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Molecular virology of Epstein–Barr virus

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Epstein–Barr virus (EBV) interacts with its host in three distinct ways in a highly regulated fashion: (i) EBV infects human B lymphocytes and induces proliferation of the infected cells, (ii) it enters into a latent phase *in vivo* that follows the proliferative phase, and (iii) it can be reactivated giving rise to the production of infectious progeny for reinfection of cells of the same type or transmission of the virus to another individual. In healthy people, these processes take place simultaneously in different anatomical and functional compartments and are linked to each other in a highly dynamic steady-state equilibrium. The development of a genetic system has paved the way for the dissection of those processes at a molecular level that can be studied *in vitro*, i.e. B-cell immortalization and the lytic cycle leading to production of infectious progeny. Polymerase chain reaction analyses coupled to fluorescent-activated cell sorting has on the other hand allowed a descriptive analysis of the virus–host interaction in peripheral blood cells as well as in tonsillar B cells *in vivo*. This paper is aimed at compiling our present knowledge on the process of B-cell immortalization *in vitro* as well as *in vivo* latency, and attempts to integrate this knowledge into the framework of the viral life cycle *in vivo*.

Keywords: Epstein–Barr virus genetics; B-cell immortalization; *in vivo* latency; virus reactivation; germinal centre; memory B cells

1. THREE MODES OF VIRUS–HOST INTERACTION *IN VIVO*

All herpesviruses have in common that, once the initial phase of virus multiplication has been resolved, they enter into a phase of lifelong persisting latency. From this state of latency the viral infection can be reactivated, thus leading to successive cycles of virus replication and re-entrance into latency *in vivo*. In contrast to other herpesviruses, Epstein–Barr virus (EBV) uses a dual strategy to ensure infection of a large number of cells for the maintenance of *in vivo* latency. On the one hand, the virus is able to drive infected cells into cell cycle and proliferation, thus increasing dramatically the number of viral-genome-carrying cells. On the other hand, the virus replicates, releases infectious virus particles and is able to initiate a new round of infection. The life cycle of the virus thus consists of at least three phases: (i) expansion of infected cells maintaining the viral genome in an episomal state, (ii) establishment of *in vivo* latency, and (iii) reactivation, replication and synthesis of viral progeny. Because of space constraints we shall deal only with the first two topics in this review. We shall first discuss B-cell immortalization and the interplay of viral and cellular gene products in this process and shall finally try to assemble our current knowledge obtained from *in vitro* studies into the framework of the virus infection *in vivo*. It is beyond the scope of this article to discuss the pathogenesis of EBV-associated diseases, and in particular of EBV-associated malignancies.

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2. EPSTEIN–BARR VIRUS GENETICS: THE EXPERIMENTAL TOOLS TO STUDY VIRUS–HOST INTERACTIONS *IN VITRO*

(a) *Mini-EBV*

The molecular analysis of EBV has been hampered by the absence of a permissive tissue culture system for propagation of the virus. It was thus impossible to generate mutants and to study the function of individual viral genes using a genetic approach. The only source of the virus were supernatants of lymphoid cell lines in which a small and varying number of cells underwent a lytic cycle. Two properties of an unusual laboratory strain of EBV (P3HR-1) were instrumental for the development of a genetic system: (i) the loss of its transforming potential through deletion of the EBV nuclear antigen (EBNA)-2 gene (Bornkamm *et al.* 1982; Jeang & Hayward 1983; Miller *et al.* 1974), and (ii) the presence within P3HR-1 virus preparations of defective virus particles carrying rearranged and repetitive viral sequences with high infectious potential for Raji cells (Cho *et al.* 1984*a*; Delius & Bornkamm 1978). The analysis of these defective genomes led to the discovery of the BZLF1 gene product as the molecular switch for lytic-cycle induction (Cho *et al.* 1984*b*; Countryman & Miller 1985; Grogan *et al.* 1987; Heston *et al.* 1982). (EBV genes are designated according to the position and orientation of the reading frame on the respective BamHI fragment, e.g. BZLF1, the first leftward reading frame on EBV BamHI-Z fragment.) The breakthrough in the development of a genetic system for EBV came with the discovery of EBV's lytic origins of replication and its packaging signal within

the terminal repeats (Hammerschmidt & Sugden 1988). Cloning the lytic origin of replication and terminal repeats onto an episomally replicating plasmid (the mini-EBV) allowed this plasmid to be replicated concomitantly with the endogenous viral DNA upon induction of the lytic cycle and to be packaged into virus particles. Any foreign sequences within the packaging size constraints of the viral genome could thus be cloned, replicated and packaged into EBV virions.

(b) *Maxi-EBV*

An even more versatile system for the genetic manipulation of the viral genome has recently been developed by cloning the complete viral genome in *Escherichia coli*. Using homologous recombination Delecluse *et al.* (1998) introduced the bacterial F-plasmid origin of replication into the B95-8 viral genome together with the gene encoding the green fluorescent protein (GFP) and propagated the complete viral genome in *E. coli*. After stable transfection of the so-called maxi-EBV plasmid into 293 cells, the lytic cycle can be induced by BZLF1 and infectious and transforming virus harvested in the supernatant. The virus not only maintained its transforming ability, but the virus titre could also be easily determined and the transforming potential quantified relative to infectivity in Raji cells. Any mutation can now be introduced into the viral genome by homologous recombination in *E. coli*. If a mutation affects a gene essential for replication and/or synthesis of viral progeny, the respective function can be provided in *trans* in a (first generation) helper packaging cell line that harbours a P3HR-1 viral genome lacking the packaging signal (Delecluse *et al.* 1999). With these tools a new era of EBV genetics has begun. Figure 1 depicts the viral B95-8 genome with the origin of replication of the F-plasmid and the *GFP* gene inserted, and describes which type of viral mutants have been generated up to now with the aid of the maxi-EBV system.

3. EPSTEIN–BARR VIRUS-INDUCED B-CELL IMMORTALIZATION

(a) *The viral genes involved in B-cell immortalization* in vitro

The ability to infect B lymphocytes and to induce their unlimited proliferation was discovered in 1967 by Henle *et al.* (Henle *et al.* 1967; Pope *et al.* 1968). It is this transforming or immortalizing property of the virus (both terms have been used synonymously in the EBV field) which has attracted most of the interest of researchers because it is regarded as a reflection of an important part of the pathogenicity and oncogenicity of the virus. Infection of primary B cells by EBV is associated with a dramatic change in the morphology and growth behaviour of the cells in culture. The cells become activated, they lose their round small-size morphology, become large and irregular in shape, develop villipodia and strongly adhere to each other to form large clumps. This phenotypic change is associated with an entry of the cells into the cell cycle and initiation of continuous proliferation with a doubling time of 20–30 h. Upon induction of proliferation, the telomere length decreases continuously with increasing cell divisions until a critical telomere length is reached. After *ca.* 100–150 population doublings the cells undergo a crisis and the vast majority of cells die. Immortal cells grow out that have stabilized their telomeres at a short length concomitantly with upregulation of telomerase activity. These cells exhibit an increased rate of chromosomal aberrations (Counter *et al.* 1994; Kataoka *et al.* 1997). Induction of telomerase activity appears not to be an early event after infection of B cells.

