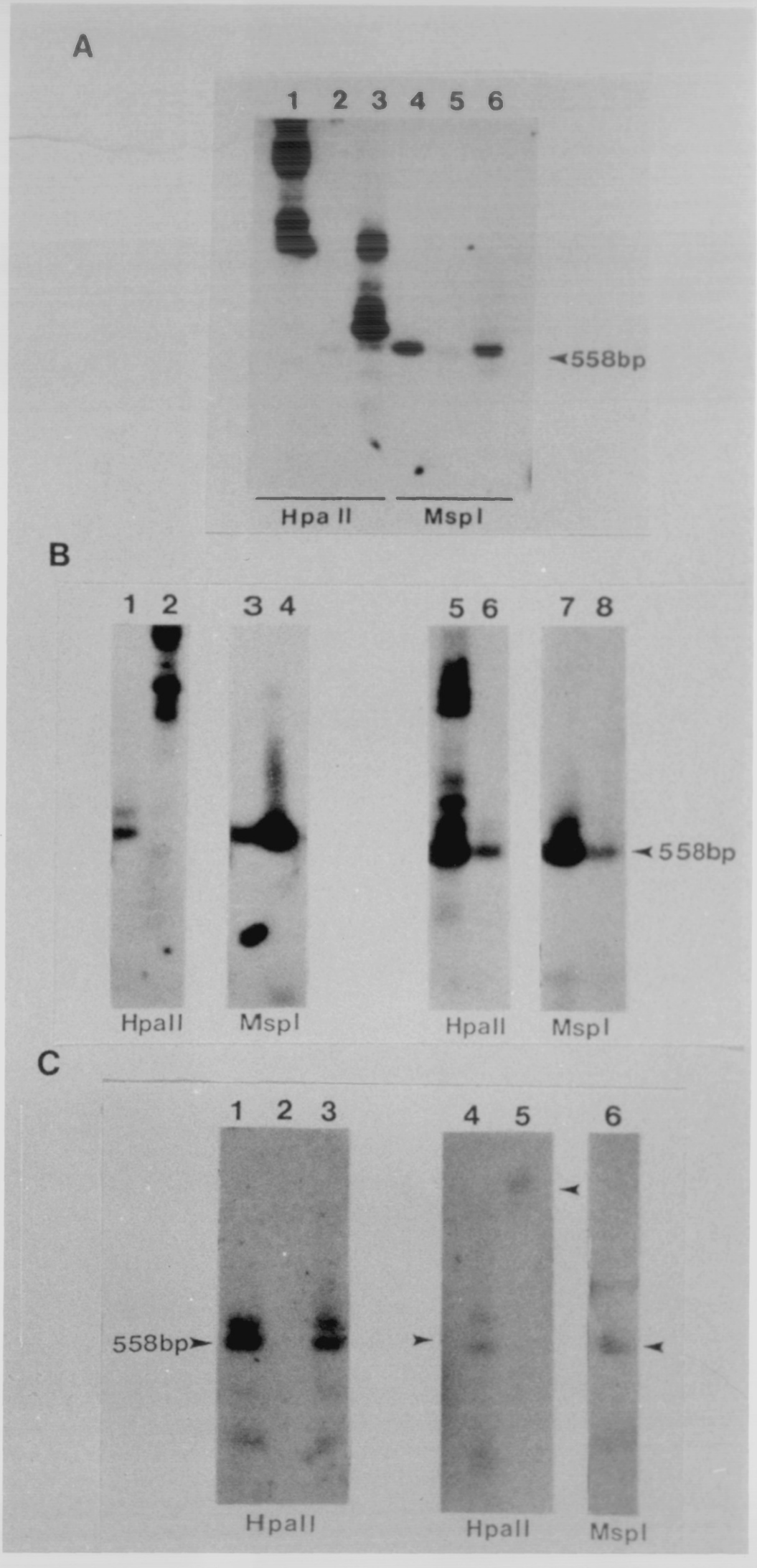

A
BamHI
BamHI
Probe
558 bp
HpaII
CpG
B
HpaII
CpG
-400
-300
-200
-100
+1
+100
GCCAAT
TATAA

Figure II.33: CpG Methylation in the BamHI W Region. Southern blots of total cellular DNA digested with either *Hpa*II (which does not cleave methylated CpG) or *Msp*I (which cleaves methylated CpG), as indicated. Each blot was probed with nick translated EBV *Bam*HI W DNA. DNA extraction, blotting and hybridization was performed according to standard protocols. **A.** C15 (tracks 1 and 4); C15-LCL (tracks 2 and 5); Eli BL (tracks 3 and 6). **B.** C15 LCL (tracks 1 and 3); C15 (tracks 2 and 4); BLMH (tracks 5 and 7); Raji (tracks 6 and 8). **C.** Maja LCL (track 1); EBV-negative tissue (track 2); tamarin lymphoma, B124 (track 3); transplant lymphoma (track 4); AIDS lymphoma (tracks 5 and 6). DNA from the C15 virus and also Eli BL virus have a polymorphism in *Bam*HI W which results in the major product of complete HpaII/*Msp*I digestion being slightly larger than the 558 bp fragment from B95-8 (Baer *et al.*, 1984). The submolar band appearing just above this in tracks B1 & 6 and C 1, 3 & 4 is a fragment extending from within the last W repeat into *Bam*HI Y.


A
1 2 3 4 5 6
558bp
Hpa II
Msp I
B
1 2 3 4 5 6 7 8
558bp
HpaII
MspI
HpaII
MspI
C
1 2 3 4 5 6
558bp
HpaII
HpaII
MspI

III DISCUSSION

1 EBNA6

It has been demonstrated here that the polypeptide encoded by the EBV BERF4 open reading frame is a nuclear protein which is synthesised in the tightly latent LCL, IB4, and early after EB virus infection of resting B cells. Numerous other EBV-containing LCLs and some eBL cell lines also express the antigen. It is, however, absent from the EBV-negative sBL lines Ramos and EB4 and is only recognised by EBV-positive sera. By all accepted criteria (see Kallin *et al.*, 1986) this identifies the protein as an "EBNA"; it has been designated EBNA6. The absence of EBNA6 from the Raji BL cell line is consistent with the fact that the EBV genome in Raji has a deletion that removes most of the BERF4 open reading frame (Polack *et al.*, 1984; Hatfull *et al.*, 1988; Figure I.1). During the course of this study three other groups have arrived at essentially similar conclusions (Ricksten *et al.*, 1988; Petti *et al.*, 1988; Shimizu *et al.*,1988); the analysis of cDNA clones has also shown that the full length EBNA6 protein is probably translated from an mRNA spliced in very similar manner to the other EBNA species. A very long primary transcript is processed to produce a mature message with a complex 5' leader sequence comprised of exons from W, Y and U regions of the genome (see Figure I.3); the polypeptide is encoded by an open reading frame created by splicing from BERF3 into BERF4 (Bodescot *et al.*, 1986; Petti *et al.*, 1988; Figure III.1).

Attempts to establish cell lines stably expressing EBNA6 have been unsuccessful in two different laboratories (Section II.3.3; L. Rymo, personal communication). These results are reminiscent of the effect which SV40 small T-antigen (ST) had on cells when it was introduced by the cotransfection of multicopy plasmids encoding the protein under the transcriptional control of a strong promoter. As with EBNA6, the cells did not proliferate to establish stable drug resistant colonies/lines (Lemieux, 1986). This problem may possibly be overcome, as it was with ST, by introducing a single copy of the gene into cells in a retrovirus vector (Noda *et al.*, 1986). In this respect, it may be significant that the only group to produce stable EBNA6 expressing cells utilized a retroviral vector and the level of protein synthesised was barely detectable by either IF or immunoblotting (Petti *et al.*, 1988). This very low level of expression apparently had no detectable effect on the growth properties of rodent cells.

Clues to the function of EBNA6 and the reasons for its apparent cytotoxicity (when synthesised in an unregulated manner) come from a comparison of its predicted amino acid sequence and tertiary structure with that of a number of known nuclear oncoproteins and transcriptional activators. The amino acid sequence (deduced from genomic and cDNA sequences; Figure III.1.A) includes a number of interesting features. There are two major repeating domains (GPPAA and PQAPYQGYQEPPA; Figure III.1.A). Variation in these (similar to findings in other EBNA species) probably accounts for the slight size polymorphism exhibited by EBNA6 (see Figure II.6A). The larger repeat element contains three glutamine (Q) residues and is arranged as three tandem copies flanked in the polypeptide by sequences also rich in glutamine. This pattern is reminiscent of the "glutamine-rich domain" identified as playing an important role in transcriptional activation by the transcription factor Sp1 (Kadonaga *et al.*, 1987; Courey and Tjian, 1988). Similar domains have also recently been identified in members of the POU family (*P*it-1, *O*ctomer-binding proteins and *u*nc-86) of mammalian transcription factors (for example: OTF-2/Oct-2, Muller *et al.*, 1988; Sheidereit *et al.*, 1988; Clerc *et al.*, 1988; and OTF-1/Oct-1, Sturm and Herr, 1988; reviewed by Robertson, 1988). The precise function of this glutamine-rich domain is unclear but it has been suggested that it may mediate in factor binding to RNA polymerase (Courey and Tjian, 1988).

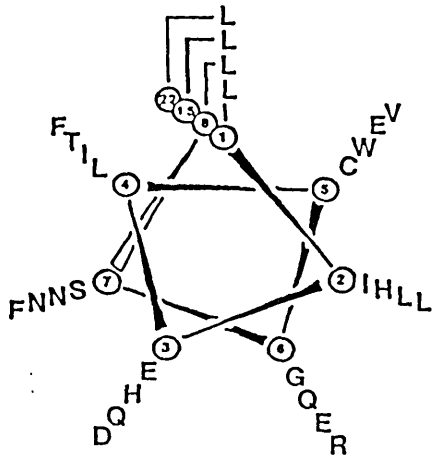
A second potentially significant feature in the EBNA6 polypeptide is a "leucine zipper" motif (Figure III.1.A and B), similar to that identified in a number of proteins again associated with the regulation of gene expression. These include the transcriptional regulators GCN4 (from yeast) and C/EBP (from rat liver cells), the nuclear oncoproteins Jun/API, Fos and Myc (Landschulz *et al.*, 1988) and the POU factors listed above. The essential features of the "leucine zipper" are four to five leucines (the fifth may be replaced by methionine) at every seventh position which, in an idealised helix, extend from one side of the helix and interdigitate with leucine side chains extending from the helix of another polypeptide. It has been suggested that in this way the "zipper" could facilitate either the formation of homodimers or heterodimeric complexes. The opposite side from the hydrophobic leucine repeat in the hypothetical helix is composed of residues with charged side chains (for instance, lysines, arginines, aspartic acids and glutamic acids) or

Figure III.1: The EBNA 6 Polypeptide. A. Amino acid sequence as predicted from cDNA and genomic DNA sequences (Petti *et al.*, 1988; Baer *et al.*, 1984)) The position of the splice from BERF3 to BERF4 is shown ▼ . The putative DNA-binding domain (amino acids 235-262) is indicated by the charged residues and the leucines of the "leucine zipper" (amino acids 263-284) are printed bold and underlined (it should be noted that in the "fifth" position in EBNA6 - amino acid 291 - is a methionine). Continuous underlining indicates the repetitive sequences and double underlining the KRPR putative nuclear localization signal. The start of the e_2-β-gal fusion polypeptide is shown ⟶ [amino acid 826]. B. "Helical wheel" analysis of the "leucine zipper" region of EBNA6 (based on Landschulz *et al.*, 1988; M. Ginsburg, ICRF, personal communication). The amino acid sequence is displayed end-to-end down the axis of a schematic α-helix. The helical wheel consists of seven "spokes", corresponding to the fit of seven amino acid residues into every two α-helical turns. The amino acid abbreviations are as follows: A, alanine; C; cysteine; D, aspartic acid; E; glutamic acid; F; phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

A

```
MESFEGQGDS RQSPDNERGD NVQTTGEHDQ DPGPGPPSSG ASERLVPEES  50
YSRDQQPWGQ SRGDENRGWM QRIRRRRRRR AALSGHLLDT EDNVPPWLPP 100
HDITPYTARN IRDAACRAVK QSHLQALSNL ILDSGLDTQH ILCFVMAARQ 150
RLQDIRRGPL VAEGGVGWRH WLLTSPSQSW PMGYRTATLR TLTPVPNRVG 200
ADSIMLTATF GCQNAARTLN TFSATVWTPP HAGPREQERY AREAEVRFLR 250
GKWQRRYRRI YDLIELCGSL HHIWQNLLQT EENLLDFVRF MGVMSSCNNP 300
AVNYWFHKTI GNFKPYYPWN APPNENPYHA RRGIKEHVIQ NAFRKAQIQG 350
LSMLATGGEP RGDATSETSS DEDTGRQGSD VELESSDDEL PYIDPNMEPV 400
QQRPVMFVSR VPAKKPRKLP WPTPKTHPVK RTNVKTSDRS DKAEAQSTPE 450
RPGPSEQSSV TVEPAHPTPV EMPMVILHQP PPVPKPVPVK PTPPPSRRRR 500
GACVVYDDDV IEVIDVETTE DSSSVSQPNK PHRKHQDGFQ RSGRRQKRAA 550
PPTVSPSDTG PPAVGPPAAG PPAAGPPAAG PPAAGPPAAG PPAAGPRILA 600
PLSAGPPAAG PHIVTPPSAR PRIMAPPVVR MFMRERQLPQ STGRKPQCFW 650
EMRAGREITQ MQQEPSSHLQ SATQPTTPRP SWAPSVCALS VMDAGKAQPI 700
ESSHLSSMSP TQPISHEEQP RYEDPDAPLD LSLHPDVAAQ PAPQAPYQGY 750
QEPPAPQAPY QGYQEPPPPQ APYQGYQEPP AHGLQSSSYP GYAGPWTPRS 800
QHPCYRHPWA PWSQDPVHGH TQGPWDPRAP HLPPQWDGSA GHGQDQVSQF 850
PHLQSETGPP RLQLSLVPLV SSSAPSWSSP QPRAPIRPIP TRFPPPPMPL 900
QDSMAVGCDS SGTACPSMPF ASDYSQGAFT PLDINATTPK RPRVEESSHG 950
PARCSQATAE AQEILSDNSE ISVFPKDAKQ TDYDASTESE LD 992
```

B



uncharged polar side chains (for instance, glutamines, threonines and asparagines) (Landschulz *et al.*, 1988; Figure III.1.B). By these criteria EBNA6 has a perfect "zipper" motif located immediately adjacent to a thirty amino acid sequence which is rich in residues with charged side chains; it is predominantly basic and consequently could be a DNA-binding domain (see Figure III.1). A similar basic DNA-binding domain is found adjacent to the "leucine zipper" in C/EBP and Fos (Landschulz *et al.*, 1988). Also, EBNA6 has been shown to bind (non-specifically) to both single and double stranded DNA (Kallin *et al.*, 1986).

