The Epstein-Barr virus (EBV) is a human B lymphotropic herpesvirus carried in a persistent state by over 90% of the world’s population. Primary infection occurs by the oral route, generally in early childhood, and is asymptomatic. When infection is delayed until adolescence or later, infectious mononucleosis results in approximately 50% of cases. Although it has been postulated that the oropharynx provides a reservoir where circulating B cells can be infected during transit through this anatomical site, evidence has been presented suggesting that EBV can also persist in the B cell (Gratama et al., 1988; Yao et al., 1989a, b). Cellular immune mechanisms are believed to be of the greatest importance in the suppression of infected cell proliferation, and the balance between continual infection and suppression results in a life-long carrier state (reviewed by Rickinson, 1986). In individuals with inherited or acquired immunodeficiency states, the proliferation of the EBV-infected B cells is not controlled and can result in polyclonal lymphoproliferative lesions as well as B cell lymphomas (Kalter et al., 1985; Young et al., 1989b; Falk et al., 1990; Gratama et al., 1991). Burkitt’s lymphoma (BL), undifferentiated nasopharyngeal carcinoma (NPC) and certain forms of Hodgkin’s lymphoma are also associated with EBV infection; however, the role played by the virus is unclear. Infection of cotton-top tamarins (Sanguinus oedipus oedipus) with large doses of EBV regularly gives rise to multiple EBV-infected B cell lymphomas appearing within 14 to 21 days (Cleary et al., 1985). Similar lymphoproliferative disease results when SCID mice, which lack mature T and B cells, are administered with EBV-infected lymphoblastoid cell lines (LCLs) (Rowe et al., 1991) or peripheral blood lymphocytes from EBV-seropositive individuals (Purtilo et al., 1991).

EBV acts as a polyclonal activator of B cells, inducing differentiation and immunoglobulin production in a T cell-independent manner (Rosen et al., 1977). Infection of human B cells with EBV in vitro results in their immortalization and the creation of an LCL (Pope et al., 1968). Thorley-Lawson & Mann (1985) proposed that the immortalization of B cells by EBV involves two processes. The first parallels the normal pathway of B cell activation (proliferation and differentiation), whereas the second involves fixing the cells at the lymphoblast stage of differentiation leading to indefinite proliferation. The immortalized cells are said to be ‘latently infected’ and the cells express only a limited number of viral genes. These latent genes code for six nuclear proteins, EBV nuclear antigens (EBNAs)-1, -2, -3A, -3B, -3C and leader protein (EBNA-LP), three membrane proteins, designated latent membrane protein (LMP)-1, -2A and -2B and two small untranslated RNA species termed the EBV-encoded RNAs (EBERs). The roles played by these viral gene products in the immortalization process are the subject of this review. Upon infection, the virus binds to the C3d (complement) receptor (Fingeroth et al., 1984) and penetration is complete in 1 to 2 h. Expression of EBNA-2 and -LP can be detected 8 to 10 h post-infection, followed by the other EBNAs. LMP-1 and -2 are not detectable until 40 to 48 and 70 h post-infection, respectively (Allday et al., 1989; Alfieri et al., 1991). The single circular genome is then amplified to approximately 50 copies per cell by about 1 to 2 weeks post-infection (Hurley & Thorley-Lawson, 1988).

Despite great progress in the understanding of the molecular biology of EBV and its effects upon B cells, it has not been possible to isolate a gene or genomic region solely responsible for immortalization. The transient induction of the activation antigen CD23 following exposure of B cells to u.v.-inactivated EBV (Gordon et al., 1986) may indicate that either some virion component is required, or that the virus-receptor interaction plays an integral part in the immortalization process. It is known that EBV infection is closely associated with B cell activation, normally triggered by the binding of antigens or mitogens to cell surface molecules. Several biological activities are required for immortalization.
Some are directed at the target cell, such as inducing B cells resting in $G_0$ phase to become competent to enter the cell cycle, and causing continuous cell division without inducing terminal differentiation. Transcription and translation of viral genes that function during latency must be facilitated, whereas expression of genes involved in viral replication must be suppressed. Studies have concentrated on the viral genes expressed immediately following infection and during latency, on the assumption that they are likely to be required for the initiation and maintenance of immortalization. It is likely that the immortalizing potential of EBV underlies the pathogenesis of infectious mononucleosis and other lymphoproliferative lesions, and possibly plays a role in the development of Burkitt's and Hodgkin's lymphomas. Since EBV possesses a large DNA genome (approximately 180 kbp) and no efficient experimental system exists for viral replication, it has been difficult to generate specific mutant viruses. Indeed, it has only been in the last few years that EBV strains containing specific mutations have been engineered and their effects studied. Prior to this, analysis of the EBV immortalizing function was limited to the investigation of naturally occurring EBV strains that are unable to immortalize B cells, or studying the effects of the EBV latent proteins when expressed individually following gene transfer into EBV-negative cell lines.