In EBV-immortalized cells only a limited number of viral genes are expressed (Rickinson & Kieff 1996). These encode six nuclear antigens, EBNA-1, -2, -3A, -3B, -3C and EBNA-LP, two membrane proteins, latent membrane protein (LMP-1) and LMP-2A (a splice variant designated LMP-2B lacks the first N-terminal exon) and two small nuclear non-polyadenylated RNAs. (For EBNA-3A, -3B and -3C the terms EBNA-3, EBNA-4 and EBNA-6, respectively, have also been used. For EBNA-LP the terms EBNA-4 and EBNA-5 have been used in the literature, and for LMP-2 the term TP-1. We follow here the terminology used in Kieff (1996).) The transforming potential of EBV is maintained within about one-third of the viral genome encompassing these genes (Kempkes *et al.* 1995*a*; Robertson & Kieff 1995). BARF0, a reading frame first reported to be expressed in nasopharyngeal carcinomas (NPCs) passaged in nude mice (Hitt *et al.* 1989) and found to be expressed also to a lesser extent in lymphoblastoid cell lines (LCLs) (Chen *et al.* 1992), is not required for B-cell immortalization (Robertson *et al.* 1994). Neither is viral interleukin-10 (IL-10), a gene of the lytic cycle with suspected B-cell growth factor activity (Swaminathan *et al.* 1993), nor BHRF1, a viral Bcl-2 homologue (Lee & Yates 1992; Marchini *et al.* 1991).

(b) *The minimal set of genes involved in B-cell immortalization is still unknown*

Not all the genes expressed in immortalized cells are in fact required for this process. The small nuclear RNAs (Swaminathan *et al.* 1991) as well as *EBNA-3B* were shown to be dispensable (Tomkinson *et al.* 1993). EBNA-LP (Hammerschmidt & Sugden 1989) and *LMP-2* also are not absolutely required (Kim & Yates 1993; Longnecker *et al.* 1993*a*,*b*), but they improve the outgrowth of virus-infected B cells, a finding that has recently been challenged for *LMP-2* (Brielmeier *et al.* 1996; Mannick *et al.* 1991; Speck *et al.* 1999). EBNA-1 is required to maintain the viral genome as an episome in proliferating cells (Lee *et al.* 1999; Yates *et al.* 1984). *EBNA-2*, *-3A* and *-3C* and *LMP-1* are required for initiation of immortalization (Cohen *et al.* 1989; Hammerschmidt & Sugden 1989; Kaye *et al.* 1993; Tomkinson *et al.* 1993) and *EBNA-2* and *LMP-1* also for its maintenance (Kempkes *et al.* 1995*b*; Kilger *et al.* 1998). It should be noted that the minimal set of viral genes required for immortalization is still unknown. The question whether EBNA-1, -2, -3A and -3C and LMP-1 are sufficient to induce and maintain proliferation has not yet been addressed experimentally. Functional redundancy among non-essential genes may allow deletion of one but not several or all of the non-essential genes simultaneously.

(c) *Viral transcription pattern upon infection of human primary B lymphocytes by EBV*

EBV uses its envelope glycoprotein (gp) 350 to attach to CD21, the receptor for complement component C3d,

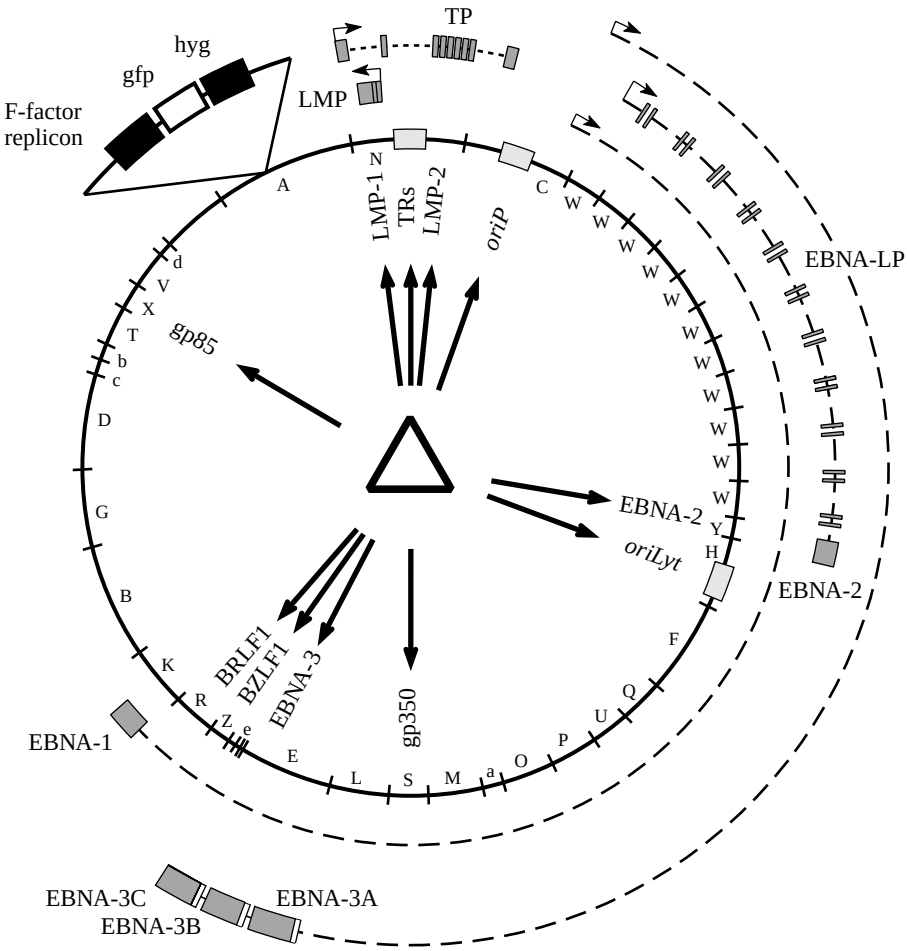

Figure 1. Schematic representation of the B95-8 viral genome cloned in *E. coli*. The F-factor replicon, the GFP gene (gfp) and the hygromycine resistance gene (hyg) were inserted into the viral genome by homologous recombination at the site of the B95-8 deletion. The *cis* elements important for replication and packaging are shown in light grey. The arrows mark the elements or genes for which deletion mutants have been generated.