Although the precise mechanism of interaction between "leucine zipper" proteins has been questioned (that is, whether interdigitation of leucine residues occurs, (O'Shea *et al.*, 1989) experimental evidence has rapidly accumulated that "zippers" do in fact mediate in the formation of both homodimers (for instance, Myc, Dang *et al.*, 1989; C/EPB, Lanschulz *et al.*, 1989) and also heterotypic complexes (for instance, Fos-Jun/API, Schuermann *et al.*, 1989; Kouzarides and Ziff, 1988; Sassone-Corsi *et al.*, 1988; Gentz *et al.*, 1989; Turner and Tjian, 1989). The Fos-Jun/API complex formed *via* this domain is required for both transactivation of API-dependent transcription and neoplastic transformation by Fos (Schuermann *et al.*, 1989), so the "leucine zipper" is clearly a functionally significant motif.

On the basis of these comparisons with known transcriptional activators and considering its nuclear distribution, a reasonable hypothesis to explain the apparent toxicity of EBNA6 is that it is a transcriptional activator which normally interacts with a cellular transcription factor *via* a "leucine zipper". If such a protein were over-expressed, and therefore present in excess, it might bind to and sequester the cellular factor and so inhibit transcription of multiple cellular genes and cell proliferation. This model, termed "squelching", has been proposed previously to explain the inhibitory effects of overexpressed VP16 (a herpes simplex virus transcriptional activator) and Gal4 (a yeast transcriptional activator) (Gill and Ptashne, 1988; Ptashne, 1988).

If EBNA6 acts as a transcriptional activator (of, as yet unidentified cellular and/or viral genes) then its synthesis very early after EBV infection of resting B cells (as they are activated to enter and traverse the cell cycle) may be important in

the initiation of immortalization. In this respect, it may be significant that a cascade of Fos-related proteins which participate in heterotypic DNA binding complexes (probably *via* "leucine zippers") are induced during the first 8 hours after (serum) stimulation of resting cells (Gentz *et al.*, 1989). The highly regulated level of expression (<5 mRNA copies per cell) which continues in LCLs suggests EBNA6 might be required in the maintenance of cell proliferation.

2 THE EBNA FAMILY

The e_2-β-gal fusion protein was used to identify human sera which recognised EBNA6 on Western immunoblots of proteins extracted from LCLs. A number of these sera (including serum RT) additionally recognised EBNAs 1 and 2 and two other polypeptides which migrate in the 140-180 kD region of SDS-polyacrylamide gels. Using a variety of these sera with different specificities (together with sera exchanged with other laboratories, M. Rowe, L. Rymo, B. Kallin and I. Joab, personal gifts), the additional EBV-associated nuclear proteins have been identified as EBNA3 (encoded by BLRF3 and BERF1) and EBNA4 (encoded by BERF2a and 2b). Although LCLs invariably express EBNAs 1, 2, 3 and 6, and generally express EBNA4 (see Section III.3), eBL cell lines are far more heterogeneous with respect to EBNA expression . The absence of EBNA2 from lines such as P3HR1 and Daudi is well documented (see Section I.4.6) and the results presented here suggest that eBL cell lines do not have an absolute requirement for EBNAs 4 or 6 expression either (see Raji, P3HR1 and Daudi, Figure II.7). However, a note of caution comes from the recent report that in eBL cell lines containing 2B-viruses (for instance, AG876 and Jijoye) homologues of EBNAs 3, 4 and 6 may be demonstrated with certain EBNA-2B specific human sera (Rowe *et al.*, 1989). Further analysis of EBNA expression in eBL cell lines with more 2B-specific sera and, when they become available, monoclonal antibodies will be required to clearly distinguish between protein polymorphism and truly restricted gene expression.

3 IMMORTALIZATION BY EBV

3.1 The Temporal Sequence of Latent Gene Expression

In this study, well-characterized human sera and murine monoclonal antibodies have been utilized to investigate expression of the EBV latent proteins at the very onset of immortalization, when resting B-cells are stimulated by viral infection to enter and traverse the cell cycle. PLL cells have been employed because they are a relatively homogenous (monoclonal) population of resting B-cells which can be very efficiently immortalized by EBV. As 60% or more can be infected and immortalized, they provide a very sensitive target population for analyzing early viral gene expression before cellular proliferation begins between 48 and 72 hours post-infection.

EBNA expression can be demonstrated by ACIF eight hours after infection of PLL cells with B95-8 virus and soon after this it can be analysed by Western immunoblotting. EBNA2 is the first antigen to be detected (8-10 hours) and the remainder of the EBNAs appear more or less synchronously between 12-18 hours post infection. By 24 hours, the latent pattern of EBNA expression found in long term LCLs is established. The appearance of EBNA2 as the first of this family to be observed is reproducible and consistent with previously published data on the infection of tonsil and cord blood B-cells (Kallin *et al.*, 1986; Moss *et al.*, 1986; Rooney *et al.*, 1989) and our own experiments with peripheral B lymphocytes (MJA and D.H. Crawford, unpublished). Whether EBNA2 is actually transcribed and translated before the other EBNA species or whether it is just more readily detected by the immunological techniques used is still unclear. Nevertheless, it seems that all the EBNAs are synthesised at, or about, the same time early after infection and long before cellular or viral DNA synthesis. Apart from the early appearance of EBNA2, no differential expression of the other individual EBNA species was apparent in any experiment performed with B95-8 virus and the PLL cells.

Although the pattern of EBNA expression is identical to that in established LCLs approximately 24 hours after infection of PLLs with B95-8 virus, LMP cannot be detected by either IF or immunoblotting at this time. It is only after 48 hours that LMP is observed. This delay in expression of LMP correlates with an observation that the cell surface adhesion molecule LFA1 (on PLL cells) is induced 48-72 hours after infection (T. Lillie, personal communication). LFA1 is known to be induced by LMP (Wang *et al.*, 1988). The findings suggest that cellular and/or virally mediated events occur after EBNA synthesis which then facilitate efficient LMP expression. One possibility is that a threshold level of EBNA2 (or any other EBNA species) acting in *trans*, either directly or indirectly by stimulating cellular activation, is necessary. Evidence for this hypothesis comes from reports that P3HR1 (EBNA2 negative) converted EBV-negative BL (sBL) lines do not synthesise LMP whereas B95-8 conversion of the same lines allows LMP expression (Murray *et al.*, 1988b). However, it should be noted that the parent P3HR1 cell line readily synthesizes LMP in the absence of EBNA2 expression. Another contributory factor may be the circularization of the EBV genome. This is thought to occur between 16 and 24 hours after infection (Hurley and Thorley-Lawson, 1988; Thorley-Lawson, 1988), that is, before LMP expression. Whereas EBNA expression probably does not require genome circularization, the LMP gene, located near the "end" of the linear EBV genome, may only be efficiently expressed after circularization has occurred and cis-acting sequences are juxtaposed to the region controlling LMP transcription. The failure of P3HR1 converted BL lines such as AW Ramos to express LMP (Figure II.29,B track 2 and Murray *et al.*, 1988b) would then be explained by the fact that the EBV genome is present only as linear molecules integrated into host chromosomes (see Teo and Griffin, 1987) where the cis-acting elements need not be correctly sited.

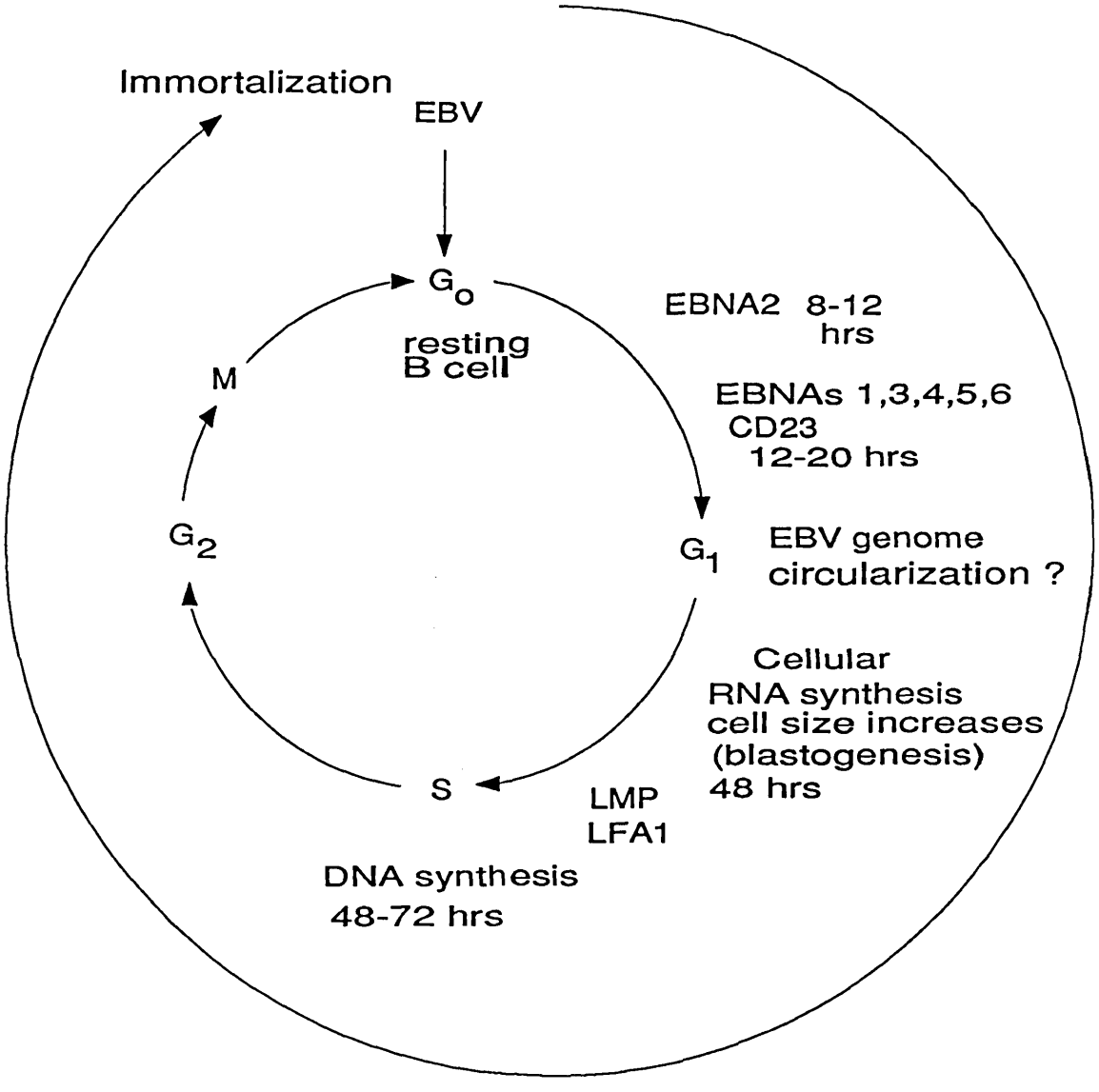
When EBV infects resting B-cells, the binding of virus to CD21 (Nemerow *et al.*, 1986) and possibly the insertion of virion associated LMP into the membrane by envelope fusion (Mann *et al.*, 1985) stimulate the cells to enter the cell cycle (Go - G_1). EBNA2 has been implicated in the progression towards S phase (Gordon *et al.*, 1986) and "superinduction" of the B cell activation marker CD23/Blast 2 (Wang *et al.*, 1987; Thorley-Lawson *et al.*, 1985). EBNA1 becomes necessary for replication

and correct partitioning of the viral episome after it has circularized and cells start to proliferate. LMP appears to be synthesized at or around the time cells start to synthesize DNA (S phase). Its expression appears to be essential for immortalization (Walls *et al.*, 1989; Section III.3.2) and for stimulating changes associated with blastogenesis (Wang *et al.*, 1988). The rôles of EBNAs 3, 4, 5 and 6 have not yet been determined but their temporal expression in cells becoming immortalized suggests that functions associated with progression through the cell cycle, including the transition from G2-M (Melchers and Lernhardt, 1985; Gordon and Guy, 1987), should be considered. On the basis of this analysis a model of the early events in B cell immortalization has been constructed (Figure III.2).

At least one of the latent proteins, EBNA4, cannot be absolutely necessary for the maintenance of cell proliferation although it may be necessary during initiation. For example, the LCL, IB4 (and two other LCLs), established with the B95-8 virus (King *et al.*, 1980; N. Sullivan, personal communication; Figure II.6), have lost the ability to express EBNA4. This suggests that EBNA4 is not only unnecessary but that failure to synthesize it may possibly give a growth advantage *in vitro*.

Previous reports (see Thorley-Lawson, 1988) suggest that P3HR1 virus infection of resting B-cells does not result in EBNA expression. The delayed and transient nature of EBNA expression seen in our experiments is in agreement with this observation. A small number of P3HR1 infected cells transiently synthesize EBNA1. These cells do not express EBNAs 2, 4 and 6 nor, apparently, LMP. Neither have we been able to consistently show expression of EBNAs 3 and 5. It is not surprising that five to six days after infection the cells die. However, it is unclear whether this failure to synthesize some of the latent proteins and start continuously cycling (immortalization) results solely from the deletion in the *Bam*HI YH region that removes the EBNA2 gene and truncates EBNA5 (Rabson *et al.*, 1982) or whether other genomic lesions are involved. In this respect it should be noted that the successful conversion of EBV negative BL cells with P3HR1 shows that even a defective, non-immortalizing virus can infect, express EBNAs 1, 3 and 5 and

Figure III.2: The Progression of EBV Infected Resting B Cells through the Cell Cycle. The main cellular events and the sequence of viral "latent" gene expression are shown. This summary is based on data from this study, Thorley-Lawson, (1988), Walls *et al.*, (1989) and T. Lillie (personal communication).


Immortalization
EBV
G_o
resting
B cell
EBNA2 8-12
hrs
EBNAs 1,3,4,5,6
CD23
12-20 hrs
G_1
EBV genome
circularization ?
Cellular
RNA synthesis
cell size increases
(blastogenesis)
48 hrs
S
LMP
LFA1
DNA synthesis
48-72 hrs
G_2
M

presumably give a growth advantage to cells which are already proliferating (see Murray *et al.*, 1988a & b). This raises the possibility that EBNA2 expression and concomitant cellular activation may be necessary for efficient expression of the other latent proteins only in resting B cells. Infection of the EBV negative B cell line, BJAB, with the P3HR1 virus used in this study results in more than 80% of cells becoming EBNA positive (by ACIF) after 24 hours (D.H. Crawford, personal communication). It is therefore tempting to speculate that the PLL cells which are permissive for EBNA expression after P3HR1 infection are those which have been spontaneously activated into the cell cycle. These may then bypass the requirement for EBNA2 in the efficient expression of other EBNA species. Recently, Rooney and colleagues have arrived at a similar conclusion (Rooney *et al.*, 1989).