**The EBV latent proteins**

**EBNA-1**

Of all the viral gene products expressed in latent infection, a function has been demonstrated most clearly for EBNA-1. This protein exhibits an $M_t$, ranging from approximately 65K to 95K and consists of a short amino-terminal region, a 20K to 40K glycine–alanine repetitive sequence flanked by arginine-rich sequences, and a highly charged acidic carboxy-terminal region (Hennessy & Kieff, 1983). EBNA-1 binds to two components of the latent cycle origin of replication, $oriP$, and is the only virus-encoded trans-acting factor required for episomal maintenance of the EBV genome (Yates et al., 1984, 1985). EBNA-1 is detectable in all EBV-infected cells, but the promoter used in BL and NPC cells is different from those used in LCLs (Sample et al., 1991; Schaefer et al., 1991; Smith & Griffin, 1992). It appears that EBNA-1 controls its own expression since it activates the $oriP$ enhancer that regulates the promoter involved in the transcription of the EBNA genes in LCLs (Sugden & Warren, 1989) and also binds to a sequence near to the promoter controlling the expression of EBNA-1 in BL and NPC cells (Sample et al., 1992). Inoue et al. (1991) identified a basic helix–loop–helix-like motif in EBNA-1, similar to those in a number of DNA-binding proteins involved in transcriptional regulation. The EBNA-1 protein binds to specific DNA sequences in the form of a dimer (Ambinder et al., 1991) and initial mutational analyses indicated that the regions of the protein important for DNA binding and trans-activation of $oriP$ are located in the carboxy-terminal third of the protein (Polvino-Bodnar et al., 1988; Ambinder et al., 1991; Inoue et al., 1991). More recently, analysis of an extensive series of deletion and point mutants have localized three separate subdomains within this carboxy-terminal region, one involved in DNA recognition and two required for dimerization of the protein (Chen, M.-R. et al., 1993).

Transfection of EBNA-1 expression constructs into EBV-negative cell lines has failed to demonstrate any effects upon cellular growth characteristics. However, expression of EBNA-1 in the B cells of transgenic mice has been shown to be associated with the development of lymphocytic lymphoma and leukaemia (Wilson & Levine, 1992). Further investigation is required, however, in order to determine whether malignant transformation is directly attributable to EBNA-1 expression.

**EBNA-2**

EBNA-2 contains a polyproline region, a glycine–arginine repeat and a highly charged acidic carboxy terminus (Dambaugh et al., 1984). It is phosphorylated on serine and threonine residues (Grasser et al., 1991) and binds non-specifically to both single- and double-stranded DNA (Kallin et al., 1986). Antigenic differences between the EBNA-2 proteins encoded by different EBV isolates have revealed two distinct EBV strains designated type A (or EBV-1) and type B (or EBV-2), represented by B95-8 and AG876 or Jijoye, respectively (Dambaugh et al., 1984; Adldinger et al., 1985; Rowe et al., 1985; Zimber-Strobl et al., 1986). Indeed, the B95-8 and AG876 EBNA-2 proteins exhibit only 53% identity at the amino acid level (Dambaugh et al., 1984). EBNA-2A and EBNA-2B exhibit $M_t$s of approximately 85K and 75K, respectively.

A number of studies have suggested that EBNA-2 is important for the immortalization of B cells. Firstly, the non-immortalizing P3HR-1 and Daudi EBV strains do not encode EBNA-2 owing to genomic deletions (Miller et al., 1974; Rabson et al., 1982; Jones et al., 1984). Secondly, superinfection of the EBV-positive Raji BL cell line (which does not produce virions) with P3HR-1 virus results in the production of recombinant, immortalizing virus. All the immortalizing recombinants analysed were shown to have recovered a functional EBNA-2 gene (Skare et al., 1985). These data, although suggestive, do not prove the importance of EBNA-2 in the immortalizing process since the recovered region also encodes the
carboxy terminus of EBNA-LP. Recently, two reports have demonstrated more directly that the EBNA-2 protein is, indeed, essential for the immortalization of B cells in vitro. In these experiments the sequences deleted in the P3HR-1 strain were reintroduced into P3HR-1 by homologous recombination. The re-aquisition of a functional EBNA-2 gene rendered the reconstituted virus capable of immortalizing B cells in vitro (Hammerschmidt & Sugden, 1989; Cohen et al., 1989). Furthermore, mutation of the EBNA-2-encoding sequences resulted in the production of non-immortalizing virus (Cohen et al., 1989). Rickinson et al. (1987) demonstrated that B cells immortalized with type B viruses grow slower, exhibit a greater sensitivity to seeding at limiting dilution and grow to lower saturation densities compared with cells immortalized with type A strains. It has also been shown that type A strains are more efficient than type B strains at inducing tumors in SCID mice (Rowe et al., 1991). Cohen et al. (1989) reintroduced either EBNA-2A or EBNA-2B genes into the P3HR-1 genome and showed that the differences in the growth phenotype of LCLs immortalized by type A and type B EBV strains is determined primarily by the EBNA-2 type expressed. Further, Cohen et al. (1991) introduced a number of mutated EBNA-2 genes into P3HR-1 and demonstrated that at least four separate domains are essential for immortalization, one within the amino-terminal third of the protein and the others in the carboxy-terminal third. In addition, an association between the viral trans-activation and immortalization functions was noted, namely, mutations in EBNA-2 which diminish or abolish B cell immortalization also diminish or abolish trans-activation of LMP-1, respectively. Furthermore, there is a direct correlation between the ability of EBV strains encoding mutant EBNA-2 proteins to immortalize B cells in vitro and to cause B cell tumours in SCID mice (Cohen et al., 1992). Further investigations have demonstrated that immortalizing virus can still be generated when a region of EBNA-2 important for trans-activation is substituted for an equivalent region of VP16 (Cohen, 1992). The re-aquisition of a functional EBNA-2 gene rendered the reconstituted virus capable of immortalizing B cells in vitro (Hammerschmidt & Sugden, 1989; Cohen et al., 1989). Further, Cohen et al. (1991) introduced a number of mutated EBNA-2 genes into P3HR-1 and demonstrated that at least four separate domains are essential for immortalization, one within the amino-terminal third of the protein and the others in the carboxy-terminal third. In addition, an association between the viral trans-activation and immortalization functions was noted, namely, mutations in EBNA-2 which diminish or abolish B cell immortalization also diminish or abolish trans-activation of LMP-1, respectively. Furthermore, there is a direct correlation between the ability of EBV strains encoding mutant EBNA-2 proteins to immortalize B cells in vitro and to cause B cell tumours in SCID mice (Cohen et al., 1992). Further investigations have demonstrated that immortalizing virus can still be generated when a region of EBNA-2 important for trans-activation is substituted for an equivalent region of VP16 (Cohen, 1992), a herpes simplex virus (HSV) structural protein which, upon infection, activates expression of the HSV immediate early genes (Batterson & Roizman, 1983).