as a route of entry into B cells, but other modes of entry are likely to exist since a virus lacking gp350 is still able to infect and immortalize B cells (Janz *et al.* 2000). The viral genome is circularized within *ca.* 16–20 h after infection (Hurley & Thorley-Lawson 1988) and gives rise to large transcripts initiated at the W promoter (Wp) of the large internal repeats by RNA polymerase II (Woisetschlaeger *et al.* 1989). *EBNA-2* and *EBNA-LP* are the first viral genes expressed after infection followed by *EBNA-1* (Allday *et al.* 1989; Rooney *et al.* 1989). Upon expression of *EBNA-1* and *-2*, transcription switches to the C promoter (Cp) and gives rise to manifold spliced transcripts coding for EBNA-LP, EBNA-2, -3A, -3B and -3C and EBNA-1 (Puglielli *et al.* 1996; Schlager *et al.* 1996; Woisetschlaeger *et al.* 1991). *OriP* the plasmid origin of replication, to which EBNA-1 is binding, acts not only as an origin of replication (Yates *et al.* 1984, 2000) but also as an EBNA-1- and EBNA-2-dependent enhancer of the Cp as well as of the *LMP-1* promoter (Gahn & Sugden 1995; Reisman & Sugden 1986). Cp transcription occludes the Wp and brings the viral genome under its own transcriptional control (Puglielli *et al.* 1996, 1997). In EBV-immortalized cells expression of the latent membrane proteins is controlled by EBNA-2. *LMP-1* and *LMP-2B* are transcribed from a bidirectional promoter responding to EBNA-2 (Johannsen *et al.* 1995; Laux *et al.* 1994*b*; Wang *et al.* 1990*b*), whereas *LMP-2A* is transcribed from a separate promoter that in B cells is stringently controlled by EBNA-2 (Zimber-Strobl *et al.* 1991, 1993).

(d) *Viral proteins involved in B-cell immortalization*

(i) *EBNA-1*

EBNA-1 was the first viral gene product identified in EBV-immortalized cells using anticomplement immunofluorescence (Reedman & Klein 1973). It is the only viral antigen associated with chromosomes during mitosis (Ohno *et al.* 1977) and is encoded by the terminal exon of a large mRNA that is initiated at the Wp or Cp and spans a region of *ca.* 100 kb (Speck & Strominger 1985). In group I Burkitt's lymphoma (BL) cells, which represent the *in vivo* phenotype of endemic BL, EBNA-1 is the only viral protein expressed and transcription is initiated from a promoter located further downstream, the Q promoter (Qp) (Schaefer *et al.* 1995). Of the viral proteins expressed in EBV-immortalized cells or EBV-positive BL cells, EBNA-1 is the only viral protein that binds directly to DNA in a sequence-specific manner (Rawlins *et al.* 1985). DNA binding is mediated by its C-terminally located DNA-binding and dimerization domain (Ambinder *et al.* 1991; Bochkarev *et al.* 1995,

1996). EBNA-1 binds to *oriP*, which consists of 20 EBNA-1-binding sites arranged in an array of multiple tandem repeats (FR, family of repeats) as well as of four sites arranged in dyad symmetry (DS element) (Rawlins *et al.* 1985; Yates *et al.* 1984). Cooperative binding of EBNA-1 to the multiple binding sites at *oriP* is required and sufficient for episomal replication of the viral genome. The FR and DS elements fulfil different functions in the process of episomal replication. The DS element acts as an EBNA-1-dependent replicator responsible for assembly of the replication machinery (Yates *et al.* 2000). Subsequent to origin recognition, all replication steps are performed by host enzymes in S phase synchronously with replication of the cellular genome. EBNA-1 binding to the FR stalls transcription in one direction. As a consequence, replication proceeds unidirectionally and terminates at the same site where it had started (Dhar & Schildkraut 1991; Gahn & Schildkraut 1989). Furthermore, EBNA-1 bound to the FR is absolutely required for accurate partitioning and segregation of replicated *oriP*-containing DNA to daughter cells, a function that can be mechanistically separated from the replication process itself (Aiyar *et al.* 1998). EBNA-1 and the FR are thus indispensable for episomal replication whereas the DS element is not. Replication in the absence of a DS element is initiated over a large zone (Norio *et al.* 2000). As already mentioned, *oriP* furthermore acts as an EBNA-1-dependent enhancer and plays a crucial role in the regulation of transcription. The only other EBNA-1-binding sites in the viral genome apart from *oriP* are located in Qp, the promoter that gives rise to *EBNA-1* transcripts in BL cells (Schaefer *et al.* 1995; Tsai *et al.* 1995). Qp is regulated by interferon-regulatory factors (Schaefer *et al.* 1997; Zhang & Pagano 1997, 1999). By binding to Qp, EBNA-1 negatively regulates its own promoter, thus ensuring that the gene is only transcribed when EBNA-1 is needed (Davenport & Pagano 1999; Sung *et al.* 1994).

Both functions of EBNA-1 in replication and transcription regulation are mediated by EBNA-1's ability to bind DNA. Linking DNA, to which it binds intra- or intermolecularly, EBNA-1 establishes firm protein–protein interactions at its DNA-binding sites (Frappier & O'Donnell 1991, 1992; Mackey *et al.* 1995; Mackey & Sugden 1997, 1999). Another hallmark of EBNA-1 is an array of glycine–alanine repeats located in the N-terminal part of the protein. These repeats were shown to prevent proteasomal degradation and peptide loading to major histocompatibility complex (MHC), class I molecules (Levitskaya *et al.* 1995). This represents a novel and unique mechanism for bypassing recognition by cytotoxic T cells and providing longevity to a foreign molecule that plays such a pivotal role for the maintenance of the viral genome in proliferating cells as well as in the establishment of *in vivo* latency.

Apart from its role in the regulation of viral replication and transcription, EBNA-1 might also affect cellular replication and transcription if EBNA-1-binding sites exist in the cellular genome. Effects of EBNA-1 on growth pattern, phenotype and tumorigenicity should become apparent from the comparison of EBV-negative BL cell lines with EBV-positive group I BL lines. However, such comparative studies have not provided any evidence for changes that might be caused by EBNA-1 (Falk *et al.* 1993; Ruf *et al.* 1999). In a model system of EBV-negative Akata cells reinfected by EBV, changes in soft-agar growth and tumorigenicity have been observed that could not be attributed to EBNA-1 (Falk *et al.* 1993; Komano *et al.* 1998, 1999; Ruf *et al.* 1999). An oncogenic potential of EBNA-1 *in vivo* expressed under the control of the immunoglobulin (Ig) heavy chain intron enhancer has, however, been reported in two transgenic mouse lines (Wilson *et al.* 1996). This finding awaits confirmation in an experimental setting that excludes position effects due to integration of the transgene. If confirmed, it may not only shed new light on EBNA-1 as a viral oncogene expressed in virtually all EBV-associated malignancies (including BL), but it may also call for novel safety considerations for the use of EBV-derived EBNA-1-expression vectors in human gene therapy.