3.2 Latent Gene Expression in Immortalizable and Non-immortalizable B Cell Subsets

After EBV infection, CD23 expression on the surface of B cells is predictive of immortalization (see Section I.1.3); however, both CD23-positive and negative fractions contain EBNA-positive cells. Therefore, to determine whether different latent proteins are expressed in those cells which immortalize and those which do not, E-negative peripheral blood B lymphocytes were separated according to CD23 expression three days after infection with B95-8 virus. Immunoblot analysis revealed that in addition to EBNA1, EBNA2 was clearly expressed in both fractions. This result is unexpected as the induction of CD23 has been specifically associated with EBNA2 expression (Wang *et al.*, 1987; Calender *et al.*, 1988). My assumption is that a subpopulation of resting B cells exists which does not respond to EBNA2 by becoming "activated" and expressing CD23; these cells may be at a different point on the B cell differentiation pathway to those susceptible to EBV-induced proliferation. Also, although EBNA2 is probably necessary for immortalization (see Sections I.1.3 and 4.6) these results clearly show it is not sufficient, at least not in all circulating B cells.

The failure of the CD23-negative population to synthesise detectable levels of EBNAs 3, 4 and 6 (although the positive population does so at the same time point) suggests that the EBNA complex can be differentially expressed in B cell subsets;

this has not been previously reported. An alternative explanation is that CD23-induction may require coordinated expression of EBNAs 2, 3, 4 and 6 in resting B cells and that the CD23-negative population results from infection with defective viruses. (It should be noted that this experiment has only been performed twice and, although the results were consistent, EBNAs 3, 4 and 6 are not always easily detected on immunoblots, particularly of proteins from small numbers of cells. Further studies are currently in progress to confirm this observation).

CD23-negative cells do not express detectable levels of LMP. Their failure to be immortalized is therefore consistent with the hypothesis that LMP plays a key rôle in inducing continuous proliferation (see Section I.4.1). The lack of expression does not, however, merely correlate with the absence of EBNA2. (EBNA2 is as abundant in the CD23-negative fraction as in the CD23-positive cells). Although EBNA2 may be required for efficient expression of LMP, clearly again it is not sufficient. As the EBV genome does not appear to circularize in the CD23-negative population of cells (Hurley and Thorley-Lawson, 1988) it is again tempting to speculate that this process may be a necessary prerequisite for LMP expression in resting B cells.

4 LATENT ANTIGEN EXPRESSION IN EBV-ASSOCIATED TUMOURS

4.1 Are the EBV Latent Proteins Involved in Tumourigenesis?

This is clearly one of the most interesting questions arising from this and various parallel studies. Unfortunately it still cannot be resolved in a very satisfactory manner. The answer to the slightly simpler question of what EBV proteins are detectable in tumours at the time of presentation is summarised in Table II.3. With respect to EBNA expression the tumours superficially fall into two categories: eBL, NPC and AIDs-associated BL express only EBNA1; tamarin and transplant-associated large cell lymphomas express most (if not all) EBNA species found in LCLs. The restricted expression in BL and NPC is associated with the methylation of DNA sequences within the promoter/enhancer in *Bam*HI W (Section III.5 for further discussion) indicating inactivation of the EBNA2 (-6) transcription

unit and suggesting EBNA1 could be transcribed from an alternative, as yet unidentified, promoter. Although the number of tumours analysed in this study is small, the results are consistent with those from groups which have investigated many specimens (Rowe *et al.*, 1986; Rowe *et al.*, 1987; Young *et al.*, 1988; Fåhraeus *et al.*, 1988; L.S. Young, personal communication). The role played by EBV latent proteins in the development of the different EBV-associated tumours is unlikely to be exactly the same in each case.

4.2 Nasopharyngeal Carcinoma (NPC)

Analysis of the NPC-related C15 revealed that in this poorly differentiated epithelial tumour only EBNA1 and low levels of LMP are expressed. The other EBNA proteins were not detected, neither was there any evidence of their corresponding transcripts (as judged by screening a large cDNA library). The expression of these genes in the LCLs, NAD-C15-STO and CBL-C15-STO, which contain the same viral genome as C15 "rescued" into B cells, show this suppression is probably due to cell specific control of gene expression. Analysis of the EBNA2 5'-flanking sequences suggest DNA methylation may be involved in the block to transcription. EBNA1 expression probably reflects the basic need for this protein in maintaining extrachromosomal viral episomes and latency. A reasonable assumption is that EBNAs 2-6 are inessential and that a restriction in their expression may actually be a prerequisite for tumour development. It is possible that tumours develop from cells at a stage of differentiation when (transcription) factors necessary for the expression of EBNA2-6 (from the *Bam*HI W promoter) are unavailable. Tumours may therefore exhibit a "frozen" stage-specific phenotype and their normal counterparts in the EBV life cycle may also express only EBNA1 (or EBNA1 and LMP). It will be interesting to discover whether expression of further EBNAs (2-6) may actually commit cells to terminal differentiation and concomitant viral replication (see Section I.2 and Figure I.2). Alternatively an expanded pattern of latent expression may simply render the tumour cells "visible" to the immune system and eradication.

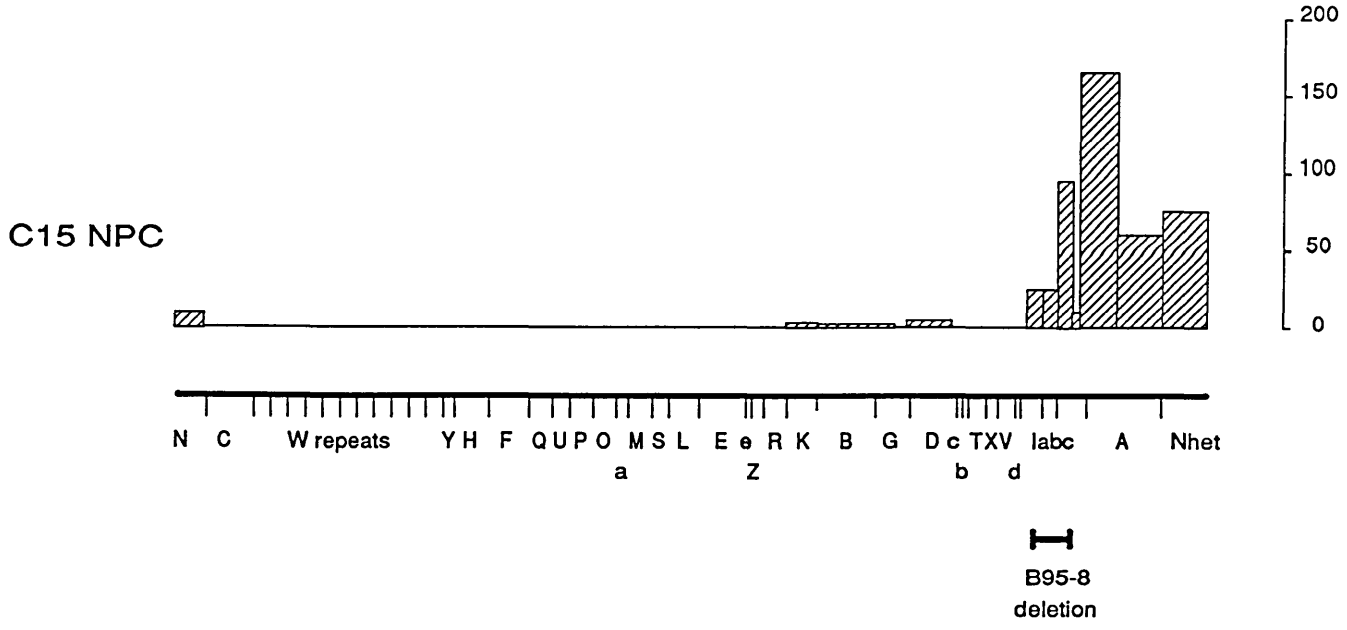
It remains to be established whether the EBV gene products which are detected in NPC (for example EBNA1, the EBERs and LMP) contribute to tumour development. EBNA1 is invariably present in NPC cells and may therefore play a role in the transformation of epithelial cells. However, numerous transfection studies have been carried out using DNA including this gene (see Section I.4.5; Griffin and Karran, 1984); none has ever shown an EBNA1-specific alteration in the growth properties of the transfected cells. The EBERs may be viewed in the same way, that is, they are always present but there is no evidence they have growth transforming potential (see Section I.1.4). LMP, on the other hand, which possesses transforming properties (see Section I.4.1), could contribute to the development of the neoplastic phenotype. However, the reports that it can only be detected in 50% of tumour biopsies (Young *et al.*, 1988; Fåhraeus *et al.*, 1988) suggest that as with EBNAs 2-6 it also may not be essential.

Preliminary analysis of the EBV-specific transcripts in the C15 NPC have revealed that previously unidentified latent genes, located at the "right hand" end of the EBV genome, could play a role in the pathogenesis of NPC (Figure III.3). Specifically, an open reading frame, (capable of encoding a 40-50 kD protein) and tentatively designated BARF0 (P. Farrell, personal communication) appears to be transcribed in C15 (Hitt *et al.*, submitted for publication; L. Karran, personal communication). If the product of this gene is consistently found in NPC biopsies it should be considered a prime candidate as a transforming function. It may be significant that this open reading frame is located in a region of the EBV genome which has been used to immortalize (by DNA-mediated gene transfer) primate (including human) epithelial cells (Griffin and Karran, 1984).

4.3 eBL and AIDs-BL

In EBV-positive BL biopsies and also early passage (group I) BL cell lines the only latent protein which has been detected is EBNA1 (see Section I.2.4 and II.6). The fact that LMP, EBNA2 and possibly the high molecular weight EBNA species (3,4, and 6) are targets for CTLs (see Section I.1.6; D.Moss personal communication) may explain why BL cells expressing these antigens have not been found *in vivo*. Such cells would become "high profile" targets for specific cytotoxic T cells. It is

Figure III.3: Pattern of "Latent" Gene Expression in the C15 NPC Tumour. A histogram (at top) shows the data obtained by hybridizing a comprehensive cDNA library (C15 NPC) generated in λgt10, from mRNA isolated from the C15 tumour, with *Bam*HI restriction fragments of EBV DNA from B95-8 and Raji cells (Arrand *et al.*, 1980), as indicated in the text (Section II.5.4) and Hitt *et al.* (submitted). The number of viral clones detected per 10^6 recombinants screened is shown on the ordinate, and their location on the EBV genome is shown relative to the *Bam*HI physical map (at bottom). The region corresponding to Ia-c is deleted in the B95-8 genome.


C15 NPC
200
150
100
50
0
N C W repeats Y H F Q U P O M S L E e R K B G D c T X V labc A Nhet
a Z b d
B95-8
deletion

still unclear, however, precisely why the tumour cells do not synthesise these antigens and whether, indeed, they ever have. The failure of BL cells to express them is paradoxical because they are probably the main effectors in EBV-induced cell proliferation *in vitro* (Figure III.3). They are the best candidates for EBV encoded oncogenes. One must assume that in cells maintained in the cell cycle by other means (for instance a constitutively active c*myc*) they are unnecessary.

There are a number of possible explanations for the absence of detectable EBNAs 2-6 and LMP. The first is that EBV infects B cells at a stage of development when only EBNA1 can be expressed (see also Section III.5) and, as with NPC, we may be seeing in the tumour *in vivo*, cells "frozen" with this phenotype. An alternative is that cells may initially express the full complement of latent genes and subsequently all but EBNA1 are "down regulated". If this is the case EBNAs 2-6 and LMP may make a transient contribution to tumour development - possibly stimulating them to enter the cell cycle. However, the mechanism by which the antigens are then "down regulated" is obscure (although it may involve DNA methylation - see Section III.5). The activation (by translocation) of c*myc* is unlikely to modulate directly EBV gene expression as there is no detectable change in the levels of either LMP or EBNAs 2-6 in LCLs constitutively expressing high levels of Myc protein following stable transfection with recombinant c*myc* plasmids (Hotchin *et al.* submitted for publication). Finally, we cannot formally exclude the possibility that pre-cancerous cells are infected by mutant (such as Daudi and Naliaki, see Section I.1.3) or methylated "epimutant" virus (see Section III.5) which are incapable of expressing a full complement of latent functions *in vivo*.

As with NPC, we are left with the question of what contribution EBV makes to the development of BL tumours. Again EBNA1 and the EBERs cannot be ruled out as effectors and the identification of novel "latent" products in NPC leads one to speculate that similar functions may be expressed in BL.

4.4 Tamarin Lymphomas and Transplant Associated Large Cell Lymphomas

Preliminary data (this study; Thomas *et al.*, 1989; L.S. Young, personal communication) suggest that the pattern of EBV latent gene expression in tamarin

lymphomas and the large cell lymphomas in immunosuppressed humans is very similar, if not identical, to that in LCLs established and grown *in vitro*. Analysis of more material (as it becomes available) is required to confirm this. Nevertheless, assuming the same genes are expressed, then it is a valid hypothesis that EBV is driving the B cells to proliferate *in vivo* in the same way as it induces LCLs *in vitro* (Figure III.2). Because of CTLs(Section I.1.6) this could only occur in experimentally infected animals (where the primary immune response is presumably overwhelmed by the massive intraperitoneal inoculum of virus) or in individuals with suppressed cell mediated immune responses. The monoclonal nature of many of these tumours (Cleary, 1986; Thomas *et al.*, 1989) is puzzling. The implication is that the outgrowth of a malignant clone into a discrete tumour nodule may require a "second event" in addition to EBV induced proliferation. If this is indeed the case one must assume that such a clone may differ from the cells producing the polyclonal hyperplasia of acute and fatal IM and also LCLs. A careful comparison of the phenotype, genotype and growth properties of these cells may lead to the identification of the cofactor(s) which induces monoclonal outgrowth and suggest what might restrict the proliferation of other EBV infected cells *in vivo*.

5 CpG METHYLATION AND EBNA EXPRESSION

Although most LCLs express all the EBV latent proteins, it is now apparent that in some tumours or cell lines freshly established from tumours, a much more restricted pattern of latent protein expression occurs (Table II.3). An analysis of the pattern of CpG methylation in the putative 5' regulatory region of the EBNA2 gene (which has been shown to function in human LCLs, Sample *et al.*, 1986; Ricksten *et al.*, 1988; Figure II.32) has revealed a tight inverse correlation between methylation of certain CpG dinucleotides in this region and EBNA2(-6) expression.

Overall, CpG is a rare dinucleotide in the DNA of higher eukaryotes (Subak-Sharpe *et al.*, 1966) and in many cases the cytidine is modified at the 5' position with a methyl group. There is now much evidence to suggest that gene transcription is correlated with the absence of methylation at sites in regulatory regions. It is generally assumed that methylation interferes with the binding of transcription

factors to DNA. This could involve alterations in chromatin structure or, alternatively, methylation of CpG may directly inhibit gene activation by sterically hindering the binding of transcription factors (for instance, as observed with the adenovirus major late transcription factor, MLTF, Watt and Malloy, 1988). From most studies it is unclear, however, whether methylation is the initial cause of gene inactivation or *vice versa* (for reviews see Doerfler, 1983; Bird, 1986; Holliday, 1987; Cedar, 1988). The control region for EBNA2 identified by Ricksten *et al.*, (1988) and the flanking sequences in the major internal (*Bam*HI W) repeat are especially rich in CpG dinucleotides (Figure II.32). This region may also regulate the expression of other EBNA species. Recently *in vitro* methylation of *Hha*I restriction sites within this region was shown to block the transcription of DNA subsequently transfected into normally permissive cells (L. Rymo, personal communication).