The importance of EBNA-2 in the immortalization process was indicated in early gene transfer experiments. Dambaugh et al. (1986) showed that the expression of EBNA-2 in rodent cells reduces their dependence on serum factors. Furthermore, infection of the EBV-negative BL cell line Loukes with a recombinant retrovirus encoding EBNA-2 induced the expression of the B cell activation antigen CD23 (Wang et al., 1987). Indeed, more recent studies have shown that transfection of EBNA-2A into EBV-negative B lymphoma cells induces higher levels of CD23 than does transfection of EBNA-2B (Wang et al., 1990a). Since a secreted cleavage product of CD23 (soluble CD23; sCD23) has been shown to act as an autocrine B cell growth factor (Swendeman & Thorley-Lawson, 1987), these data are consistent with the observation that B cells immortalized with type B EBV strains grow slower, exhibit a greater sensitivity to seeding at limiting dilution and grow to lower saturation densities compared with cells immortalized with type A strains. Trans-activation by the EBNA-2 protein accounts for the observed induction of CD23 following conversion of EBV-negative BL lines with the prototype B95-8 strain but not with P3HR-1 virus (Calender et al., 1987). Induction of c-fgr expression in converted cells has also been shown to be dependent upon expression of the EBNA-2 protein (Knutson, 1990). In addition to the trans-activation of cellular genes, transfection of EBNA-2 expression constructs into P3HR-1-converted B lymphoma cell lines has also been shown to result in the trans-activation of both the EBV LMP-1 (Abbot et al., 1990; Wang et al., 1990b) and LMP-2 (Zimmer-Strobl et al., 1991) genes. Furthermore, EBNA-2 has been shown to trans-activate the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) (Scala et al., 1993). The precise mechanism of this effect is unknown; however, the observation that intact NF-κB and Sp1 binding sites are required for trans-activation suggests that the effect is mediated via these transcription factors.

**EBNA-LP**

The *M* of EBNA-LP (also termed EBNA-5) ranges between 22K and 70K. The majority of the protein consists of repeated peptides since all but its carboxy terminus is encoded by exons derived from the large internal repeat sequences in the viral genome. EBNA leader protein is so designated because it is encoded by the 5' leader sequences of bicistronic mRNAs specifying the other EBNAs. The translational initiation codon for EBNA-LP is created by a splicing event that occurs near the 5' end of the message (Sample et al., 1986; Speck et al., 1986; Rogers et al., 1990). The non-immortalizing P3HR-1 and Daudi strains of EBV encode mutant EBNA-LP proteins owing to the deletion of the two exons that normally encode its carboxy terminus (Rabson et al., 1982; Jones et al., 1984). In fact, it has recently been shown that the carboxy terminus of the Daudi EBNA-LP species are determined by the alternative splicing of exons encoded downstream of the genomic deletion (Ring et al., 1992).

EBNA-LP is highly phosphorylated (Petti et al., 1990) and has been shown to co-localize in the cell with the retinoblastoma gene product, p105<sup>ret</sup> (Jiang et al., 1991). The recent demonstration that EBNA-LP binds to the...
p105th and p53 proteins when they are expressed as glutathione-S-transferase fusion proteins (Szekely et al., 1993) may suggest that EBNA-LP interacts with these tumour suppressor proteins in vivo. It should be noted, however, that no interaction between EBNA-LP and the p105th and p53 proteins has yet been demonstrated in extracts of EBV-infected cells. Both Hammerschmidt & Sugden (1989) and Mannick et al. (1991) have shown that mutant EBVs, lacking the last two exons of the EBNA-LP gene, immortalize B cells with reduced efficiency. Moreover, it has been suggested that, despite B cells infected with EBV lacking these exons having extended life in vitro, they are not, in fact, immortal (Allan et al., 1992).