(ii) *EBNA-LP*

cDNA sequencing revealed an additional open reading frame in some of the multiply spliced EBNA mRNAs with coding potential for a protein with N-terminal repeats of 66 amino acids derived from two exons in the W repeats, and 45 C-terminally located unique amino acids derived from two exons located in the long unique region (Bodescot *et al.* 1984; Speck *et al.* 1986). Antipeptide antibodies verified the existence of this protein, which is highly variable in size due to variations in the number of W-repeat exons (Dillner *et al.* 1985; Finke *et al.* 1987; Sauter *et al.* 1988). Depending on the usage of Wp or Cp, the AUG translation initiation codons are generated by differential splicing, as well as the frequency with which mRNAs carry an AUG translation initiation codon in front of the EBNA-LP open reading frame (Rogers *et al.* 1990). EBNA-2 and EBNA-LP are the first viral genes expressed after infection of primary human B cells with EBV (Allday *et al.* 1989; Rooney *et al.* 1989). EBNA-LP appears to be important for the initiation of B-cell immortalization, since mutant viruses lacking the two C-terminal exons have a reduced potential to immortalize B cells *in vitro* (Hammerschmidt & Sugden 1989; Mannick *et al.* 1991). EBNA-LP cooperates with EBNA-2 in transcriptional regulation and enhances the transactivation potential of EBNA-2 for its target genes (Harada & Kieff 1997; Nitsche *et al.* 1997; Peng *et al.* 2000). The structure of the EBNA-LP protein and its helper function for EBNA-2 transactivation are conserved amongst non-human primate lymphocryptoviruses (LCVs) (Peng *et al.* 2000). A role for EBNA-LP in cell-cycle regulation is suggested by the finding that EBNA-LP phosphorylation is dependent on the cell-cycle stage (Kitay & Rowe 1996) and that EBNA-LP, together with EBNA-2, can induce cyclin D2 and cell-cycle activation in primary human B cells pre-activated by stimulation of CD21 through gp350 treatment (Sinclair *et al.* 1994). Whether cyclin D2 is a direct or indirect target gene of EBNA-2 and EBNA-LP is still not known. EBNA-LP was reported to co-localize with p53 and retinoblastoma (Rb) proteins (Szekely *et al.* 1993) but there is no evidence that the function of p53 and Rb is modified due to EBNA-LP binding (Allday *et al.* 1995; Inman & Farrell 1995). EBNA-LP was described as localizing to the nuclear compartment, defined by accumulation of the promyelocytic leukaemia (PML) gene

product (Szekely *et al.* 1996). This nuclear compartment has gained great interest because it is disrupted in a human malignancy, acute PML. It is suspected to play a role in many physiological processes including transcription, formation of chromatin structure, cellular growth control, differentiation and apoptosis, but a comprehensive picture as to its function(s) has not yet emerged (Matera 1999; Seeler & Dejean 1999).

(iii) *EBNA-2*

Deletion of the EBNA-2 reading frame in P3HR-1 virus

The EBNA-2 reading frame is located close to the junction of the large internal repeats to the long unique region. Deletion of the EBNA-2 reading frame in P3HR-1 virus is associated with a loss of the immortalizing capacity of the virus while infectivity of the virus for Raji cells is maintained (Miller *et al.* 1974). Immortalizing virus, with the deletion repaired, could be rescued either by superinfection of Raji cells with P3HR-1 virus (Fresen *et al.* 1978; Skare *et al.* 1985) or by reconstituting the deletion of P3HR-1 virus with cloned fragments spanning the deletion and harbouring the *EBNA-2* gene (Cohen *et al.* 1989; Hammerschmidt & Sugden 1989). The *EBNA-2* gene of the B95-8 prototype codes for a protein of 487 amino acids with two repeat structures, a polyproline stretch of 42 amino acids (59–100) and an arginine–glycine repeat of 16 amino acids (341–356). Analysis of the viral genome harboured by the parental Jijoye line, from which the P3HR-1 line had been obtained by single-cell cloning (Hinuma *et al.* 1967), revealed the surprising finding that the *EBNA-2* genes of Jijoye and B95-8 differ from each other and share only *ca.* 50% sequence homology (Adldinger *et al.* 1985; Bornkamm *et al.* 1982). Although strain differences between EBV type 1 (B95-8 prototype) and type 2 (Jijoye and AG876 prototypes) (Dambaugh *et al.* 1980) have also been found in other genes (Sample *et al.* 1990), the difference in the transforming ability of EBV type 1 and type 2 strains (Rickinson *et al.* 1987) have been assigned to the differences in the *EBNA-2* genes (Tomkinson & Kieff 1992). A detailed deletional and mutational analysis of EBNA-2 by Kieff and co-workers has revealed that many regions of the molecule can be deleted without destroying the transforming capacity except three: (i) several proline residues from the polyproline stretch and the neighbouring region, (ii) the region encompassing the recombination signal-binding protein Jκ (RBP-Jκ)-interaction domain (amino acids 281–336), and (iii) the transactivation domain (amino acids 426–462) (Cohen *et al.* 1991; Harada *et al.* 1998; Tong *et al.* 1994; Yalamanchili *et al.* 1996).

EBNA-interacting proteins

EBNA-2 is a transactivator of many cellular and viral genes lacking the ability to bind to DNA directly. The EBNA-2 response elements in the *LMP-1*, *LMP-2* and Cp promoters are relatively complex and consist of 87, 81 and 50 bp, respectively. A cellular protein mediating binding of EBNA-2 to its response element was first described in the *LMP-2A* promoter (Zimber-Strobl *et al.* 1993) and later shown also to have binding sites in Cp and the *LMP-1* promoter (Laux *et al.* 1994*b*; Ling *et al.* 1993*a*). This protein has been identified as RBP-Jκ, and is also known as CBF-1 (Cp-binding factor 1) (Grossman *et al.* 1994; Henkel *et al.* 1994; Waltzer *et al.* 1994; Zimber-Strobl *et al.* 1994). It is an ubiquitously expressed protein highly conserved in evolution with homology in *Drosophila* (Suppressor of Hairless, Su(H)) (Furukawa *et al.* 1992; Schweisguth & Posakony 1992) and *Caenorhabditis elegans* (Christensen *et al.* 1996). Its name is misleading since it does not bind to the Ig Jκ sequence. *Drosophila* genetics provided a clue to its physiological function. RBP-Jκ–Su(H) is a DNA-binding protein and the downstream target of the cell-surface receptor Notch (Artavanis-Tsakonas *et al.* 1999). Ligand binding to its receptor induces intracellular cleavage of Notch (Kidd *et al.* 1998; Lecourtois & Schweisguth 1998; Schroeter *et al.* 1998; Struhl & Adachi 1998). The clipped-off intracellular domain of Notch migrates to the nucleus, binds to RBP-Jκ and converts this repressor (Hsieh & Hayward 1995) into an activator and switches on target genes (Jarriault *et al.* 1995). EBNA-2 and activated Notch interact with similar regions in the RBP-Jκ molecule and fulfil similar functions (Hsieh *et al.* 1996; Sakai *et al.* 1998). EBNA-2 may therefore be regarded as a constitutively active functional homologue of Notch.