There are a number of ways to explain the origin of the methylation pattern observed in BL, NPC and AIDS-related lymphoma; consistent with all of them is the notion that EBNA 2 expression is not required for, and may even be incompatible with, tumour development: The first hypothesis assumes a cell was infected by an EB virion carrying viral DNA that was methylated in *Bam*HI W. This genome would behave as an EBNA 2 negative "epimutant" (Jego and Holliday, 1986; Holliday, 1987) unable to express the antigen. Maintenance methylases (and immune selection, if this failed) would then fix the methylation pattern in the developing tumour. Such "epimutant" viruses would not immortalize resting B-cells or consequently be detected in *in vitro* transformation assays. Consistent with this view is the observation that viruses defective for EBNA 2 expression and immortalizing ability (e.g. P3HRI) can infect and be latently maintained in proliferating cells of tumour origin (Murray *et al.*, 1988a & b). Also it has been shown that a fraction of EBV virion DNA is indeed methylated (Perlmann *et al.*, 1982; Diala and Hoffman, 1983). Methylation of bovine papilloma virus (BPV) DNA has also been reported and, in the context of our studies it may be of interest that *in vitro* methylation of BPV DNA can reduce its transformation efficiency (Christy and Scangos, 1986). An equally attractive hypothesis is that "normal" unmethylated EBV infects a cell at a stage of differentiation where transcription factor(s) required for EBNA 2 expression are missing. In the absence of transcription complexes the "silent" promoter/enhancer region would be available for *de novo* methylation. There have now been a number

of reports in which methylation follows transcriptional inactivation (Niwa *et al.*, 1983; Lock *et al.*, 1987; Enver *et al.*, 1988; Grosschedl and Marx, 1988) supporting this as a valid assumption. The pattern would again be fixed by maintenance methylases. A third possibility, for which there is no obvious precedent but which cannot be discounted, is that EBV infects a pre-cancerous cell type which has a hyperactive methylation system and that *de novo* methylation of *Bam*HI W occurs before a transcription complex can be formed, thus blocking EBNA 2 expression.

The phenotypic "drift" seen in Burkitt's lymphoma cells grown *in vitro*, with its concomitant expression of EBNA 2 (and other latent proteins) (Section I.2.4; Rowe *et al.*, 1987), may reflect a general decline in maintenance methylation releasing the block for expression of the EBNA 2 gene. Methylation in cultured cells has been shown elsewhere to be unstable (Pollack *et al.*, 1980; Wigler *et al.*, 1981; Reis and Goldstein, 1982). Alternatively, a change in the phenotype of the cells upon growth in culture could lead to cellular maturation and the synthesis of stage specific transcription factors which are insensitive to methylation (see for example Kelley *et al.*, 1988; Holler *et al.*, 1988). In either case EBNA 2 expression *in vitro* probably gives cells a growth advantage and in the absence of immune selection such a population would soon predominate. It was reported by Kintner and Sugden (1981) that EBV episomes maintained latently in LCLs become progressively methylated. The results presented here suggest that the CpG-rich promoter/enhancer region in the *Bam*HI W repeats must be relatively protected from methylation in these cells. This would be completely consistent with the notion that EBNA2 transcription is an absolute requirement for proliferation of LCLs and certain EBV-associated tumours.

6 CONCLUSIONS

In brief, EBV functions involved in immortalizing B lymphocytes *in vitro* include six nuclear (EBNAs 1-6) and two membrane (LMP and TP) proteins. At least seven of these (the EBNAs and LMP) are initially expressed while EBV-infected resting cells enter the cell cycle and are then induced to complete mitosis. Preliminary data (this study; Rooney *et al.*, 1989 and C. Rooney, personal

communication) suggest that EBNA2 expression may initiate a cascade of viral (and cellular) gene expression which promotes and sustains the continuous proliferation of some B cells.

Although the precise mode of action of the viral nuclear proteins in this activation pathway is unclear, it is likely to depend on their interaction with cellular factors. An analysis of the amino acid sequence and predicted tertiary structure of one EBNA polypeptide (EBNA6) suggests, that in this case, complex formation could be mediated *via* a "leucine zipper": such complexes may then activate the transcription of cellular and/or viral genes. Studies are currently in progress to determine if the leucine repeat domain in EBNA6 is functionally significant.

The long-term presence of antibodies to all the EBNAs in the serum of many normal individuals suggests that the antigens can be continuously synthesized, *in vivo*, during (normal) asymptomatic viral persistence. It is not yet clear, however, whether their synthesis occurs primarily in B lymphocytes, in squamous epithelium or both. Nevertheless, tamarin and transplant-associated B cell tumours (which express the range of EBV proteins found in LCLs *in vivo*) show that, under circumstances where cellular immunity is overwhelmed, LCL-like cells can and do proliferate unchecked. The expression of EBNA1, but not the other EBNAs, in eBL and NPC cells remains a largely unresolved paradox. The data presented here and by others (I. Ernberg and L. Rymo, personal communications) on CpG methylation of certain viral DNA sequences, suggest that transcription of EBNAs 2-6 may be prevented in these tumours. The mechanism for this inactivation has still, however, to be established.

IV MATERIALS

1. CHEMICAL ABBREVIATIONS

AET	2-amino-ethylisothiouronium bromide
APTG	p-amino-phenyl-β-thiogalactoside
(d)ATP	(deoxy) Adenosine triphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CFB	Complement Fixation Buffer
(d)CTP	(deoxy) Cytosine triphosphate
DAB	Diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
(d)GTP	(deoxy) Guanosine triphosphate
HEPES	N-2,hydroxyethylpiperazine-N'-2,ethane sulphonic acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
β-NAD	Nicotinamide adenine dinucleotide
NP-40	Nonidet P-40
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol

RNA	Ribonucleic acid
RNAase	Ribonuclease
RNAasin	Ribonuclease inhibitor
RT	Room temperature
SAM	S-adenosyl-L-[methyl-^{3}H] methionine
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TEMED	*N,N,N',N'*,tetramethyl ethylenediamine
TRIS	Tris(hydroxymethyl) aminomethane
TTP	Thymidine triphosphate
U	Unit
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

2. REAGENTS AND EQUIPMENT (Chemicals were of Analar quality and supplied by BDH unless otherwise stated)

Acrylamide:	Biorad
Agarose:	Seakem
Antibiotics:	Bacterial culture - Sigma
	Cell culture - Flow
	Actinomycin D (Calbiochem)
	Geneticin (G418) (Calbiochem)
	Hygromycin (Calbiochem)
Autoradiography:	X-ray film - Kodak XAR-5
	- Fuji X-Omat
	Intensifying screens - Dupont Lightning Plus
BSA:	Pentex fraction nuclease free (BRL)
	Fraction V (Sigma)
Buffers:	TRIS, HEPES, (Sigma)
Caesium Chloride:	Koch Light
Carrier DNA:	Calf Thymus DNA (Sigma). Dissolved to 10 mg/ml, sonicated and extracted twice with buffered phenol. After ethanol precipitation, DNA was dissolved in TE (pH 7.5) and stored at 4°C.
Chromatography:	G75 Sephadex (Pharmacia Fine Chemicals)
	Oligo-(dT) cellulose (BRL)
	APTG-agarose (Sigma)
	Disposocolumns (Bio-Rad)
Detergents:	N,lauryl sarcosyl (Serva)
	Sodium dodecyl sulphate (BDH)
	Triton X 100 (Sigma)
	Nonidet NP40 (BDH)
	Tween 20 (BDH)
DNA Binding:	Nitrocellulose, 0.45 μM (Schleicher and Schuell)
	DEAE paper, NA45 (Schleicher and Schuell)
	Biotrap (Schleicher and Schuell)

Enzymes:	Restriction Enzymes (Biolabs, Boehringer or BRL)
	Intestinal Alkaline Phosphatase (Boehringer)
	DNase I (Worthington Biochemicals)
	E. coli DNA polymerase I (PL Biochemicals, Pharmacia)
	Klenow DNA polymerase I (Amersham)
	Kornberg DNA polymerase I (Boehringer)
	E. coli Ligase (Biolabs)
	Lysozyme (Sigma)
	Proteinase K (Sigma)
	RNase A (Sigma)
	RNase T1 and T2 (Sigma)
	T4 DNA Ligase (Biolabs)
	T4 polynucleotide kinase (PL Biochemicals, Pharmacia)
	T4 DNA polymerase (PL Biochemicals, Pharmacia)
	Reverse Transcriptase (Super RT, Anglian Biotechnology)
	RNase H (BRL)
	*Eco*RI methylase (Biolabs)
Formamide:	Fluka, deionized over Amberlite (Biorad)
	Aliquoted and stored at -20°C.
Macaloid:	Steetley Minerals, Ltd. Autoclaved and washed several times in sterile 50 mM Tris-HCl (pH 7.5).
	Stored at 4°C.
Miscellaneous:	DAB (Sigma)
	IPTG (Sigma)
	Lambda packaging kits (Amersham and Stratagene)
	Linkers, *Eco*RI (PL Biochemicals, Pharmacia)
	β-NAD (Sigma)
	Oligo-$(dT)_{20}$ (PL Biochemicals, Pharmacia)
	Powdered milk (Marvel, Cadbury)
	RNAsin (P & S Biochemicals)
	Nucleoside triphosphates (PL Biochemicals, Pharmacia)
	X-gal (Boehringer)

Phenol:	Fisons. Redistilled and stored at -20°C. Before use, neutralized by buffering twice with 500 mM Tris-HCl (pH 8.0), 50 mM EDTA and twice with distilled water. Stored at 4°C.
Tissue Culture:	DMEM (Gibco or Flow) FCS (Gibco or Flow) RPMI (Flow) Plastic ware (Nunc or Falcon)

3. GENERAL SOLUTIONS

PBS

0.8% (w/v) NaCl
0.02% (w/v) KCl
0.02% (w/v) Na_2HPO_4
0.15% (w/v) KH_2PO_4

SSC

150 mM NaCl
150 mM sodium citrate

Denhardt's solution

0.02% (w/v) BSA

0.02% (w/v) polyvinlpyrrolidone
0.02% (w/v) Ficoll 400

Phenol/Chloroform

49.5% (v/v) buffered phenol (pH 8.0)
49.5% (v/v) chloroform
1% (v/v) isoamyl alcohol
0.1% (w/v) 8-hydroxyquinoline

L-Broth

1% (w/v) bacto-tryptone
0.5% (w/v) yeast extract
1% (w/v) NaCl

Psi-Broth

2% (w/v) bacto-tryptone
0.5% (w/v) yeast extract
10 mM NaCl
20 mM $MgSO_4$
5 mM KCl

TE

10 mM Tris
1 mM EDTA
pH adjusted with HCl

Versene

0.02% EDTA (w/v) in PBS (pH7.2)

2 x TY

1.6% Tryptone
1% yeast extract
0.8% NaCl

SM

100 mM NaCl
10 mM $MgSO_4$
50 mM Tris-HCl (pH 7.5)
0.01% (w/v) gelatin

4. BACTERIA AND PHAGE

E. coli K12 strain JM101 [*Sup*E, *thi*, del(*lac*$^-$ *pro*AB), (F', *tra*D36, *Pro*AB, *lac*I^{q}ZΔM15)]. (Yanisch-Perron *et al.*, 1985).

E. coli K12 strain TG2 (JM101 *Rec*A1). (T. Gibson, personal communication).

E. coli K12 DH1 [F$^-$, *end*A1, *hsd*R17 (rk$^-$, mk$^+$), *Sup*E44, *thi*-1, λ-, *rec*A1, *gyr*A96, *rel*A1]. (D. Hanahan, 1985).

E. coli K12 BNN93 (*hsd*R$^-$, *hsd*M$^+$, *Sup*E, *thr*, *leu*, *thi*, *lac*Y1, *ton*A21).

E. coli K12 BNN102 [BNN93 *hfl*A150 (chr::Tn*10*)].

λgt10 [λsr*I*k1°b527 sr*I*k3°imm^{434} (sr*I*434$^+$) sr*I*k4° sr*I*k5°]

(for BNN93, BNN102 and λgt10 see Huynh *et al.*, 1985)

5. CELLS

CELL LINE	TYPE	REFERENCE
Human		
IB4	LCL	King *et al.*, (1980)
2C9	LCL	K. Lam, RPMS, (personal communication)
Maja	LCL	J. Bodmer, ICRF, (personal communication)
674	LCL	N. Sullivan, ICRF, (personal communication)
874	LCL	N. Sullivan, ICRF, (personal communication)
NAD-C15-STO	LCL	Fåhraeus *et al.*, (1988)
CBL-C15-STO	LCL	Fåhraeus *et al.*, (1988)
Ramos	EBV-negative BL	Klein *et al.*, (1975)
EB4	EBV-negative BL	Epstein *et al.*, (1966)
Raji	EBV-positive BL	Pulvertaft, (1965)
P3HR1	EBV-positive BL	Hinuma *et al.*, (1967)
Daudi	EBV-positive BL	Klein *et al.*, (1968)
BLMH	EBV-positive BL	D.Kundu, RPMS, (personal communication)
AW-Ramos	P3HR1-virus-converted Ramos	Klein *et al.*, (1975)
Putko	P3HR1-K562 fusion	Klein *et al.*, (1980)
Eli	EBV-positive BL	Rowe *et al.*, (1987)

Primate		
CV1	Monkey fibroblast	Harris and Sutherland (1964)
Cos7	Monkey fibroblast expressing SV40 LT-antigen	Gluzman, Y. (1981)
Rodent		
Balbc/3T3 (Clone 24)	Mouse fibroblast	D.Critchley, Leicester (personal communication)

6. SERA AND ANTIBODIES

SERUM/ ANTIBODY	SPECIFICITY	ORIGIN	SOURCE
Human sera			
RT	EBNAs 1,2,3,4,6	Normal Donor	MJA
062	EBNAs 1,2,3,4,6	NPC patient	M. Ng, Hong Kong
MS	EBNAs 1,2,6	Normal Donor	M. Rowe, Birmingham
RM	EBNAs 1,2,3,5,6	Normal Donor	G. Miller, Newhaven
ST	EBNAs 1,2	Sjögren patient	P. Venables London
WC	EBNAs 2,3	Chronic Mononucleosis patient	T. Schooley, Boston
MJ	EBV-Negative	Normal Donor	D. Crawford, RPMS
Monospecific Antibodies			
α107	EBNA 1	Affinity purified from rabbit serum	J. Dillner, Stockholm

Monoclonal Antibodies

α-β-Gal	β-Galactosidase	Mouse	J.Burke, Sussex
CS1-4	LMP	Mouse	M.Rowe, Birmingham
MHM6	CD23(Blast-2)	Mouse	M. Rowe Birmingham

Commercial Antibodies

Anti-human IgG-peroxidase conjugate	Dako or Nordic
Anti-rabbit immunoglobulin-peroxidase conjugate	Dako
Anti-mouse immunoglobulin-peroxidase conjugate	Dako
Anti-human complement C3-FITC conjugate	Nordic
Anti-human IgG-FITC conjugate	Nordic
Rabbit anti-mouse immunoglobulins	Dako
Rabbit anti-mouse IgG-Dynabeads	Dynal

V METHODS

1. TISSUE CULTURE TECHNIQUES, VIRUS INFECTIONS AND DNA MEDIATED GENE TRANSFER

1.1 Cell Culture

All lymphocyte derived cell lines were grown in suspension, in RPMI medium supplemented with 10% mycoplasma screened and heat inactivated foetal calf serum in a 5% CO_2:95% air humidified incubator at 37°C. All established and non-established adherent cells were grown, on plastic tissue culture dishes, in DMEM supplemented with 10% FCS in a 10% CO_2:90% air humidified incubator at 37°C. On reaching confluence these cells were washed with versene, freed from the surface of the dish by the addition of a small volume of versene containing 0.25% trypsin (1-5 min, 37°C), and replated, at an appropriated dilution, onto new dishes containing fresh medium. All growth medium was supplemented with 100 units/ml Penicillin, 100 μg/ml Streptomycin and 2 mM Glutamine.