**EBNA-3A, -3B and -3C**

The proteins in the EBNA-3 family (alternatively known as EBNAs 3, 4 and 6) range in size from approximately 140K to 180K. There is only very limited similarity in their predicted primary amino acid sequences, although their hydrophobicity profiles and the distributions of charged amino acids show resemblances. All three proteins contain repeated domains at their carboxy termini, are phosphorylated and are similarly distributed throughout the nucleus in latently infected cells (Petti et al., 1990). Type A and B strains of EBV exhibit 84%, 80% and 72% amino acid identity in their EBNA-3A, -3B and -3C sequences, respectively (Sample et al., 1990).

There is increasing evidence that EBNA-3C, like EBNA-2, functions as a trans-activator of both cellular and viral genes. Transfection of an EBNA-3C expression construct into an EBV-negative B lymphoma cell line has been shown to result in the upregulation of CD21, the EBV receptor (Wang et al., 1990a). Furthermore, expression of EBNA-3C in the Raji cell line (which harbours an EBV genome that is deleted for most of the EBNA-3C open reading frame) induces an upregulation of LMP-1 and the cellular proteins CD23 and vimentin (Allday et al., 1993). EBNA-3C is predicted to contain a basic leucine zipper motif (Allday et al., 1993), a feature consistent with a direct role as a trans-activator protein. EBV strains with stop codons inserted into the EBNA-3A, -3B and -3C open reading frames have recently been generated by homologous recombination. Attempts to immortalize primary B cells with these recombinants have shown that EBNAs-3A and -3C are essential for the outgrowth of LCLs (Tomkinson et al., 1993), whereas EBNA-3B is dispensable (Tomkinson & Kieff, 1992b).

Similar technology has also been used to show that the substitution of the P3HR-1 (type B) EBNA-3 genes for those encoded by a type A strain has no effect upon B cell growth (Tomkinson & Kieff, 1992d). This is consistent with the earlier demonstration that the differences in the growth phenotype of LCLs immortalized by type A and type B EBV strains are determined primarily by EBNA-2 (Cohen et al., 1989).

**LMP-1**

The Mr of LMP-1 ranges between 58K and 63K, depending upon the strain of EBV. The protein is predicted to consist of a short hydrophilic amino terminus, six hydrophobic transmembrane domains and a long hydrophilic acidic carboxy terminus. Both the 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 reverse turn loops on the external surface of the cell (Liebowitz et al., 1986; Thorley-Lawson & Israelsohn, 1987). The predicted secondary structure of LMP-1 shows similarity to ion channels, the β-adrenergic and rhodopsin receptors (Fennewald et al., 1984), and the protein encoded by the mas oncogene (Sugden, 1989). LMP-1 is phosphorylated predominately on serine, but also on threonine residues (Baichwal & Sugden, 1987; Mann & Thorley-Lawson, 1987) and is linked to the cytoskeletal matrix by association with vimentin (Liebowitz et al., 1987; Mann & Thorley-Lawson, 1987). Since only 30 to 50% of cells in LCLs express LMP-1 at any one time (Mann et al., 1985; Rowe et al., 1987), it has been suggested that its expression may be cell cycle-related (Boos et al., 1987). LMP-1 has a very short half-life of 2 to 3 h (Baichwal & Sugden, 1987; Mann & Thorley-Lawson, 1987) owing to specific cleavage of the carboxy terminus, which releases a 25K fragment into the cytoplasm and leaves a 35K fragment in the membrane (Moorthy & Thorley-Lawson, 1990). LMP-1 has been detected in the Epstein–Barr virion and may, therefore, play an active role in the initial events of immortalization, possibly providing an early activation signal for the B cell (Mann et al., 1985). Indeed, it has recently been demonstrated that transient expression of LMP-1 in primary B cells is associated with cellular activation and DNA synthesis (Peng & Lundgren, 1992). In contrast to the early expression of the EBNA proteins, newly synthesized LMP-1 is not detectable until 40 to 48 h post-infection, after which the cells undergo blast formation. The finding that LMP-1 expression is induced by EBNA-2 (Abbot et al., 1990; Fahraeus et al., 1990b; Wang et al., 1990b) may indicate that in the context of primary B cell infection, EBNA-2 is required for LMP-1 expression. BL cells and early passage BL cell lines (that is, those retaining the original BL cell phenotype) do not express LMP-1 (Rowe et al., 1987) indicating, however, that this protein is not important in the maintenance of the BL tumour. The role played by LMP-1 in the development of NPC is unclear since this
protein is detected in only approximately 50% of NPC biopsies (Fahraeus et al., 1988; Young et al., 1988).