Mammalian cells have four Notch homologues and at least as many ligands. Despite some common properties and target genes, activated Notch-1 is unable to substitute for EBNA-2 either in induction or in maintenance of B-cell proliferation (Höfelmayr *et al.* 2001). Binding of EBNA-2 to RBP-Jκ is necessary but not sufficient for transactivation of the EBNA-2-regulated *LMP-2A* promoter. Other factors involved in promoter activation have not yet been identified (Meitinger *et al.* 1994). In the *LMP-1* promoter, PU.1 (or Spi-B binding to the same site) is additionally involved in transactivation by EBNA-2 (Johannsen *et al.* 1995; Laux *et al.* 1994*b*). The region in EBNA-2 interacting with RBP-Jκ has been mapped to amino acids 316–326 (Ling *et al.* 1993*a*; Yalamanchili *et al.* 1994), a region highly conserved among the EBNA-2 genes of EBV type 1, type 2 and the baboon lymphocryptovirus (LCV) *Herpesvirus papio* (Ling *et al.* 1993*b*). It is one of the three regions of EBNA-2 that is absolutely necessary for immortalization (Cohen *et al.* 1991; Harada *et al.* 1998; Tong *et al.* 1994; Yalamanchili *et al.* 1996). How EBNA-2 is recruited to PU.1 or Spi-B in the *LMP-1* promoter is still an unresolved issue. An EBNA-2-deletion mutant lacking the RBP-Jκ-interaction domain is unable to immortalize but is able to activate the *LMP-1* promoter (Sjoblom *et al.* 1995; Yalamanchili *et al.* 1994). At present we are left with the paradox that the regions of EBNA-2 required for expression of the *LMP-1* promoter have been reported not to be essential for immortalization. Using protein-purification regimens or a two-hybrid screen a number of additional EBNA-2-interacting proteins have been identified. The transactivation domain of EBNA-2 was shown to interact with the transcription machinery, i.e. TFIIB, TFIIE, TFIIH, TAF40 and RPA70 (Tong *et al.* 1995*a*,*b*,*c*) and with histone acteyltransferases p300, CBP and PCAF (Wang *et al.* 2000). EBNA-2 furthermore interacts with hSNF5/Ini1, a member of the family of chromatin remodelling proteins (Wu *et al.* 1996). A function of EBNA-2 apart from transcriptional regulation in RNA transport or splicing is suggested by the fact that EBNA-2 as well as EBNA-3C interact with DP103, a DEAD box protein and RNA helicase (Grundhoff *et al.* 1999).

EBNA-2 target genes

As discussed above, the identification of viral target genes has been fundamental for the elucidation of the biochemistry and function of EBNA-2. Our knowledge is, however, still very limited concerning cellular target genes of EBNA-2. Since EBNA-2 regulates the expression of almost all other viral genes it is impossible to discriminate which of the many cellular genes that are upregulated in EBV-immortalized cells are direct target genes of EBNA-2. Kempkes *et al.* (1995*b*) have described a conditional system for EBNA-2 that allows discrimination between the direct and indirect effects of EBNA-2. They fused EBNA-2 to the hormone-binding domain of the oestrogen receptor thus rendering the function of EBNA-2 dependent on the presence of oestrogen. The *EBNA-2* fusion gene was used to complement the EBNA-2 defect of P3HR-1 virus in B-cell immortalization. Primary human B-cell lines were obtained that proliferate in the presence but not the absence of oestrogen. Since the function of EBNA-2 is regulated by oestrogen at a post-translational level, it is possible to add oestrogen to hormone-deprived cells in the presence of protein synthesis inhibitors and to study the pattern of transcripts that are direct targets of EBNA-2. This approach has identified the viral *LMP-1* and *LMP-2* genes as well as the cellular *c-myc* gene as direct target genes of EBNA-2 (Kaiser *et al.* 1999). Notably, activated *notch-1*, if expressed to a comparable level, does not share this important function with EBNA-2 (Höfelmayr *et al.* 2001). A systematic search for EBNA-2 target genes using array technologies is underway.

(iv) *EBNA-3A, -3B and -3C*

EBNA-3A, -3B and -3C are members of a family of nuclear proteins composed of 944, 937 and 992 amino-acid residues, respectively, encoded by tandemly arranged genes located in the middle of the linear viral genome. The proteins of the EBNA-3 family were first detected by Western blotting using human sera from patients with rheumatoid arthritis (Dillner *et al.* 1986; Kallin *et al.* 1986; Rowe *et al.* 1987; Sculley *et al.* 1984; Sculley *et al.* 1986). They are encoded by alternatively spliced transcripts initiated at the Cp and are each composed of a small 5′- and a large 3′-exon. EBNA-3A, -3B and -3C differ in type 1 and type 2 strains but these differences do not affect the immortalizing ability of the virus (Sample *et al.* 1990; Tomkinson & Kieff 1992). EBNA-3A and -3C are required for immortalization whereas EBNA-3B is not (Tomkinson *et al.* 1993). EBNA-3B is one of the dominant primary targets for recognition of immortalized cells by cytotoxic T cells (Rickinson & Moss 1997) and might be counterselected if it were a non-essential gene for the viral life cycle *in vivo*. In patients with post-transplant lymphoproliferative disease (PTLD) treated with EBV-specific T cells, viral escape mutants have been selected *in vivo* that have deleted the immunodominant EBNA-3B gene (Gottschalk *et al*. 2001).

The EBNA-3B and -3C proteins were reported to participate in transcriptional regulation of *CD40*, *CD77* and vimentin genes, and of the *LMP-1* and *CD21* genes, respectively (Allday *et al.* 1993; Silins & Sculley 1994; Wang *et al.* 1990*a*). EBNA-3A, -3B and -3C proteins were shown to inhibit transcriptional activation of EBNA-2-responsive promoters (Le Roux *et al.* 1994) by preventing RBP-Jκ and EBNA-2–RBP-Jκ complexes from binding to their cognate RBP-Jκ-binding sites (Bain *et al.* 1996; Johannsen *et al.* 1996; Radkov *et al.* 1997, 1999; Robertson *et al.* 1995; Waltzer *et al.* 1996; Zhao *et al.* 1996). The EBNA-3 proteins are thus believed to counterbalance and fine-tune the action of EBNA-2. They may, however, not only act as negative regulators of EBNA-2. They may harbour intrinsic transcription-activation functions that need to be unmasked (Cludts & Farrell 1998; Marshall & Sample 1995). EBNA-3C, but not -3A or -3B, was found to cooperate with EBNA-2 in the induction of the *LMP-1* promoter through PU.1 (Zhao & Sample 2000). EBNA-3C has furthermore been described to counter the action of cyclin-dependent kinase inhibitor p16/INK4A, to functionally inactivate Rb in a similar way to the viral proteins human papillomavirus E7 and adenovirus E1A, and to override a number of cell-cycle checkpoints (Parker *et al.* 1996, 2000; Wade & Allday 2000).