1.2 Storage of Cells

Stocks of cells were stored in liquid nitrogen by resuspending approximately $1x10^7$ cells in 1ml of 90% FCS + 10% DMSO.

1.3 Cell counting and Viability

Ten microlitres of cell suspension were mixed with 10 μl of 0.25% Trypan blue solution (Flow) and counted microscopically in a new, improved Neubeaur counting chamber. Viable cells remained unstained whereas the dead cells took up blue dye.

1.4 Preparation of Human Lymphocytes

Peripheral blood was obtained from normal healthy donors. Heparin treated blood cells were diluted 1:1 (v/v) in RPMI/2% FCS and layered on an equal volume of Ficoll-hypaque (Pharmacia fine chemicals). After centrifugation at 800g for 20

minutes the mononuclear cells which form a band at the interface were removed with a Pasteur pipette and then washed twice with RPMI/2% FCS. Mononuclear cells were counted before use.

1.5 Rat Embryo Fibroblasts

Primary rat embryo fibroblasts were obtained from the ICRF Cell Production Unit.

1.6 Leukaemic Cells

Blood samples were obtained from a patient (not undergoing treatment) with chronic B cell leukaemia which was diagnosed as prolymphocytic leukaemia (PLL) by clinical, morphological and phenotypical criteria (Melo *et al.*, 1986). Mononuclear cells were separated by centrifugation over Ficoll-hypaque and washed twice in RPMI/2% FCS (plus antibiotics). Since the leukaemic cells constituted at least 90% of the total peripheral blood mononuclear cells, enrichment for B-lymphocytes was not carried out. The PLL cells were stored in aliquots at -80°C until required for use.

1.7 Separation of T Cells from Mononuclear Cells by AET-Sheep Red Blood Cell (SRBS) Rosetting

Mononuclear cells were resuspended in washing medium at 10^7 cells /ml and equal volumes of 4% AET treated SRBC (a gift from D.H. Crawford) and FCS were added. The suspension was centrifuged at 200gfor 10 minutes and incubated on ice for at least 1 hour to allow rosetting of the T cells with the AET-SRBC. The pellet was then gently resuspended and the solution underlayed with an equal volume of cold Ficoll-paque. After centrifuging at 800g for 15 minutes at 4°C, the non-rosetted (E-negative) interphase cells were removed and washed in RPMI/2% FCS.

1.8 EB Virus

Supernatants from a culture of B95-8 marmoset LCL were used as a source of infectious B95-8 virus (Miller *et al.*, 1972). Virus was concentrated by ultracentrifugation (17,000g for 90 minutes at 4°C) and titrated by immortalization of umbilical cord blood mononuclear cells (transforming titre of approximately 10^{-6}) (Moss and Pope, 1972). Virus from P3HR1 supernatants was also concentrated by ultracentrifugation and titrated by scoring EBNA positive cells after infecting the EBV negative BL line, BJAB, (80% EBNA positive cells were obtained after 24 hours).

1.9 Infection of Lymphoctyes with EBV

Up to 10^7 lymphocytes were pelleted and resuspended in 1ml RPMI/10% FCS containing the virus sample. Cells were incubated at 37°C for 1 hour to allow adsorption. The cells were washed in RPMI/2% FCS, then plated out in RPMI/10% FCS. The point at which the cells were diluted for plating out was taken as time zero in all experiments.

1.10 Separation of EBV Infected E-Negative Peripheral Blood Mononuclear Cells on the Basis of CD23 Expression

EBV infected E-negative cells were incubated with MHM6 murine monoclonal antibody (Rowe *et al.*, 1982) at 4°C for 30 minutes in plastic Universal tubes. Cells were washed 3 times and prewashed magnetic microbeads coated with rabbit anti-mouse IgG, (Dynabeads, Dynal A.S.) were added at 10 μl/10^6 cells (the beads were previously well washed). The mixture was incubated at 4°C for 10 minutes with occasional gentle mixing. Cells were gently resuspended, a drop was placed on a glass slide to assess the formation of rosettes microscopically and the tube placed in a Dynamagnet (Dynal). The CD23-positive cells bound to the microbeads aligned against the magnet leaving the unbound CD23-negative cells in the supernatant. The latter was collected and the procedure was repeated. The cells in each fraction were spun down and the purity of the CD23-negative fraction was

judged prior to use by taking 10 μl of cell suspension on a glass slide and counting the percentage of rosettes under the microscope. This was generally less than 1%.

1.11 DNA-Mediated Gene-Transfer: Calcium Phosphate Method

This was performed by the calcium phosphate co-precipitation method of van der Eb and Graham (1980). The cells were trypsinised and replated at a density of approximately 5 x 10^5/5 cm dish 24 hours before transfection, and then refed with 4.5 ml of fresh medium 4 hours pre-transfection.

A total of 10 μg of DNA (a combination of plasmid and sonicated salmon sperm DNAs) were added to 465 μl of HeBS [70 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 5 mM glucose, 20 mM HEPES, (pH 7.05)] in a sterile bijou. The gradual addition of 25 μl of a sterile 2.5 M $CaCl_2$ solution produced a very fine white precipitate (within 20 minutes or less). This was added to the tissue culture medium. The cells were then incubated under standard conditions for between 4 and 16 hours before the medium was removed, the cells washed with versene, and 5ml of fresh medium were added.

1.12 DNA-Mediated Gene-Transfer: Electroporation

Experiments were performed using a "Gene Pulser™" apparatus (Biorad) fitted with a Capacitance Extender module (Biorad), all essentially according to the manufacturers instructions. 1-5 x 10^6 cells were pelleted and resuspended in 800 μl of PBS or fresh medium containing 2-10 μg of plasmid DNA. After being transferred to plastic cuvettes containing electrodes set 0.4 cm apart, the cell + DNA suspension was left on ice for 10-20 minutes. Each suspension was then "pulsed" at a single voltage and capacitance setting. At least four voltage settings, typically 0.05, 0.1, 0.2 and 0.4 kV were used at each capacitance (125, 250, 500 and 960 μFD). After "pulsing" the cuvettes were left on ice for 10 minutes and the cells were then transferred to, and cultured in, fresh medium.

1.13 Generation of Drug-Resistant Cell Lines: Adherent Cells

Cells were transfected, as described above with 5 μg of recombinant plasmid or salmon sperm DNA and 1 μg of pSV2Neo (Southern and Berg, 1982). The cells were trypsinised 72 hours post-transfection and replated, at a density of 5 x 10^5 cells /5 cm dish in medium containing Geneticin (400 - 800 μg/ml). The medium was replaced every 4 days, and generally widespread cell death was observed after 7-10 days of selection. Drug resistant colonies, visible after 12-14 days, were isolated using cloning rings after 3-4 weeks and, where possible, expanded into cell lines in medium containing 100 μg/ml Geneticin.

1.14 Generation of Drug-Resistant Cell Lines: B Cells

Cells were electroporated, as described above, with 5 μg of recombinant plasmid DNA and 1 μg of SV2Hyg (J. Sinclair, personal communication). 72 hours after electroporation cells were spun down, washed and resuspended at a density of 5 x 10^5/ml in 2 ml flat bottom wells in medium containing Hygromycin B (500 μg/ml). The medium was replaced every 4 days and wells were periodically examined for outgrowth of drug resistant clumps of cells.

1.15 Indirect Immunofluorescence (IF) Staining for EBNA

Adherent cells were grown on multichamber microscope slides (Lab-Tek, Miles Scientific) or 5 cm plastic dishes and cytospins were made from B-cell suspensions (200g for 5 minutes). All cells were washed twice with complement fixation buffer (CFB) and fixed for 5 minutes in a 1:1 (v/v) mixture of acetone and methanol at -20°C. Slides (or dishes) were air dried and if necessary stored at -20°C. Before incubation with human serum (EBV-positive or EBV-negative, as control) cells were washed in CFB. The first antibody was applied (20 μl/spot, diluted 1/10 in CFB) and incubated in a damp box for 1 hour at 37°C. Slides or dishes were washed 3 times for ten minutes (stirring or shaking) with CFB and then carefully dried around the cell spot with a tissue (Kleenex). Twenty microlitres of antihuman IgG (λ-chain) - FITC (Dako) diluted (1/100) in CFB were then applied to each spot and this was incubated at RT for 1 hour in a moist, dark atmosphere.

After washing 3 times, as above, specimens were mounted in PBS/Glycerol (1:1). The stained slides (or dishes) were kept at 4°C until examined under a UV microscope (Zeiss). EBNA-positive cells could be recognised by their apple-green, granular, nuclear fluorescence.

1.16 Anti-Complement Immunofluorescence (ACIF) Staining for EBNA

(Reedman and Klein 1973)

Cytospins were prepared and fixed as above. Slides were rinsed in CFB and 10 μl of human serum was diluted with 80 μl of CFB and 10 μl of complement to give a final dilution of 1/10. This was applied to the cell spots and incubated for 1 hour at 37°C in a moist atmosphere. (The source of complement used was serum from a healthy donor known to contain no antibodies against EBV related antigens; this was always stored at -70°C). (At this point an optional complement reinforcement step was sometimes used (Klein *et al.*, 1976): 20 μl of complement diluted 1:10 with CFB was added to each cell spot and incubated at 37°C for a further 40 minutes). Slides were washed 3 times as for IF, then 20 μl of goat anti-human complement C3 fluorescein conjugate (Nordic), diluted 1:20 with CFB, was added to each spot. Slides were incubated at room temperature for 1-2 hours in a moist atmosphere and then washed 3 times in CFB, mounted in PBS/glycerol and examined as above.

1.17 Indirect Immunofluorescence (IF) Staining for LMP

Cells were washed twice in complement fixation buffer (CFB), cytospun onto glass slides, air dried and fixed in methanol:acetone (1:1) at -20°C for 5 minutes. First layer antibody was applied (CS1-4 culture supernatant used neat) for 1 hour at 37°C in a moist atmosphere. Slides were washed 3 times for 10 minutes in CFB and then carefully dried around the cell spot. Twenty microlitres of anti-mouse Ig FITC (Sigma) diluted (1/100) in CFB were then applied to each spot and incubated at RT for 1 hour in a moist, dark atmosphere. After washing 3 times, slides were mounted, stored and examined as described above.

2. GENERAL MOLECULAR BIOLOGICAL TECHNIQUES

2.1 Restriction Endonuclease Digestion of DNA

DNA was digested under the conditions prescribed by their commercial suppliers. Typically, digestion was carried out for 2-3 hours at 37°C with an excess (5-10 units/ μg DNA) of enzyme. When Plasmid DNA from small scale "minipreparations" (see below) was digested, 5-10 μg RNAase A were added.

2.2 Dephosphorylation of DNA (Removal of 5'-Terminal Phosphate Groups from DNA)

Restriction endonuclease-digested DNA was dephosphorylated by incubation at 37°C for 60 minutes with calf intestinal alkaline phosphatase, CIP (Boehringer, 0.5 U/pmol ends) in buffer containing 10 mM Tris-HCl (pH 8.0), 1mM $MgCl_2$. The enzyme was then inactivated by the addition of an equal volume of 10mM Tris-HCl (pH 8.0) 1mM EDTA, 0.1% w/v SDS and heating at 68°C for 45 minutes. This was followed by two extractions with buffered phenol and ethanol precipitation to recover DNA.

2.3 Ligation of DNA Fragments

Ligations generally contained insert and vector DNA at a ratio of 3:1. Reactions were usually carried out in 10 μl containing 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 0.5 μl of T4 DNA ligase (400,000 units/ml). They were incubated overnight at 14°C.

2.4 Culture of Bacteria

Bacteria of the DH5 and TG2 strains of *E.coli* were grown on L-Broth containing 100 μg/ml of Ampicillin. LBA plates were prepared from L-Broth containing 1.2% bacto-agar (Difco) and Ampicillin (100 μg/ml) and poured in 9 cm plastic Petri dishes (Sterilin).

2.5 Preparation of Competent Bacteria (Hanahan, 1985)

A single bacterial colony was transferred into 5 ml of Psi-Broth, and shaken overnight at 37°C, before being used to inoculate a further 100ml of Psi-Broth, prewarmed in a 1 litre glass flask. The culture was then incubated at 37°C with vigorous shaking until an OD_{550} of approximately 0.48 was achieved (2-2.5 hours). The cells were then transferred to a sterile 50 ml tube (Falcon 2070), pelleted (Denley BR401, 2,500 rpm, 0°C), and resuspended in 30 ml of ice-cold RF-1 [100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM KOAc, 10 mM $CaCl_2$, 15% (w/v) glycerol (pH 5.8), filter sterilised]. Following incubation at 4°C for 20 minutes, the cells were pelleted (Denley BR401, 2,500 rpm, 0°C) and resuspended in 8 ml of RF-2 [10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% (w/v) glycerol (pH 6.8), filter-sterilised]. The bacteria were then aliquoted into 1.8 ml cryotubes (Nunc 3-63401), and maintained at 4°C for 20 minutes, before snap-freezing in a dry-ice/ethanol bath and storage at -70°C. Both DH1 and TG2 were routinely prepared in this way.