LMP-1 expression has been shown to induce morphological transformation in human keratinocytes (Fahraeus et al., 1990a) and to inhibit differentiation of epithelial cells (Dawson et al., 1990). In addition, LMP-1 expression in the epidermis of transgenic mice is associated with the development of hyperplastic dermatosis (Wilson et al., 1990). These observations suggest that LMP-1 expression could play a role in the development of NPC by predisposing the cells to malignant transformation. The expression of LMP-1 can transform immortal, but non-tumorigenic, rodent fibroblasts into cells which are tumorigenic in nude mice. Transfected cells exhibit loss of contact inhibition and anchorage dependence and acquire the ability to grow in soft agar (Wang et al., 1985; Baichwal & Sugden, 1988). It has recently been shown that all three regions of LMP-1, that is, the amino terminus, the transmembrane domain and the carboxy terminus, are essential for transforming activity in Rat-1 fibroblasts (Moorthy & Thorley-Lawson, 1993a). Recently, Moorthy & Thorley-Lawson (1993b) have shown that the ability of LMP-1 to transform rodent fibroblasts is dependent upon phosphorylation of the molecule. This suggests that the function of LMP-1 is regulated by phosphorylation, another feature LMP-1 shares with members of the β-adrenergic receptor family (Lefkowitz & Caron, 1988). Hammerschmidt et al. (1989) have shown that LMP-1 is toxic when expressed in rodent fibroblasts at high levels.

LMP-1 expression in EBV-negative BL cells has profound effects on the cell surface phenotype. LMP-1-expressing cells exhibit increased homotypic adhesion due to increased expression of the cell adhesion molecules lymphocyte function-associated antigen 1 (LFA-1) and intercellular cell adhesion molecule 1 (ICAM-1). Induction of LFA-3 also results in increased adhesion to T cells. There is an increase in acid production, cell size and plasma membrane ruffling. Expression of the B cell activation molecule CD23 and the transferrin receptor is increased, as is the intracellular level of free calcium (Wang et al., 1988). Recently, cooperation has been demonstrated between LMP-1 and EBNA-2 in the upregulation of CD23 (Wang et al., 1990a). These properties of LMP-1 are consistent with the hypothesis that this protein plays a key role in EBV-induced B cell proliferation in vitro. Furthermore, the detection of LMP-1 and cell adhesion molecules in B cell proliferations that develop in transplant recipients and AIDS patients (Young et al., 1989) is strongly suggestive of a role of LMP-1 in lymphoproliferation in vivo. Hammerskjold & Simurda (1992) reported that LMP-1 transactivates the HIV-1 LTR via the induction of NF-κB activity. Furthermore, their observations that transformation-defective LMP-1 mutants fail to transactivate the HIV-1 LTR strongly suggest that the induction of NF-κB activity plays a key role in EBV-mediated cell proliferation.

LMP-2A and -2B

The genes encoding LMP-2A and -2B have also been named terminal protein 1 and 2 (TP-1, TP-2) genes because they are transcribed across the terminal repeat sequences that are fused together upon infection of cells to generate the intracellular episomal form of the viral genome (Laux et al., 1988; Sample et al., 1989). The two messages consist of different 5' exons and eight common exons and are predicted to encode nearly identical proteins differing only in the length of their hydrophilic amino termini. LMP-2A and -2B are 54K and 40K in size, respectively. Both proteins are predicted to possess 12 highly hydrophobic membrane-spanning domains and are localized to patches in the plasma membrane of infected cells, in close association with LMP-1 (Longnecker & Kieff, 1990). The LMP-2 proteins are phosphorylated on serine, threonine and tyrosine residues (Longnecker et al., 1991) and have been shown to interact with lyn, lyn and Etc-family tyrosine kinases in EBV-infected B cells (Burkhardt et al., 1992). These interactions suggest that the LMP-2 proteins play a role in transmembrane signal transduction. Three specific LMP-2 mutants have been generated by homologous recombination in order to investigate the importance of these proteins in B cell immortalization. One mutation introduced a nonsense codon in the first LMP-2A exon, thereby interrupting LMP-2A expression (Longnecker et al., 1992). A second mutation introduced a nonsense codon that resulted in the truncation of both the LMP-2A and -2B proteins after the fifth transmembrane domain (Longnecker et al., 1993a), whereas the third mutant was generated by the deletion of the first five transmembrane domains of LMP-2A and -2B (Longnecker et al., 1993b). None of these mutations had any discernible effects on the ability of the EBV mutants to immortalize B cells in vitro. Despite this clear demonstration that LMP-2A and -2B are dispensable for B cell immortalization, expression of these genes in latently infected B cells in vitro (Longnecker & Kieff, 1990) and peripheral blood B cells in vivo (Qu & Rowe, 1992) is suggestive of a role for these proteins in viral infection. Indeed, the demonstration of LMP-2 gene expression in nasopharyngeal carcinoma (Brooks et al., 1992; Busson et al., 1992), Hodgkin's lymphoma (Pallesen et al., 1991; Murray et al., 1992) and peripheral T cell lymphomas (Chen, C.-L. et al., 1993) is strongly suggestive of a role for these proteins in malignant transformation. The precise role remains unclear, but
Miller et al. (1993) have speculated that the LMP-2 proteins may modulate the calcium-mobilizing effects of LMP-1, thereby providing a mechanism by which reactivation of EBV is down-regulated. This hypothesis remains to be tested.

Other EBV gene products possibly playing a role in the immortalization process

In addition to the EBNAs and LMPs, two small untranslated polymerase III-transcribed RNA species, EBERs 1 and 2, are expressed in LCLs (Jat & Arrand, 1982). They are predominately located in the nucleus of infected cells and are similar in primary and secondary structure to the cellular U6 small nuclear RNA suggesting a role for them in RNA processing (Glickman et al., 1988). They behave like the adenovirus-associated (VA) RNAs in their ability to complex to the cellular La antigen (Lerner et al., 1981). The EBERs are detectable in B cells from about 36 h post-EBV infection and persist in high abundance throughout latent infection (Rooney et al., 1989; Alfieri et al., 1991). A recent report has shown that deletion of the EBER genes fails to affect the ability of EBV to immortalize B cells in vitro (Swamminathan et al., 1991).