(v) *LMP-1*

Transforming potential for fibroblasts, in vivo *oncogenicity and target genes*

The *LMP-1* gene, located close to the right-hand terminus of the linear viral genome, is transcribed leftwards and encodes an integral membrane protein of 386 amino acids. It consists of an internally localized short hydrophilic N-terminus, six hydrophobic transmembrane domains separated from each other by short turns and an internal cytoplasmic domain of about 200 amino acids. LMP-1 is localized in patches on the plasma membrane, reminiscent of growth-factor receptors stimulated by ligand binding (Liebowitz *et al.* 1986). *LMP-1* is the only known EBV gene with transforming potential for rodent fibroblasts (Wang *et al.* 1985). *LMP-1*-expressing rodent fibroblasts grow in low serum concentration, acquire anchorage-independent growth in soft agar and become tumorigenic. In primary B cells, *LMP-1* induces DNA synthesis and upregulation of CD23, CD40 and CD54 (Peng & Lundgren 1992), and, when expressed from the Ig heavy chain intron enhancer in transgenic mice, it induces B-cell lymphomas after one year of age (Kulwichit *et al.* 1998).

Upon transfection into EBV-negative BL cells, LMP-1 is cytostatic if expressed at high levels (Floettmann *et al.* 1996; Gahn & Sugden 1993; Hammerschmidt *et al.* 1989). When expressed at low levels, it induces a number of phenotypic and functional changes such as upregulation of adhesion molecules and activation markers (e.g. CD23, CD39, CD40, CD54 and CD58) (Wang *et al.* 1988, 1990*a*), upregulation of haemopoietic growth-factor receptors (Devergne *et al.* 1996*b*), restoration of immunological functions, i.e. coordinate upregulation of peptide transporters and HLA (human leucocyte antigen) class I and class II molecules (Cuomo *et al.* 1990; de Campos-Lima *et al.* 1993; Rowe *et al.* 1995; Zhang *et al.* 1994), and induction of cyclin D2 (Arvanitakis *et al.* 1995) and of stress-response genes preventing apoptosis such as *bcl-2*, *A20*, and *bfl-1* (D'Souza *et al.* 2000; Henderson *et al.* 1991; Laherty *et al.* 1992). LMP-1 downregulates Bcl-6, a gene frequently translocated in human B-cell lymphomas and physiologically involved in germinal-centre formation (Carbone *et al.* 1998) and suppresses the senescence-induced expression of p16INK4a (Yang *et al.* 2000). Many of these functions have been attributed to LMP-1's

ability to induce nuclear factor kappa B (NF-κB) (Cahir-McFarland *et al.* 2000; Feuillard *et al.* 2000; Hammarskjold & Simurda 1992), but the molecular link between LMP-1 and induction of a given gene is in many cases still missing. When expressed in epithelial cells, LMP-1 inhibits cell differentiation in keratinocyte raft cultures *in vitro* (Dawson *et al.* 1990), causes morphological transformation (Fahraeus *et al.* 1990), and induces aberrant keratin expression and severe epidermal hyperplasia in transgenic mice (Wilson *et al.* 1990). Among the molecular targets of LMP-1 in epithelial cells are matrix metalloproteinase MMP-9 (Takeshita *et al.* 1999; Yoshizaki *et al.* 1998) and the epidermal growth factor receptor (Miller *et al.* 1997), which are suspected of playing an important role in tumour development and invasion.

Requirement for immortalization

Conditional expression of LMP-1 from a tetracycline-regulated promoter revealed that continuous LMP-1 expression is required for proliferation of EBV-immortalized cells (Kilger *et al.* 1998). Constitutive expression of LMP-1 in the absence of functional EBNA-2 can rescue cell survival but not proliferation, similar to stimulation of the cells with CD40 ligand (Zimber-Strobl *et al.* 1996). A detailed mutational analysis of LMP-1 has shown that the N-terminal cytoplasmic region, the transmembrane domain and the neighbouring 44 amino acids of the cytoplasmic C-terminal domain are required for B-cell immortalization (Kaye *et al.* 1993, 1995), of which amino acids 185–211 appear to be essential (Izumi *et al.* 1997). The function of the C-terminal 155 amino acids can be complemented through co-cultivation of infected cells on a fibroblast feeder layer. It is not essential for initial outgrowth of EBV-infected cells but is required for growth at low-cell density and establishment of long-term cultures. A similar phenotype was observed when only two tyrosine residues in the C-terminal part were mutated (Izumi & Kieff 1997). No difference in the immortalizing potential compared with wild-type was observed with mutants lacking amino acids 232–351 (Izumi *et al.* 1999*a*).

LMP-1-interacting proteins

A first step towards elucidating the molecular mechanism of action of LMP-1 was the observation that LMP-1 activates NF-κB (Hammarskjold & Simurda 1992). Analysis of proteins interacting with the C-terminal cytoplasmic domain of LMP-1 revealed an association with molecules called TRAFs (tumour necrosis factor (TNF) receptor-associated factors) that bind to the C-terminal domains of several members of the TNF-receptor family including CD40 and participate in the activation of NF-κB (Mosialos *et al.* 1995). These findings initiated an intensive genetic and biochemical analysis of LMP-1. The NF-κB-activating function could be narrowed down to two separate regions in the C-terminal part of the molecule, the C-terminus activation region, CTAR-1 (amino acids 194–231) and CTAR-2 (amino acids 351–386) (Huen *et al.* 1995) that appear to cooperate in the same signalling complex (Floettmann *et al.* 1998). CTAR-1 can associate with TRAF-1, TRAF-2, TRAF-3, and TRAF-5 through a PxQxT/S motif (Brodeur *et al.* 1997; Devergne *et al.* 1996*a*; Sandberg *et al.* 1997). TRAF-1 and TRAF-2 seem to be involved in transmitting a positive and TRAF-3 in transmitting a negative signal. CTAR-2 comprises the eight C-terminal amino acids. CTAR-2 does not interact with TRAFs directly. It recruits TRAF-2 and the signalling complex through TRADD (TNF receptor-associated death domain protein). TRADD binds to CTAR-2 not via its death domain, as it does when binding the TNF receptor I, but through its N-terminus (Izumi *et al.* 1999*b*; Kieser *et al.* 1999).

An important function of both CTAR-1 and CTAR-2, mediated by TRAF-2 as a common denominator, is the induction of NF-κB through sequential activation of NIK (NF-κB-inducing kinase), IKK-α and IKK-β (inhibitor of κB kinase), and IκB-α (inhibitor of κB) (Sylla *et al.* 1998). Additional pathways downstream from CTAR-1 as well as CTAR-2 are activation of p38 stress kinase and its target ATF-2 (Eliopoulos *et al.* 1997) and downstream of CTAR-2 but not CTAR-1, induction of AP-1, the heterodimeric complex composed of Fos and Jun (Kieser *et al.* 1997). Activation of p38 kinase is mediated by TRAF-2, whereas the involvement of TRAF-2 in the activation of JNK1 (c-jun N-terminal kinase) is still disputed (Eliopoulos *et al.* 1999; Kieser *et al.* 1999). A third activation domain (CTAR-3) located between CTAR-1 and -2 has been defined in the C-terminal part of LMP-1 through interaction with Janus-activated kinase (JAK)3. Interaction with JAK3 induces tyrosine phosphorylation and activation of STAT (signal transducer and activation of transcription) factors (Gires *et al.* 1999). Figure 2 summarizes which cellular proteins interact with LMP-1 and which pathways are engaged.