2.6 Transformation of Competent Bacteria (Hanahan, 1985)

Competent cells were partially thawed at RT, then incubated at 4°C (5 minutes). A 200 μl aliquot of competent cells was mixed with DNA (<100 ng, in a volume <50 μl) in a cryotube (Nunc 3-63401), and maintained at 4°C for 20 minutes. The bacteria were then heat-shocked by incubation at 42°C for 2 minutes, followed by chilling on ice for a further 2 minutes. Four volumes of Psi-Broth were added, and the culture was incubated at 37°C, with gentle shaking, for 60 minutes. Aliquots of the transformation mixture (100-400 μl) were spread onto 9 cm LBA plates, which were inverted and incubated overnight at 37°C.

2.7 Selection of Transformants Containing Recombinant "Bluescribe" Plasmids

The cloning vector "Bluescribe" (Stratagene) includes a polylinker sequence with multiple cloning sites in the *lac*Z gene. This means that a blue-white colour selection can be accomplished by plating on LBA plates covered with a thin layer (3 ml/9 cm plate) of LB-agar containing 40 μg/ml X-gal and 5mM IPTG. After growing colonies for 12-18 hours at 37°C, plates were routinely transferred to 4°C

for 1-2 hours to enhance the blue colour. *E.coli* (TG2) containing "Bluescribe" vectors which include most inserts grow much better than those containing vectors without inserts. Thus recombinant colonies are not only white on Xgal/IPTG plates, they are larger than the blue non-recombinants.

2.8 Small Scale Preparation of Plasmid DNA (Birnboim and Doly, 1979; Birnboim, 1983)

Cells from individual bacterial colonies were transferred, using a sterile toothpick, to a sterile plastic universal containing 5 ml of LBA. After overnight incubation at 37°C on a shaking platform, the bacteria were pelleted (Centra 4X, 4,000 rpm for 5 minutes). The supernatant was removed by aspiration and the pellet resuspended in 150 μl of Solution I [1-2% (w/v) glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0)] and transferred to a microfuge tube (Starstedt) and left at RT for 5 minutes. Three hundred microlitres of solution II (0.2 M NaOH, 1% SDS) were then added and after mixing well this was left on ice for 5 minutes. Solution III [5 M KOAc, (pH 5.0)] was chilled on ice and 225 μl were added and the suspension was chilled on ice for a further 15 minutes. The precipitate was pelleted (10 minutes, microfuge, RT) and the supernatant poured into a clean tube. The latter was extracted with phenol and precipitated with ethanol. The pellet was redissolved in 40-50 μl TE (pH 8.0).

2.9 Large Scale Preparation of Plasmid DNA (Birnboim and Doly, 1979)

A single bacterial colony was transferred, using a sterile inoculating loop, into a sterile universal (Sterilin) containing 10 ml of LBA. Following overnight incubation at 37°C on a shaking platform, the culture was used to inoculate 1 L of LBA in a 2 L flask, which was then shaken for a further 16 hours at 37°C. The bacteria were pelleted (Sorvall GS-3, 6,000 rpm, 5 min, 4°C), resuspended in 40 ml of 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 5 mg/ml freshly-prepared lysozyme, and incubated at RT for 10 minutes. Following the sequential addition of 80 ml of 0.2 M NaOH, 1% SDS (5 min, 4°C), and 40 ml of 3 M NaOAc (pH 4.8) (10 min, 4°C), the precipitated cellular debris was removed by centrifugation (Sorvall GS-3, 6,000 rpm, 5 min, 4°C). The DNA in the supernatant

was then precipitated by the addition of 0.6 volumes of propan-2-ol (30 mins, -20°C), and pelleted by centrifugation (Sorvall GS-3, 7,000 rpm, 15 min, 4°C), before resuspension in 10 ml of TE (pH 8.0). The solution was phenol-extracted once, and the DNA reprecipitated by the addition of 2.5 volumes of ethanol (3 hours, -20°C). The DNA was then pelleted (Sorvall HB-4, 10,000rpm, 30 mins, 4°C), and resuspended in 10 ml of TE (pH 8.0), before the addition of 10 g of CsCl, and 1 ml of ethidium bromide (5 mg/ml in dH_2O). This solution was loaded into a Quick-Seal ultracentrifuge tube (Beckman), and centrifuged (Beckman Ti 70.1, 47,000 rpm, 48 hours, 18°C) to separate supercoiled (Form I) plasmid DNA from other plasmid forms and contaminating high-molecular weight cellular DNA. The Form I DNA was removed using a hypodermic syringe, extracted five times with CsCl-saturated propan-2-ol, diluted with two volumes of dH_2O, and precipitated by the addition of 2.5 volumes of ethanol (3 hours, -20°C). The plasmid DNA was then recovered by centrifugation (Sorvall HB-4, 10,000 rpm, 30 mins, 4°C), resuspended in TE (pH 8.0), and adjusted to a concentration of 1 mg/ml following quantitation by measurement of OD_{260}.

2.10 Quantitation of Nucleic Acids

Optical absorptions of nucleic acid samples at 260 nm and 280 nm wavelengths were determined. An OD reading of 1.0 at 260 nm corresponds to 50 μg/ml of double-stranded DNA and 40 μg/ml of RNA. The OD 260/280 ratio (nucleic acid/protein) provides an estimate of the purity of the preparations and should be 1.8 and 2.0 for pure DNA and RNA, respectively.

2.11 Isolation of Total Cellular DNA (Gross-Bellard *et al.*, 1973)

Cells were harvested and washed three times in ice-cold PBS. Tumour specimens were teased apart. Approximately 10^8 cells were resuspended in a minimal volume of PBS and 20 ml of lysis buffer [10 mM Tris-HCl, (pH 8.0), 1mM EDTA, 5mM NaCl, 0.5% (w/v) SDS and 100 μg/ml Proteinase K] was added. The lysate was incubated at 37°C for 16 hours and then extracted twice with buffered phenol. The aqueous phase was dialysed extensively against TE (pH 8.0) and digested with 50 units/ml of T1 RNAase and 0.5 units/ml T2 RNAase for 4 hours at

37°C. The preparation was then extracted twice with buffered phenol and dialysed as before. DNA was ethanol precipitated in the presence of 0.3 M NaOAc, recovered by centrifugation (Sorvall HB-4, 10,000 rpm, 30 min, 4°C), resuspended in TE (pH 8.0) and adjusted to a concentration of about 1 mg/ml.

2.12 Agarose Gel Electrophoresis

Agarose gels (0.8-1.2%) were prepared by adding agarose powder to 200 ml of E buffer [40 mM Tris-acetate (pH 7.5), 2 mM EDTA] and heating to 100°C (microwave, 5 min). After cooling to 50°C, and the addition of ethidium bromide to a final concentration of 0.5 μg/ml, the gel was cast on a horizontal glass plate (20 cm x 15 cm). Once set, the gel was transferred to a flat bed electrophoresis chamber containing 1800 ml of E buffer with 0.5 μg/ml ethidium bromide. DNA samples were mixed with 0.1 volumes of loading buffer [1.25% SDS, 2.5 mM EDTA, 37.5% (w/v) sucrose, 0.15% bromophenol blue] and loaded on to the gel. The DNA fragments were then electrophoresed towards the anode (40 mA, 16 hours, RT); migration was visualised under longwave UV light, and photographed using Polaroid Land 57 film.

Rapid analysis of DNA samples was performed using minigels (10 cm x 8 cm) formed by dissolving the appropriate amount of agarose in 50 ml of TBE buffer (90 mM Tris-borate, 90 mM boric acid). Electrophoresis was performed in TBE buffer (60 mA, 1 hour or less). DNA restriction fragments of known molecular weight (generally *Hin*dIII digested λ or *Hae*III digested ΦX174) were used as size markers.

2.13 Isolation and Purification of DNA Fragments

Following restriction enzyme digestion and agarose gel electrophoresis, it was sometimes necessary to isolate DNA fragments for subcloning or preparation of specific radiolabelled probes. DNA was isolated in one of three ways:

i. Dialysis Elution (McDonnell *et al.*, 1977)

A gel slice containing the DNA band was excised and placed inside a dialysis bag containing E buffer and 1% nuclease free BSA. The bag was placed in an

electrophoresis tank and the DNA electroeluted from the gel slice at 100 volts for 1-2 hours. The polarity was reversed for 15-20 seconds prior to removing the bag (this allowed bound DNA to detach from the dialysis tube wall). The DNA solution was then extracted twice with buffered phenol and ethanol precipitated.

ii. NA45 Elution (adapted from Dretzen *et al.*, 1981)

NA45 paper (DEAE membrane, 0.45 μ, Schleicher and Schull) was cut into small strips (1 cm x 2 cm) and soaked in 2.5 M NaCl, TE (pH 8.0) for about 60 minutes at room temperature. The strips could then be stored in 1 mM EDTA at 4°C. A strip was rinsed in electrophoresis buffer and then inserted into a slot, at 90° and immediately adjacent to the band of DNA to be purified. The gel was then rotated through 90° and the DNA electrophoresed (70-100 mA for 10-60 min) onto the membrane. The NA45 was removed and rinsed briefly in sterile distilled water, then cut in very small pieces and covered with 1.5 M NaCl, TE (pH 8.0) in a microfuge tube. This was incubated at 68-70°C for 20-30 min and the paper was then re-eluted with another 200 μl. The eluates were pooled and extracted with buffered phenol, then precipitated with ethanol. (This method was generally used for DNA fragments of less than 1kbp).

iii. Electrodialysis using a "BIOTRAP"

A gel slice containing the DNA band was excised and placed in the BIOTRAP (Scheicher and Schuell) according to the manufacturer's instructions. After electroelution at 100 V for 1-4 hours the DNA sample was recovered in a volume of 150-200 μl of elution buffer (usually TBE Buffer) and precipitated with ethanol prior to use.

Following recovery by all three methods the purity and quantity of DNA was determined by resuspending in TE (pH 8.0) and running a small aliquot on another agarose gel.

2.14 Preparation of Radioactively-Labelled DNA Probes: Nick Translation

Nick translation reactions (Rigby *et al.*, 1979) were routinely performed using 50 ng of DNA in a reaction mixture containing 50 mM Tris-HCl (pH 7.8), 5 mM

$MgCl_2$, 10 mM β-mercaptoethanol, 50 μg/ml BSA, 2 μM dATP, 2 μM dGTP, 2 μm TTP and 50 μCi of α-(^{32}P)-dCTP (Amersham International, 2-3,000 Ci/mmol), and 20 U of DNA polymerase I (Boehringer) in a total volume of 50 μl. Following incubation at 14°C for 3 hours, labelled DNA was separated from unincorporated radionucleotides by passing the reaction mixture over a 3 ml Dispo-column containing Sephadex-G75 (Pharmacia). The labelled DNA was eluted with TE (pH 8.0), 0.1% SDS; 0.2 ml fractions were collected and monitored for radioactivity using a mini-monitor. The four fractions with the highest activity were used for hybridization.

2.15 Transfer of DNA to Nitrocellulose (Southern, 1975)

Following electrophoresis through agarose gels, DNA was denatured by immersing a gel in 0.5 M NaCl, 0.5 M NaOH for 60 min at RT, with gentle agitation. The gel was then neutralised in 0.5 M NaCl, 1 M Tris-HCl (pH 7.6) (3 hours, RT, with gentle agitation), and transferred to a glass plate, covered with 3MM paper soaked in 20 x SSC, dipping into a reservoir of 20 x SSC. The gel was surrounded with Saran Wrap, and covered with nitrocellulose (Schleicher and Schuell), pre-soaked in deionised H_2O, followed by several layers of 3MM paper. Finally, a stack of paper towels was placed on top of the 3MM paper, and weighted down with a 1 Kg lead weight. The DNA was allowed to transfer by capillary action to the nitrocellulose (overnight), the towels and 3MM paper were removed, the nitrocellulose washed in 5 x SSC (5 min, RT), air-dried, and baked, under vacuum, at 80°C for 2 hours.

2.16 Hybridization

Baked nitrocellulose filters were sealed into plastic bags or plastic cassettes containing 10-20 μl of prehybridization buffer [5 x Denhardt's solution, 6 x SSC, 0.1% (w/v) SDS, 1.5 mM sodium pyrophosphate, 25 mM NaH_2PO_4] and incubated at 68°C for at least 4 hours. The buffer was then replaced with hybridization buffer (pre-hybridization buffer, containing nick-translated DNA and 2 mg of sonicated calf thymus DNA, denatured by placing in a boiling water bath for 8 minutes).

2.17 Preparation of Cytoplasmic RNA (Favalora *et al.*, 1980)

5 x 10^8 lymphocytes were pelleted and washed three times in ice-cold Tris-saline [0.8% (w/v) NaCl, 0.038% (w/v) KCl, 0.1% (w/v) Na_2HPO_4, 0.1% (w/v) dextrose, 0.3% (w/v) Tris-base and 0.00015% (w/v) phenol red]. The cell pellet was resuspended in 10 ml of ice-cold lysis buffer [140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, (pH 8.0), 0.5% (v/v) Nonidet NP40, 0.15% (w/v) Macaloid], vortexed and underlayed with an equal volume of 24% (w/v) sucrose, 1% (v/v) NP40 in lysis buffer. After 5 minutes on ice, the tube was centrifuged at 8,000 rpm for 20 minutes at 4°C in a Sorvall HB4 rotor. The upper cytoplasmic phase was removed and added to an equal volume of 2 x PK buffer [200 mM Tris-HCl (pH 7.5), 25 mM EDTA, 300 mM NaCl, 2% SDS and 400 μg/ml Proteinase K], incubated at 37°C for 30 minutes and extracted with an equal volume of phenol/chloroform. The aqueous phase was then ethanol precipitated, recovered by centrifugation (Sorvall HB-4, 10,000 rpm, 60 minutes, 4°C) and resuspended in 1 mM EDTA (pH 7.0).

2.18 Oligo-(dT) Cellulose Chromatography

Oligo-(dT) cellulose (Bethesda Research Laboratories) was suspended in Binding Buffer [10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1% sarkosyl] and a 1 ml column prepared in a 5 ml syringe with a GF/C filter at the bottom. The column was washed with 20-30 volumes of Elution Buffer [5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS] and then equilibrated with 20-30 volumes of Binding Buffer. The cytoplasmic RNA [dissolved in 1 mM EDTA (pH 7.0) to a concentration of 1-2 mg/ml] was heat denatured at 70°C for 1 minute and rapidly chilled on ice. An equal volume of 2 x Binding Buffer was added to the RNA and it was passed over the column three times. The column was then washed with 10 ml of Midwash Buffer [100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% SDS]. Finally, the poly A^+ RNA was eluted stepwise with 8-9 ml of Elution Buffer, which had been heated to 60°C. The poly A^+ RNA was precipitated with 0.3 M NaOAc (pH 5.3), and three volumes ethanol. RNA was stored in 1 mM EDTA (pH 7.0), or distilled water at -70°C.