Pearson et al. (1987) characterized the protein encoded by the BHRF-1 open reading frame as an early lytic cycle antigen. However, a number of groups have shown that the BHRF-1 appears in highly spliced EBNAs-like messages (Bodescot & Perricaudet, 1986; Pearson et al., 1987; Austin et al., 1988; Ring et al., 1992), suggesting that the BHRF-1-encoded protein may be expressed in latently infected B cells. The predicted BHRF-1 translation product has a potential hydrophobic amino-terminal signal sequence, a putative external or luminal domain of approximately 150 amino acids and a 21 residue hydrophobic potential transmembrane domain. The protein has an Mr of 17K and subcellular fractionation studies indicate nuclear, mitochondrial and microsomal association (Pearson et al., 1987). The BHRF-1-encoded protein possesses regions of significant similarity to the proto-oncogene product bcl-2 (Cleary et al., 1986) and to the yeast mitochondrial protein, cytochrome oxidase 1 (Marchini et al., 1991). Recently, two groups have demonstrated using homologous recombination that mutation of BHRF-1, however, fails to compromise the ability of EBV to immortalize B cells in vitro (Marchini et al., 1991; Lee & Yates, 1992).

Wei & Ooka (1989) demonstrated that the expression of the BARF-1 open reading frame induced morphological change, anchorage-independent growth and tumorigenic transformation in established murine fibroblast cell lines. In addition, the 33K BARF-1-encoded protein was also demonstrable in tumour tissue resulting from subcutaneous administration of transformed cells to newborn rats. There is, however, no evidence that BARF-1 is expressed in latently infected B cells and, therefore, it is unlikely to play a role in the proliferation of B cells.

Recently, a family of RNA species has been identified that are transcribed from the strand of the EBV genome opposite to that encoding lytic viral functions such as the DNA polymerase and two putative glycoproteins (Hitt et al., 1989; Gilligan et al., 1991; Karran et al., 1992). These species have been termed "complementary-strand transcripts" (Karran et al., 1992). This feature of their expression may indicate a role in the maintenance of viral latency. The transcripts were originally found in a latently infected nude mouse-passaged NPC, but have more recently been demonstrated in EBV-infected BL cell lines, LCLs (Karran et al., 1992), Hodgkin's disease biopsy samples (Deacon et al., 1993), EBV-induced tamarin lymphomas (Zhang et al., 1993) and peripheral T cell lymphomas (Chen, C.-L., et al., 1993). Proteins encoded within these messages have been immunoprecipitated using sera from NPC patients (Gilligan et al., 1991), indicating that they may be expressed in vivo; however, it is, as yet, unknown whether they play a role in immortalization. The ability to detect these transcripts in type I BL cells (in which EBNA-2, -3A/B/C and -LP are not expressed) (Brooks et al., 1993) may suggest that they are involved in the malignant transformation of B cells.

The roles played by the EBV latent proteins in the immortalization process

The binding of EBV to the C3d receptor and subsequent infection of the cell triggers a cascade of events leading ultimately to the immortalization of the cell. A number of effects are apparent even before viral genes are expressed including phosphorylation of the C3d receptor and the tyrosine kinase p56^ck (Cheung & Dosch, 1991). The immortalization of primary B cells, however, requires the expression of several viral genes. The first viral proteins to be expressed in B cells upon EBV infection, namely EBNA-2 and -LP (Allday et al., 1989; Rooney et al., 1989; Alfieri et al., 1991), are believed to play critical roles in the early stages of the immortalization process.