LMP-1 is a constitutively active receptor of the TNF-receptor family

The hypothesis that LMP-1 acts as a constitutively active receptor has been put forward because LMP-1 forms patches at the membrane like such receptors and interacts with signalling molecules that bind to the intracytoplasmic part of receptors of the TNF-receptor family. Formal proof that LMP-1 indeed acts as a constitutively active receptor was provided by domain-swapping experiments (Floettmann & Rowe 1997; Gires *et al.* 1997). Fusion of the N-terminal cytoplasmic and transmembrane domain of LMP-1 to the intracytoplasmic part of CD40 rendered CD40 activation constitutive and abolished the requirement for a ligand. Inversely, fusion of the extracellular and transmembrane part of the nerve growth factor receptor or of CD2 to the C-terminal cytoplasmic part of LMP-1 rendered signalling through LMP-1 dependent on ligand or antibody binding. Formation of an oligomeric signalling complex at the inner side of the membrane is the critical event. This was elegantly shown by mutant constructs allowing conditional cross-linking of LMP-1 at the cytoplasmic side of the membrane (Gires *et al.* 1997; Kieser *et al.* 1997).

Despite many similarities LMP-1 and CD40 have distinct functions

The functional similarity between LMP-1 and CD40 has been widely demonstrated in many different assays (Eliopoulos *et al.* 1996; Zimber-Strobl *et al.* 1996). There are, however, also distinct differences. Biochemically, the most obvious difference is that LMP-1 signalling involves TRADD binding whereas CD40 signalling does not. On

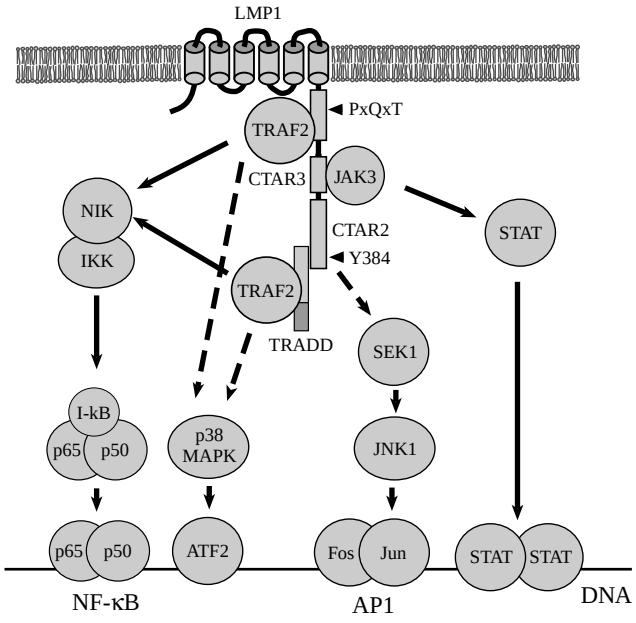

Figure 2. Schematic representation of the signalling pathways engaged by LMP-1 and the proteins interacting with its C-terminal cytoplasmic domain.

the other hand CD40, but not LMP-1, recruits TRAF-6 directly to its cytoplasmic domain. Functional similarities as well as important differences were observed in CD40 knock-out mice in which the CD40 gene was substituted by an LMP-1 transgene. Loss of CD40 is associated with a severe immunodeficiency characterized by the absence of germinal centres and a secondary IgG antibody response (Kawabe *et al.* 1994). The LMP-1 transgene expressed from an Ig heavy chain promoter and enhancer promoted Ig class switching to IgG1 in CD40 knock-out mice but failed to induce germinal-centre formation and affinity maturation during the antibody response. In CD40 wild-type mice, the LMP-1 transgene induced extrafollicular B-cell differentiation and inhibited rather than induced germinal-centre formation but it did not block the synthesis of high-affinity antibodies (Uchida *et al.* 1999). These data show that LMP-1 and CD40, even though they share some properties, have clearly distinct functions.

(vi) *LMP-2A and -2B*

LMP-2A

LMP-2A and -2B are two related integral membrane proteins which share their 12 transmembrane domains and the short C-terminal tail. LMP-2A carries an additional hydrophilic N-terminal domain of 119 amino acids with eight tyrosine residues encoded by a separate first exon. Circularization of the viral genome through the terminal repeats is a necessary step for the formation of the *LMP-2A* and *-2B* transcription units (Laux *et al.* 1988, 1989; Sample *et al.* 1989). The promoters and the respective coding and non-coding first exons of the *LMP-2A* and *-2B* genes are located close to the right-hand end of the linear viral genome and flank the *LMP-1* transcription unit, whereas the shared exons 2 to 8 are located at the opposite end of the viral genome and become juxtaposed by its circularization. Both promoters of *LMP-2A* and *-2B* respond to EBNA-2.

LMP-2A associates with phosphotyrosine kinases and interferes with B-cell-receptor signalling

A clue to the function of LMP-2A has been provided by the observation that LMP-2A shares several properties with molecules involved in B-cell-receptor signalling. It is not only phosphorylated at tyrosines, it is also tightly associated with several phosphotyrosine kinases (PTKs) through their SH2 domains and shares the so-called immunoreceptor tyrosine-based activation motif (ITAM) $(YXXL/I)_2$ with signal-transducing subunits of antigen- and Fc-receptors in various cells of the haemopoietic system (Beaufils *et al.* 1993; Burkhardt *et al.* 1992; Longnecker *et al.* 1991; Reth 1989). Similar to LMP-1, the molecule forms aggregates at the membrane, mimicking an activated receptor. Critical to the function of LMP-2A is the binding of Lyn, a src family tyrosine kinase expressed in B cells, to the phosphorylated tyrosine residue 112 of LMP-2A that is phosphorylated by Lyn or another PTK (Fruehling *et al.* 1998). Lyn binding induces phosphorylation at tyrosines 74 and 85, the two tyrosine residues of the ITAM motif, as a prerequisite for recruitment of Syk, another tyrosine kinase with two SH2 domains (Fruehling & Longnecker 1997). As a consequence of Lyn and Syk binding, the baseline PTK activities of Lyn and Syk are substantially reduced and surface Ig (sIg) cross-linking fails to activate Lyn, Syk, PI3-kinase (phosphoinositol-3-kinase), PLC-γ_2 (phospholipase C-γ_2), Vav, Shc and MAP (mitogen-activated protein) kinase. Mediated by this inhibition of signalling through the B-cell receptor, Ca^{2+} mobilization and entry into the lytic cycle is blocked upon B-cell receptor cross-linking through anti-sIg. Syk, PI3-kinase, PLC-γ_2, and Vav are constitutively tyrosine phosphorylated, and their tyrosine phosphorylation does not change following sIg cross-linking. LMP-2A is thus assumed to block signalling by desensitizing the receptor through its constitutive activation. Regarding induction of the lytic cycle, this inhibition can be bypassed by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate plus Ca^{2+} ionophore (Miller *et al.* 1993, 1994, 1995). Mutation of tyrosine 112 completely abolishes the recruitment of PTKs at the cytoplasmic tail of the LMP-2A molecule as well as LMP-2A's ability to inhibit B-cell-receptor signalling (Fruehling *et al.* 1998).