3 DIDEOXY CHAIN TERMINATION DNA SEQUENCING. (Bankier *et al.*, 1987)

3.1 Preparation of Competent JM101

An overnight culture of JM101 was grown in 2 x TY broth at 37°C. This was then diluted 1/50 with 2 x TY and shaken vigorously at 37°C for a further 3 hours. The cells were pelleted (Centra 4X, 4,000 rpm at room temperature for 5 minutes), resuspended in 1/2 the original culture volume in 50 mM $CaCl_2$ and left on ice for 20-30 minutes. After repelleting as above cells were *gently* resuspended in 1/10 volume of 50 mM $CaCl_2$ then stored at 4°C for no longer than 24 hours.

3.2 Transfection with Recombinant M13mp8

Two to three microlitres of ligation mix were added to 300 µl of competent JM101 in a sterile bijou tube and this was then left on ice for 45 minutes. The mixture was heat shocked at 42°C for 90 seconds (or at 37°C for 2-3 minutes) and top agar [0.8% (w/v) agar, 1% (w/v) tryptone, 0.8% (w/v) NaCl; 3 ml/transfection], kept at 50°C and supplemented with 30 µl 2% (w/v) X-gal in DMF, 30 µl 2.5% (w/v) IPTG and 200 µl fresh JM101 cells (overnight culture diluted, then shaken at 37°C for several hours) was added. After thorough mixing, this was poured onto an H-plate [1.4% (w/v) agar, 1% (w/v) tryptone, 0.8% (w/v) NaCl in a 9 cm plastic Petri dish]. Plates were incubated, inverted, at 37°C overnight.

3.3 Phage Growth and DNA Template Purification

White plaques were transferred with sterile toothpicks to individual sterile bijou tubes containing 2 ml of a 1/100 dilution of a JM101 overnight bacterial culture grown in 2 x TY. This was incubated, with vigorous shaking, for 4.5-5.5 hours (*no longer*) and the suspension was then transferred to a microfuge tube. The bacteria and bacterial debris were pelleted by centrifugation (5 minutes at room temperature in a microfuge) and the supernatant was decanted to a new tube (1 ml supernatant contains approximately 10^{12} pfu and can be kept as a phage stock). The phage were precipitated by incubation on ice for 15-20 minutes with 200 µl of 20%

(w/v) PEG/2.5 M NaCl and pelleted by spinning for 10 minutes at room temperature in a microfuge. The bulk of the supernatant was removed by aspiration and after a further 1 minute centifugation the remainder was removed. The phage pellet was redissolved in 100 μl TE (pH 8.0) and left at room temperature for 10 minutes. After 30 seconds vortexing, the suspension was extracted with *freshly* buffered phenol (pH 8.0). The DNA was precipitated with 0.3 M NaOAc, (pH 5.3) and ethanol, pelleted and washed with cold 95% ethanol before being air dried and redissolved in 30 μl of TE (pH 8.0).

3.4 Sequencing dNTP, ddNTP Mixes and Chase Mix

Stock dNTP (N) mixes were made up according to the following table and stored at -20°C.

	A	G	C	T
0.5 mM dATP	1	20	20	20
0.5 mM dGTP	20	1	20	20
0.5 mM TTP	20	20	20	1
TE pH 8.0	5	5	5	5
(volumes in μl)				

Stock ddNTP mixes: 0.1 mM ddATP, 0.15 mM ddGTP, 0.05 mM ddCTP and 0.4 mM dTTP were stored at -20°C. Each mix (A, G, C or T for a single chain termination reaction) was made fresh on the day by adding 8 μl ddNTP to 8 μl of the appropriate N mix. (This provided sufficient for six M13 clones and was stored on ice until required).

Stock chase mix: 0.5 mM TTP, 0.5 mM dCTP, 0.5 mM dGTP and 0.5 mM dATP was stored at -20°C.

3.5 Annealing Primer

A mixture of 8 μl template M13 DNA, 1 μl 17-mer primer (0.2 pmol, 1 ng, Collaborative research) and 1 μl of 10 x TM Buffer [100 mM Tris-HCl (pH 8.0), 100

mM $MgCl_2$] were added to a microfuge tube. This was allowed to anneal by heating to 60°C (in an electric oven) for 60 minutes. Tubes were briefly spun in a microfuge to drive down the condensation. The template/primer mix for each clone was then aliquoted into 4 capless Starstedt tubes (2.2 μl/tube).

3.6 Chain Elongation/Termination Reaction

Using a Hamilton rapid dispenser (PB600-1 fitted with a 1710 gas tight syringe and lure tip for fitting disposable yellow tips) 2 μl of A mix was added to the *side* of all A reaction tubes. G, C and T mixes were similarly dispensed. Klenow enzyme [0.125 units/μl (1-2 units /clone)] was added to freshly made Klenow mix (30-60 μCi α-(^{32}P)dCTP, 5.5 μl 10 x TM Buffer 4.5 μl 100 mM DDT and (x μl of) dH_2O to a final volume of 55 μl). This was then immediately dispensed (2 μl/tube) to each reaction. The reaction tubes were mixed by centrifugation and then left for 5 minutes at room temperature followed by 10 minutes at 37°C. Chase mix (2 μl/tube) was added and the tubes were incubated for a further 15-20 minutes at 37°C. Reactions were stopped by the addition of 4 μl of formamide dye mix [0.1% (w/v) xylene cyanole FF, 0.1% (w/v) bromophenol blue, 10 mM EDTA (pH 8.0) in deionised formamide] and immediately before loading onto a sequencing gel the samples were heated at >95°C for 3 minutes (uncovered to allow evaporation and concentration of the sample).

3.7 Acrylamide Gel Electrophoresis and Autoradiography

After denaturation, 1-3 μl of sample/well were loaded onto an 8 M urea, 6% polyacrylamide gel. Gels were run with 1 x TBE Buffer in both the top and bottom reservoir troughs at a constant current of 28-30 mA for 2-3 hours until the BPB dye had just run off the end of the gel. After completion of electrophoresis, gels were allowed to cool for 10 minutes before separating the glass plates. Gels were acid fixed in 10% acetic acid for 10-15 minutes, then rinsed in water. After draining and drying well with paper towels (Kim wipes) a careful transfer of the gel to Whatman 3MM paper was performed and the gel covered in Saran wrap for drying under vacuum for 30-50 minutes at 80°C. The film was exposed in an X-ray cassette to Fuji RX X-ray film overnight.

4. PROTEIN AND IMMUNOCHEMICAL METHODS

4.1 Induction and Extraction of β-Galactosidase and β-Galactosidase Fusion Products (adapted from Koenen *et al.*, 1985)

A streak of TG2 cells was transferred, using a sterile inoculating loop, into 10 ml of LBA. Following overnight incubation at 37°C on a shaking platform, the culture was used to inoculate a 1 L flask containing 400 ml of LBA. This was incubated with vigorous shaking for a further 4-5 hours at 37°C, transferred to room temperature and β-galactosidase expression was induced by the addition of 0.4 mg/ml IPTG. Induction was continued, with gentle shaking, for up to 4 hours at room temperature. After pelleting (Centra X, 4,000 rpm at room temperature for 5 minutes) cells were resuspended in 4 ml Buffer A [10 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 200 mM NaCl, 1.0 mM EDTA, 1 mM DTT]. The cell suspension was sonicated three times for 1 minute in an ice/NaCl bath (MSE, Soniprep 150, 18 micron setting). The cell debris was then pelleted (Beckman L8-M ultracentrifuge, 25,000 rpm in a SW55Ti rotor at 4°C for 1 hour). The supernatant was removed, snap-frozen on dry ice and stored at -70°C until further use.

4.2 Substrate Affinity Chromatography (Koenen *et al.*, 1985; adapted from Steers *et al.*, 1971)

Approximately 3 ml of the TG2 cytoplasmic extract (see above) was applied to a 1.5 ml p-amino-phenyl-β-D-thiogalactoside agarose (Sigma) column, previously equilibrated with buffer A (above). The column was then washed thoroughly with 50-100 ml buffer A [until no detectable protein (as judged by OD_{280}) was found in the column flow-through]. β-Galactosidase products were eluted with 3 bed volumes of 100 mM sodium borate (pH 10.05) and immediately dialysed against PBS (5 hours with one change) and then aliquoted for storage at -70°C until use. The purity of the preparation was judged by SDS-polyacrylamide gel electrophoresis. (All manipulations in this purification were carried out in a 4°C cold room).

4.3 Protein Extraction from Cells and Tumours

Cells were washed twice with cold PBS, pelleted (1,000 rpm, 5 minutes at 4°C) and lysed by the addition of SDS sample buffer [2% (w/v) SDS, 80 mM Tris-HCl (pH 6.8), 0.35 M 2-mercaptoethanol, 10% (v/v) glycerol, 0.025% (w/v) bromophenol blue]. Generally 100 µl of buffer was used for 3 x 10^6 cells. Samples were transferred to microfuge tubes and sonicated for 1-3 x 10 seconds (MSE Soniprep 150, 14 micron setting) to shear DNA. The tubes containing lysate were placed in a boiling water bath for 3 minutes and the lysates clarified by centrifugation in a microfuge for 5 minutes.

Tumour samples were teased apart with scissors, scalpel and forceps in an approximately equal volume of RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 and 0.1% SDS]. A similar volume of SDS-sample buffer was added and the lysates were then sonicated, boiled and clarified as above.

4.4 SDS-Polyacrylamide Gel Electrophoresis (adapted from Laemmli, 1970)

An acrylamide stock (30% acrylamide, 0.8% Bis-acrylamide) was made up and stored (wrapped in bacofoil) at 4°C. From this stock gels were poured according to the following table:

	7.5%	10%	5% (stacking)
Acrylamide stock	7.5	10	1.67
1 M Tris, pH 8.8	11.2	11.2	-
1 M Tris, pH 6.8	-	-	1.25
10% (w/v) SDS	300 µl	300 µl	100 µl
dH_2O	11.2	8.7	7.0
10% (w/v) Ammonium persulphate (fresh)	100 µl	100 µl	50 µl
TEMED	20 µl	20 µl	10 µl

(volumes in ml unless otherwise indicated)

Gels were generally cast in plates separated by 1.5 mm spacers. The proteins (extracts from 5 x 10^5 - 5 x 10^6 cells/track) were electrophoresed for 2-4 hours at 36-40 mA (constant current) in buffer containing 25 mM Tris-base, 192 mM glycine, 0.1% (w/v) SDS. If gels were not to be immunoblotted they were fixed and stained in 50% methanol, 10% glacial acetic acid, 0.1% (w/v) PAGE Blue G90 or Coomassie Brilliant Blue R250 (BDH), then destained in 10% methanol, 7% glacial acetic acid. Prestained molecular weight proteins (BRL) were used as standards.

4.5 Western Immunoblotting (adapted from Burnette, 1981)

Following electrophoresis SDS-polyacrylamide gels were transferred to 100 ml of transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol) containing 0.1% SDS in a plastic lunch box and allowed to equilibrate for 30 minutes. Proteins were then electrotransferred to nitrocellulose (0.45 μM Schleicher and Schuell) in a Transblot tank (water cooled with an LKB 2219 multitemp II cooling system) containing 2.5 litres of transfer buffer. Electrophoresis was carried out at 8 volts/cm electrode separation (48 volts) overnight with continuous stirring of the transfer buffer.

Nitrocellulose filters were briefly rinsed in PBS (pH 7.2), 0.05% Tween 20 (PBS-Tween), and then "blocked" by incubating in PBS-Tween containing 5% powdered milk (Marvel, Cadbury) for 30 minutes at room temperature or longer (as convenient) at 4°C. After blocking, the first antibody was applied at the appropriate dilution in PBS-Tween-milk and incubated, with gentle shaking, for 4-5 hours at room temperature or overnight at 4°C. The first antibody was then poured off and filters washed 3 x 20 minutes, with vigorous shaking, in PBS-Tween. The second antibody (for all blots probed with human serum this was anti-human IgG-peroxidase conjugate, Nordic) was diluted 1/1000 in PBS-Tween-milk and incubated with the filter for 30 minutes whilst shaking gently. The second antibody was poured off and the filters again washed (3 x 20 minutes) in PBS-Tween with vigorous shaking. Filters were then transferred to 10 ml of substrate mix [0.4 mg/ml 3,3-diamino benzidine tetrahydrochloride (Grade II, Sigma), 0.012% H_2O_2 in 50 mM Na_2HPO_4 and 25 mM citric acid, (pH 5.0)]. Colour was allowed to develop for 10-60 seconds and filters were washed with a large volume of flowing water and dried

between sheets of 3MM paper. For blots probed with the murine monoclonal antibodies CS1-4 (Rowe *et al.*, 1987), when the excess first antibody had been washed off, filters were incubated for 1 hour, with rabbit anti-mouse Ig (Dako) diluted 1/1000 in PBS-Tween-milk. They were again thoroughly washed (as above) and finally incubated for 2-3 hours with ^{125}I labelled, affinity purified Protein A (Amersham) diluted to a concentration of 0.1 μCi/ml in PBS-Tween-milk. After 3 x 40 minutes washing the filters were blotted dry and wrapped in Saran wrap and exposed to X-ray film (Kodak XAR or Fuji RX) for 1-10 days at -70°C.

4.6 Sequential Extraction of Nuclear Proteins (Sculley *et al.*, 1983)

Cells were collected by centrifugation (1000 rpm, 5 minutes, 4°C), washed twice with PBS and then resuspended in nuclei isolation buffer [0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4), 0.1 mM PMSF] containing 0.5% (w/v) Triton X-100 (500 μl of buffer/200 μl of packed cells). They were left on ice for 5 minutes and collected by centrifugation at 800g for 5 minutes. This process was repeated twice more and the final pellet represented *purified nuclei* (total nuclear fraction). The pooled supernatants represented the *cytosol fraction.*

Purified nuclei were resuspended in 0.4 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.1 mM PMSF (300 μl of buffer/200 μl of packed nuclei) and incubated at 4°C for 5 minutes. The nuclei were then collected by centrifugation. This extraction was repeated once again. The two extracts were pooled and the combined preparation was clarified by centrifugation (microfuge, 10 minutes, 4°C). The supernatant represented *nuclear extract I.* The 0.4 M NaCl extracted nuclei were resuspended in a solution containing 5 M urea (deionised), 2 M NaCl, 0.5 mM NaH_2PO_4, 0.5 mM Na_2HPO_4 (pH 6.8) (300 μl/200 μl of original cells). This was sonicated on ice for 10-20 seconds (MSE, Soniprep 150, 14μ setting). The solution was then allowed to stand on ice for 10 minutes and insoluble material was pelleted (microfuge, 10 minutes at 4°C). The pellet was re-extracted with another 300 μl of urea buffer and the supernatants combined. This represented the *Chromatin fraction* (*nuclear extract II*). All fractions were mixed with an equal volume of SDS-sample buffer (and sonicated if necessary), boiled and analysed by SDS-PAGE and immunoblotting (see above).