In addition to the absence of EBNA-2 or prototype EBNA-LP genes in the non-immortalizing P3HR-1 and Daudi EBV strains, it has been reported that P3HR-1 is also unable to direct the expression of LMP-1 and EBNA-1, -3A, -3B and -3C when infecting cord blood-derived B cells (Rooney et al., 1989). This suggests that EBNA-2 and/or the prototype EBNA-LP proteins are important for the expression of the other latent proteins.
When B cells are infected with immortalizing strains of EBV, transcription of the EBNA-2 and -LP genes is initiated from one promoter (designated Wp) in the early stages of infection and subsequently there is a switch to another (Cp) later on (Woisetschlaeger et al., 1990; Alfirevi et al., 1991; Rooney et al., 1992). This switch of promoter usage does not occur in P3HR-1-infected B cells owing to the inability of P3HR-1 to encode EBNA-2 (Woisetschlaeger et al., 1991; Rooney et al., 1992). Sung et al. (1991) have, in fact, identified an EBNA-2-responsive enhancer element within Cp. The finding that mutant EBNA-LP is the only viral latent protein expressed in P3HR-1-infected umbilical cord B cells (Rooney et al., 1989) suggests that EBNA-2 may not only be responsible for promoter switching, but that it is, indeed, required for the expression of the other EBNAs in primary B cells. The absolute requirement for EBNA-2 in immortalization may, therefore, be due to its trans-activating effects upon these other EBNA genes. Alternatively, or in addition, EBNA-2 may exert its essential role through its induction of the viral LMPs (Abbot et al., 1990; Zimber-Strobl et al., 1991). EBNA-2 and LMP-1 act synergistically to induce the cellular protein CD23 (Wang et al., 1990a). The finding that only CD23-positive B cells are capable of being immortalized (Thorley-Lawson & Mann, 1985) suggests that CD23 induction is an essential component of the immortalization process, presumably by acting as a B cell growth factor (Swendeman & Thorley-Lawson, 1987). Furthermore, the observation that the degree of LMP-1 trans-activation is less dependent upon which EBNA-2 type is encoded by the virus than CD23 trans-activation is (Cohen et al., 1991a) may suggest that the induction of the cellular CD23 protein is of greater importance in the immortalization process than the induction of the viral LMP-1 protein. However, in the context of infection of chronic lymphocytic leukaemia cells (CLL) with the B95-8 strain of EBV, expression of all of the EBNA proteins and high levels of CD23 is insufficient to immortalize the cells (Walls et al., 1989). The precise mechanism by which EBNA-2 trans-activates viral and cellular genes is presently unclear. The observation that EBNA-2 requires intact NF-κB- and Sp1-binding sites for trans-activation of the HIV-1 LTR (Scala et al., 1993), however, suggests that the effect of EBNA-2 is mediated via these, and possibly other, transcription factors. The recent demonstration of EBNA-2 in specific protein–DNA complexes (Zimber-Strobl et al., 1993) suggests that a direct mechanism, involving DNA binding, may exist for at least some genes.

Gordon et al. (1986) demonstrated that despite being unable to progress through to S-phase, P3HR-1-infected B cells enter the G_{\text{1}} stage of the cell cycle whereas cells exposed to u.v.-inactivated virus remain in G_{\text{0}}, indicating that a P3HR-1-encoded function is responsible for the progression. Since mutant EBNA-LP is the only viral latent protein detectable in P3HR-1-infected umbilical cord B cells (Rooney et al., 1989). EBNA-LP may be responsible for this effect. If this is indeed the case, then it is likely that the sequences encoded by the large internal repeat, i.e. those unaffected by the P3HR-1 genomic deletion, are important. In this respect, it is interesting that the binding of EBNA-LP to the tumour suppressor proteins p105^{\text{N}} and p53 appears to be mediated by such repeated sequences (Szekely et al., 1993). If further studies do indeed demonstrate an interaction between EBNA-LP and these proteins in vivo, one could postulate that EBNA-LP acts like the oncoproteins of the small DNA tumour viruses by inactivating their growth-suppressing functions, either by stimulating their degradation (Scheffner et al., 1990) or by allowing the release of active transcription factors from complexes with p105^{\text{N}} (reviewed by Nevins, 1992).

Despite the demonstrations that EBNA-2 and -LP play important roles in immortalization, it is likely that at least some of the other latent proteins contribute to the establishment of the immortalized state. For example, the ability of EBNA-3C to trans-activate LMP-1 expression (Allday et al., 1993) may be crucial in the overall process. EBNA-1 is clearly required for the extrachromosomal maintenance of the viral genome. It is possible that, like EBNA-2 and -3C, it also trans-activates growth stimulatory functions in the infected cell. The finding that LMP-1 is expressed in prolymphocytic leukaemia cells, which are immortalized by EBV, but not in CLL which are not, despite expression of all the EBNA proteins (Walls et al., 1989), suggests that LMP-1 expression is a prerequisite for immortalization. The structural similarities between LMP-1 and the β-adrenergic and rhodopsin receptors may suggest that LMP-1 associates with G proteins and is involved in the production of secondary messengers by effector molecules such as adenylate cyclase or phosphodiesterase. In turn, this could lead to the activation of protein kinases and subsequently modulate the expression of cellular genes by the phosphorylation of transcription factors (Gonzalez & Montminy, 1989). Hammarskjold & Simurda (1992) speculated that the induction of NF-κB activity by LMP-1 occurs via a calcium-dependent kinase, since cells stably transfected with LMP-1 expression constructs exhibit increased levels of intracellular calcium (Wang et al., 1988). The demonstration that transformation-defective LMP-1 mutants are incapable of inducing NF-κB activity (Hammarskjold & Simurda, 1992) strongly suggests a role for NF-κB in growth deregulation. Indeed, a chromosomal translocation involving an NF-κB gene, with the concomitant activation of its expression, has been reported in three
cases of human B cell lymphoma (Neri et al., 1991). The induction of transcription factor activity is one possible mechanism behind the induction of CD23 expression by LMP-1 (Wang et al., 1988). It should be stated, however, that no evidence exists to support this hypothesis. A major difference between the LMP-1 and the β-adrenergic/rhodopsin receptors is that LMP-1 lacks the initial transmembrane domain resulting in an intracellular rather than an extracellular amino terminus. It is possible that the absence of an extracellular domain results in a protein that constitutively transmits a proliferative signal to the cell without the need for binding of a growth factor. The observation that ectopic expression of LMP-1 is associated with the expression of the apoptosis-inhibiting protein bcl-2 (Henderson et al., 1991) may suggest that LMP-1 contributes to the immortalizing process by indirectly promoting cell survival. It is possible, however, that the observation simply reflects the selection of bcl-2-expressing cells due to a greater ability to resist the toxic effects of LMP-1 (Hammerschmidt et al., 1989).

The interaction between LMP-2A and src-family tyrosine kinases such as fyn and lyn suggests that LMP-2A plays a role in growth regulation in view of the prominent role of tyrosine kinases in growth factor receptor-mediated transmembrane signalling in B cells. Physiological triggering of B cells stimulates the activation of receptor-associated tyrosine kinases, resulting in phosphorylation and activation of cellular proteins including phospholipase C. Subsequent phosphoinositide hydrolysis and calcium mobilization lead to transcriptional activation and ultimately to B cell proliferation (reviewed by Cambier et al., 1987). Recently, Miller et al. (1993) have demonstrated that expression of LMP-2A in EBV-negative B lymphoma cell lines blocks calcium mobilization. Furthermore, they showed that co-expression of LMP-1 reversed this block. These observations implicate LMP-2A as a negative modulator of normal signal transduction in B cells and that it may function to down-regulate the EBV-induced activation state by repressing the activating properties of LMP-1. The close physical association between LMP-1 and LMP-2 in the plasma membrane (Longnecker & Kiell, 1990) may be required for such a moderating effect. The precise role played by the LMP-2 proteins in latently infected B cells is unclear since neither is required for immortalization to occur in vitro (Longnecker et al., 1992, 1993a,b). However, Miller et al. (1993) have postulated that a block of receptor-mediated calcium mobilization by LMP-2A could be a means for inhibiting reactivation of the viral lytic cycle in vivo.

Recently, Birkenbach et al. (1993) have identified a number of cellular mRNA species induced upon EBV infection by using subtractive hybridization techniques. In addition to previously reported ones, namely CD21 and vimentin, and others believed to be upregulated as a consequence of EBV-mediated activation such as annexin VI and the myristylated alanine-rich protein kinase C substrate (MARCKS), two novel putative G protein-coupled peptide receptors (designated EBI-1 and EBI-2) were shown to be upregulated. EBI-1 is predicted to be highly similar to the interleukin 8 receptors, whereas EBI-2 is most closely related to the thrombin receptor. These similarities suggest that EBI-1 and -2 act through G proteins in the production of secondary messengers. The observation that expression of the G protein-coupled serotonin 1c receptor in immortalized rodent fibroblasts results in ligand-dependent malignant transformation (Julius et al., 1989) may suggest that EBI-1 and -2 are involved in EBV-mediated B cell proliferation. These proteins, therefore, warrant further investigation. Studies should be carried out in order to determine the mechanism by which EBV infection induces the expression of these putative receptor proteins.

The function of the ‘complementary-strand transcripts’ should be investigated further since their expression in NPC tissue (Hitt et al., 1989; Gilligan et al., 1991), peripheral T cell lymphomas (Chen, C.-L. et al., 1993), and type I BL cell lines (Brooks et al., 1993) may indicate that they encode a function important in malignant transformation. Smith et al. (1993) reported a limited degree of sequence similarity between a portion of one of the open reading frames in these transcripts and a region of EBNA-2 required for immortalization. The authors postulated that the protein encoded by these transcripts may provide an EBNA-2 like function in cells such as NPC and BL, which do not express the EBNA-2 protein. Since it is presently unknown whether this particular open reading frame is actually translated in EBV-infected cells, the significance of this reported similarity is unclear. Attempts should be made, firstly, to express these transcripts in EBV-negative B cells and, secondly, to inactivate the genes involved by homologous recombination (in a manner similar to that used for the other latent genes), in order to determine whether their expression is important for the immortalization of B cells.

Using the techniques of DNA transfection and homologous recombination, it is clear that of the EBV-encoded latent gene products, EBNA-1, -2, -3A, -3C and -LP and the LMP-1 are required for the immortalization of B cells in vitro, whereas the EBERs, EBNA-3B and LMP-2A and -B are dispensable. It is presently unknown whether other factors such as proteins encoded by the ‘complementary-strand transcripts’ are required for continued B cell proliferation. The precise manner by which EBV immortalizes B cells is unknown; however, a number of virus-encoded proteins and virus-induced
cellular proteins exhibit structural or biochemical properties similar to those functioning in signal transduction pathways. These include growth factors (sCD23), growth factor receptors (LMP-1, EBI-1/2), tyrosine kinases (fgr, lck), tyrosine kinase-associated proteins (LMP-2A and -2B) and transcription factors (EBNA-1, -2, -3C, NF-κB).

Elucidation of the mechanism by which EBV achieves control but may also lead to the identification of potential antiviral drug targets and ultimately to more effective treatment for EBV-associated diseases.

Note added in proof. Kaye et al. (Proceedings of the National Academy of Sciences, U.S.A. 90, 9150–9154) have recently generated EBV viruses expressing mutant LMP-1 proteins and shown that LMP-1 is indeed essential for the immortalization of B cells.

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References


Young, L. S., Alferi, C., Hennessy, K., Evans, H., O’Hara, C.,