4.7 Immunoaffinity Purification of Anti-EBNA6 Antibodies.
(adapted from Smith and Fisher, 1984; Allday *et al.*, 1988)

Approximately 500 μg of e_2-β-galactosidase fusion protein were electrophoresed through a 7.5% SDS-polyacrylamide gel and electrotransferred to nitrocellulose. After probing with the RT serum and staining for peroxidase activity as in other Western blots (see above) the filter was kept wet in distilled water. The band(s) corresponding to the fusion protein were excised and cut into small fragments. Specific antibodies were eluted from strips using 5 mM glycine-HCl (pH 2.3), 500 mM NaCl, 0.5% (v/v) Tween 20, 100 μg/ml BSA (300 μl/cm of nitrocellulose) and immediately neutralised by making the solution 50 mM with respect to Na_2HPO_4.

5. cDNA CLONING AND ANALYSIS (adapted from Watson and Jackson, 1985; Huynh *et al.*, 1985; Kaiser and Murray, 1985)

5.1 First Strand cDNA Synthesis

Cytoplasmic poly(A^+) RNA from the B95-8 marmoset B cell line was prepared as described above. For a 100 μl reaction, 5 μg of this polyadenylated RNA in 10 μl dH_2O, was heated for 5 minutes at 65°C. The tube was then chilled on ice and the RNA transferred to another tube containing 4 μg of Actinomycin D (Calbiochem) (the solvent, 80% ethanol, had been evaporated by air drying). The following reaction mix was then added: 20 μl of 5 x RT buffer [250 mM Tris-HCl, (pH 8.3 at 42°C), 40 mM $MgCl_2$, 250 mM KCl] 20 μl of 5 x d(ACTG)TPs [a mixture of each deoxynucleoside triphosphate at 5 mM in Tris-HCl (pH 7.5)], 10 μl of 200 μg/ml oligo$(dT)_{12-18}$ (Pharmacia), 1 μl of 1 M DTT (filtered), 60 units of RNAsin (P & S Biochemicals, 100 units of reverse transcriptase (Anglian Biotechnology, super RT), 10 μCi α(^{32}P)dCTP (Amersham, 3,000 Ci/mmol) and dH_2O to 100 μl. The tube was incubated at 42°C for 2 hours and the reaction stopped by the addition of 5 μl EDTA (pH 8.0), 2.5 μl of 10% SDS, 50 μl of freshly buffered phenol and 50 μl of chloroform. The phases were separated by centrifugation (microfuge, 5 minutes, 4°C) and the aqueous phase removed and

extracted with ether. The heteroduplex RNA-DNA was precipitated by adding an equal volume (100 μl) of 4 M NH_4OAc and 4 volumes of ethanol, and incubation on dry ice for 15 minutes. The precipitate was pelleted (microfuge, 20 minutes, 4°C), resuspended in 50 μl TE (pH 8.0) and reprecipitated. The pellet was dried under vacuum.

5.2 Second strand cDNA Synthesis

The RNA-DNA pellet from the first strand reaction was resuspended in 28 μl dH_2O and to this were added: 25 μl of 4 x Pol buffer [0.4 M Hepes, (pH 7.6), 16 mM $MgCl_2$, 0.27 M KCl, 62 mM 2-mercaptoethanol] 2.5 μl of 5 x d(ACTG)TP, 1 μl of 15 mM β-NAD (Sigma), 10 μCi of $\alpha(^{32}P)$dCTP, 2.5 μl of *E. coli* DNA ligase (9 units, New England Biolabs), 150 units *E. coli* DNA polymerase (New England Biolabs), 1 unit RNAse H (BRL). The volume was adjusted to 100 μl with dH_2O. The reaction mixture was then incubated for 1 hour at 14°C and then 1 hour at room temperature. A 2 μl aliquot was removed to check that the cDNA synthesis was efficient (see below) and the reaction was stopped by adding 5 μl of 0.5 M EDTA (pH 8.0), 2 μl of 10% SDS, 50 μl of freshly buffered phenol (pH 8.0) and 50 μl of chloroform. After vortexing to mix, the phases were separated by centrifugation (microfuge 5 minutes, room temperature); the aqueous phase was then removed and precipitated with 0.3 M NaOAc and 3 volumes of ethanol.

5.3 Assay for cDNA synthesis by Agarose Gel Electrophoresis

cDNA samples were mixed with an equal volume of TE (pH 8.0) and 1/5 volume of DNA loading buffer and run through a 1.2% agarose gel adjacent to ^{32}P-end labelled size standards (obtained from D. King). After electrophoresis, the gel was placed between Saranwrap and multiple-layers of 3MM paper and pressed flat with a 1 kg lead weight. The dried and flattened gel was then covered in Saranwrap and exposed overnight to X-ray film (Kodak, XAR) to visualise the distribution of species which had incorporated ^{32}P.

5.4 Preparation of the cDNA for Ligation

i. "Filling in" Reaction:

The homoduplex cDNA was resuspended in 25 μl of dH_2O and to this were added 1 μl of 5 x d(ACGT)TPs, 1 μl of 2 mg/ml BSA (Pentex, nuclease free), 9 units of T4 DNA polymerase and 3 μl of 10 x T4 polymerase buffer [300 mM Tris acetate (pH 7.9), 660 mM KOAc, 100 mM MgOAc, 1 mg/ml BSA (Pentex, nuclease free), 5 mM DTT]. The reaction mix was incubated for 30 minutes and extracted with 60 μl of phenol/chloroform [1:1 (v/v)]. After phase separation, the aqueous phase was ether extracted, pelleted, dried and resuspended in 4.8 μl of dH_2O.

ii. Methylation of *Eco*RI Sites:

To the cDNA (4.8 μl) were added 1.0 μl of S-adenosyl-L-(methyl-^{3}H) methionine (1 μCi, Amersham), 2.0 μl of 5 x methylase buffer [500 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0)], 2.0 μl of 2 mg/ml BSA and 0.2 μl (6 units) of *Eco*RI methylase (New England Biolabs). The reaction mixture was incubated at 37°C for 20 minutes, extracted with phenol/chloroform, extracted and precipitated as above.

iii. Ligation of Linkers to cDNA:

The methylated pellet was resuspended in 5 μl of kinase buffer [10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP] with 5 μl of kinased linkers (500 ng, Pharmacia, for kinasing reaction, see below), 5 mM ATP and 0.5 μl of T4 DNA ligase (New England Biolabs, 400 units/ml) and incubated overnight at 14°C.

iv. Digestion of Linkered DNA:

The ligated cDNA/linkers were heated at 65°C for 10 minutes and briefly spun in a microfuge to collect the condensate. To this were added 1.5 μl of 1 M NaCl (filtered), 5.6 μl dH_2O and 140 units of *Eco*RI (Boehringer) and the mixture was incubated for 2 hours at 37°C. After digestion 20 μl of TE (pH 8.0) were added and two phenol and three ether extractions were performed. The resulting aqueous phase was precipitated with ethanol in the presence of 2 M NH_4OAc three times (twice at -20°C and once at room temperature). The final pellet was washed twice with 70% ethanol (chilled to -20°C), then resuspended in 5 μl of TE (pH 8.0). A

1 μl aliquot was run on a 1% agarose gel (as described above) to check the quality and quantity of recovered cDNA.

5.5 Kinasing Linkers

Dephosphorylated linkers (5 μg in 5 μl, Pharmacia) were mixed with 5 μl of fresh 10 x kinase buffer [10 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP] and 36 μl of dH_2O. One microlitre of γ-(^{32}P)ATP (10 μCi, Amersham) and 3 μl of T4 polynucleotide kinase (15 units, Boehringer) were added and the reaction mix incubated at 37°C for 1 hour. Aliquots (5 μl) were then stored at -20°C. The success of the kinasing reaction was assessed by ligating an aliquot of the linkers, then subsequently (after heat inactivation) digesting a portion. The reaction products were assessed by electrophoresis on a 6% acrylamide-7 M urea sequencing gel (see above).

5.6 Ligation of Vector to cDNA

One microlitre of cDNA (approximately 100 ng) was copreciptated with 5 μg of *Eco*RI digested λgt10 (obtained from M. Jones). After pelleting and air drying, the DNA was resuspended in a 5 μl ligation mix [60 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM ATP and 200 units T4 ligase]. This was incubated overnight at 14°C.

5.7 *In vitro* Packaging and Plating out Hybrid Phage

Recombinant λgt10 were packaged using a commercial (Amersham) *in vitro* packaging kit based on the method of Hohn (1979). 1 μl of a 5 μl ligation was included in each packaging reaction. Each packaging extract was then diluted to 0.5 ml with SM buffer [100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 0.01% (w/v) gelatin]. This diluted extract was termed "the library". The fraction of phage which were recombinants (that is clear plaque-forming) and the percentage of those which contained insert fragments (as opposed to only linkers) was determined by plating a small aliquot (0.01%) of the "library" on *E. coli* strain BNN93 (C600) on LB-plates (see below) and preparing plate lysate DNAs from 12-24 independent

clear-plaque phage (see below). This DNA was then analysed by agarose gel electrophoresis after *Eco*RI and also *Bgl*II/*Hin*dIII digestion.

5.8 Preparation of Plate Lysate λ DNA

Independent plaques picked with sterile toothpicks were dissolved in 500 µl of SM buffer (see above) plus 10-20 µl of $CHCl_3$. A 5 µl aliquot of phage was mixed with 200-300 µl plating cells [an overnight culture grown in LB medium, (pH 7.5), 0.2% (w/v) maltose, pelleted and resuspended in 1/2 the original culture volume in 10 mM $MgSO_4$, 0.2% (w/v) maltose] and added to 3 ml agarose top [LB medium, 0.7% (w/v) agarose (SeaKem), 10 mM $MgSO_4$ and 0.2% (w/v) glucose] which was kept liquid at 46°C. This mixture was then poured on an agarose-glucose plate [LB medium, 1.5% (w/v) agarose, 10 mM $MgSO_4$ and 0.2% (w/v) glucose] and incubated (not inverted) 4-6 hours at 37°C. The plates were then cooled at 4°C for 60 minutes and incubated overnight at 4°C with 5 ml of SM buffer. The supernatant was removed and 760 µl were removed for DNA extraction and the remainder was stored (+ $CHCl_3$) at 4°C. The 760 µl aliquot was spun (microfuge, 3 minutes, room temperature) and then after transfer to a clean tube incubated at 37°C for 30 minutes with RNAaseA (10 µg/ml) and DNAaseI (10 µg/ml). An equal volume of 20% PEG-6000, 2 M NaCl was added and then, after thorough mixing, the tube was left on ice for 2 hours. The phage were pelleted (microfuge, 10 minutes, room temperature) and the supernatant removed by aspiration. The spinning and aspiration were repeated and the pellet was resuspended in 2-300 µl of SM buffer. This was vortexed and adjusted to 0.5% SDS, 20 mM EDTA and 100 µg/ml Proteinase K, then incubated at 37°C for 30 minutes. The DNA was twice extracted with an equal volume of phenol/chloroform and ethanol precipitated in the presence of 0.3 M NaOAc. The pellet was redissolved in 50 µl TE (pH 8.0). The DNAs were digested with *Bgl*II/*Hin*dIII and *Eco*RI in the presence of 10 µg/ml RNAase and analysed by agarose gel electrophoresis.

5.9 Plating Out λgt10 for Screening and /or Amplification

Aliquots of packaging mixture (or amplified library) containing 10^4-5 x 10^4 λ (pfu) were mixed with 200-300 μl of *E. coli* BNN102 (C600-*Hfl*) plating cells and incubated at 37°C for 20 minutes. Approximately 7 ml of melted top agarose (LBM + 0.7% agarose), at 46°C, was added to each aliquot and the mix was poured onto a 142 mm diameter LB-agar plate (containing 20 mM $MgSO_4$). Plates were incubated at 37°C for 6-8 hours (plaques were not allowed to fuse). The plates were transferred to 4°C and left for a further hour. Nitrocellulose filter lifts were taken and processed as described below. The library was amplified by overlaying each cooled 142 mm plate with 12 ml of chilled SM buffer and allowing these to stand overnight at 4°C. The overlay solution containing phage was removed from each plate with a Pasteur pipette, the plates were rinsed with a further 4 ml of SM buffer and all the overlays were pooled and $CHCl_3$ (to 5%) was added. After 15 minutes gentle shaking at room temperature to mix and equilibrate, the debris was removed by spinning (4,000 rpm, 5 minutes, 4°C). Libraries were then stored in aliquots (containing 0.3% $CHCl_3$) at 4°C and, for long term storage, with 7% DMSO, frozen at -70°C.

5.10 Preparation of Filter Lifts for Hybridization (Allday and Jones, 1988)

Routinely (for library screening) 50,000 phage were plated onto LB-agar (+ 20 mM $MgSO_4$) in 142 mm diameter plates. When plaques had developed the plates were left at 4°C for 60 minutes. Duplicate filter lifts were taken and left to air dry. The filters were then stacked between pieces of Whatman 3MM paper and placed into envelopes of 3MM paper (< 20 per envelope) and these were wrapped in aluminium foil. Water was brought to the boil in a standard kitchen pressure cooker, the packets of filters placed on a stand inside the pressure cooker above the level of the water and autoclaved at full pressure for 3 minutes. The pressure weight was removed with forceps to allow the steam to escape rapidly, and the filters were removed immediately and spread out to dry (alternatively the whole packet was dried under a hot lamp). The filter, after baking *in-vacuo* at 80°C for 1-2 hours, were prehybridised and then hybridised as described above.

5.11 Plaque Purification and Subcloning into Bluescribe

After primary screening, phage plugs corresponding to a region of hybridization were removed from the agar plates using a 2 mm cork borer. Each plug was soaked in 500 μl of SM buffer (+ 10-20 μl $CHCl_3$) overnight at 4°C. The pfu was determined (generally found to be about 5 x 10^7 pfu/ml) and between 100-1000 plaques were plated out on 90 mm LB-plates (as above) for secondary screening. Following this it was generally possible to purify a single plaque, prepare DNA by the plate lysate technique (above) and determine the insert size. *Eco*RI digested recombinant λ DNA was then ligated to *Eco*RI digested Bluescribe (Stratagene) or *Eco*RI digested M13mp8 and recombinants selected as described in previous sections.

6. HANDLING TUMOUR MATERIAL

6.1 Passage of the C15 Tumour (Busson *et al.*, 1988)

C15 tumour fragments (approximately 2 mm^3) were initially transported in RPMI + 5% FCS + antibiotics (approximately 10 hours at 37°C). These were then inoculated by skin incision subcutaneously into the backs of six Swiss nude mice (ICRF, London). The incisions were sutured with sterile 9 mm wound clips, (Autoclip, Clay Adams). Tumours were recovered after 5 to 7 weeks, fragmented into 2 mm^3 pieces and used for subsequent passages. Alternatively they were snap-frozen and stored at -70°C or used in the experiments described.

6.2 Human Tumour Biopsy and Autopsy Material

All human tumour material was teased apart with scalpel and scissors, homogenized, then solubilised in either SDS-sample buffer or DNA lysis buffer under Category 3 containment conditions prior to analysis in the open laboratory.

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APPENDIX

Publications associated with the study presented in this thesis:

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