Regarding the expression of LMP-2A in NPC, it is interesting that LMP-2A interacts and functionally interferes in epithelial cells with other PTKs signalling to cell adhesion-initiating pathways such as Csk (Scholle *et al.* 1999).

In transgenic mice LMP-2A acts as a constitutively active surrogate B-cell receptor

The *in vitro* data clearly demonstrated that LMP-2A interferes with B-cell-receptor signalling. However, the data did not provide an answer to the question whether LMP-2A only inhibits signalling or is also able to provide a positive signal by acting as a constitutively active surrogate B-cell receptor. This important issue has now been resolved by studying LMP-2A's action in transgenic mice.

The B-cell receptor not only acts as a receptor for antigen, in an unbound state or interacting with low-affinity ligands, it also provides a survival signal to B cells. Thus, the B-cell compartment is totally wiped out and B cells carrying a functional B-cell receptor are no longer

synthesized if the transmembrane domain of the Igl heavy chain is deleted (Lam *et al.* 1997). Similarly, B-cell development is blocked at the pro-B-cell stage in Rag-1-deficient mice lacking the machinery for Ig rearrangement (Mombaerts *et al.* 1992). In two transgenic mice lines expressing LMP-2A under the control of the Ig heavy chain intron enhancer, LMP-2A inhibited V-DJ_H but not V-J_κ rearrangement and provided a survival signal to $CD19^+IgM^-$ B cells thus enabling these cells to colonize the peripheral lymphoid organs. When crossed to the recombinase-deficient Rag-$1^{-/-}$ background, LMP-2A again rescued survival and differentiation of the cells into peripheral $CD19^+IgM^-$ cells (Caldwell *et al.* 1998). In three other transgenic lines, LMP-2A expression was probably not high enough to shut down heavy chain rearrangement. In these mice LMP-2A did not interfere with normal B-cell development. When these transgenic B-cell-receptor-positive mice were crossed to Rag-$1^{-/-}$ animals, LMP-2A was, however, able to rescue survival and differentiation of pro-B cells into peripheral $CD19^+IgM^-$ cells (Caldwell *et al.* 2000). These data provide compelling evidence that LMP2A not only interferes with normal B-cell-receptor signalling, but can also act as a constitutively active surrogate B-cell receptor. This is an extremely important finding regarding the pathogenesis of Hodgkin's disease, a malignancy of B cells lacking a functional B-cell receptor in which LMP-2A is consistently found to be expressed (Niedobitek *et al.* 1997*b*).

Consistent with these findings, LMP-2A and -2B proved not to be required for *in vitro* B-cell immortalization (Longnecker *et al.* 1992, 1993*a*,*b*). In keeping with a role of LMP-2A in the maintenance of latency and prevention of entry into the lytic cycle, LMP-2A transcripts have been consistently found in latently infected peripheral memory B cells *in vivo* (Qu & Rowe 1992). It is an interesting question how LMP-2A transcription is brought about in memory cells in the absence of EBNA-2. The known LMP-2A promoter is under stringent control of EBNA-2 in B cells but cellular factors such as activated Notch might take over the role of EBNA-2. Alternatively, it is also conceivable that transcription of LMP-2A is initiated from another still unknown promoter whose activity does not depend on EBNA-2 and RBP-Jκ.

LMP-2B

The *LMP-2B* promoter is a bidirectional promoter sharing its EBNA-2 response element with *LMP-1* (Laux *et al.* 1994*a*,*b*). LMP-2B protein is postulated to exist based on transcription and sequence data, but the protein has never been visualized due to the absence of appropriate reagents. Based on predicted structural considerations of the protein, *LMP-2B* is supposed to form aggregates with *LMP-2A*. It should dampen the action of *LMP-2A* because it lacks the N-terminal domain involved in PTK recruitment and signalling. Given the likely significance of *LMP-2A* for the pathogenesis of EBV-associated malignancies and for the establishment and maintenance of *in vivo* latency, it will be important to learn more about *LMP-2B*.

(vii) *Epstein–Barr virus small RNAs*

The small non-polyadenylated nuclear RNAs (EBERs) are by far the most abundant transcripts in EBV-transformed cells. They are transcribed by RNA polymerase III, are located in the nucleus and associate with the ribosomal protein L22, a protein called EAP (EBER-associated protein), and La, a protein which is recognized by serum of patients with systemic lupus erythematosus (Glickman *et al.* 1988; Toczyski *et al.* 1994; Toczyski & Steitz 1991, 1993). Because of their sequence similarity to adenovirus VA1 and VA2 and to cellular U6 RNA, it is not surprising that functional similarities between these RNAs and the EBERs have been observed (Bhat & Thimmappaya 1983). VA1 and VA2 RNA are part of adenovirus strategy to counter the antiviral effect of interferon. They inhibit the activation of an interferon-induced kinase which phosphorylates protein synthesis initiation factor eIF-2α and blocks translation (Wold *et al.* 1994). U6 RNA on the other hand is present in ribonucleoprotein (RNP) particles which play an essential role in splicing. Given the complex splicing of viral transcripts in EBV-transformed cells, this might suggest a role for the EBERs in the regulation of splicing. However, there is no evidence that the EBERs do play a role in growth transformation. Virus mutants lacking the EBERs are transformation competent (Swaminathan *et al.* 1991). The inhibitory effect of interferon on B-cell outgrowth after the infection of primary cells is not affected by the presence or absence of the EBERs in the infecting virus. Furthermore, the EBERs do not abolish the inhibitory effect of interferon for vesicular stomatitis virus replication in LCLs (Swaminathan *et al.* 1992). Likewise, the EBERs are not required for the splicing and expression of viral RNAs and proteins essential for growth transformation. An interesting observation regarding the function of the EBERs has recently been reported (Komano *et al.* 1999). These authors reported that EBERs, if introduced into EBV-negative Akata cell clones, are able to inhibit apoptosis, to upregulate Bcl-2 and to induce colony growth in agar. These observations, however, can hardly be generalized since EBV-positive BL cases do express EBERs and yet, they do not express Bcl-2 and have an equally high susceptibility to undergo apoptosis in response to chemotherapy as EBV-negative BL cases.

(e) *Cellular genes induced by EBV during B-cell immortalization*

Induction of proliferation of normal cells is brought about by signalling molecules (soluble molecules such as hormones or growth factors or matrix or cell-borne molecules) and their interaction with specific receptors, which deliver a signal into the cell, transmit it into the nucleus and convert it into a new transcriptional programme and a proliferative response. As learnt from the lesson of oncogenes, malignant growth is a consequence of genetic changes which, on the one hand, abrogate negative control mechanisms and, on the other, render the signalling process constitutive. This is achieved by short cuts introduced into the signal-transduction pathway at one or the other level from the membrane to the nucleus.

Since different proliferative signals have to converge into a common programme leading to entry into S phase, proteins controlling these checkpoints, such as Rb and p53, are particularly good targets for viruses (papillomaviruses, SV40, adenovirus) to hijack the cellular control mechanisms.

Even though EBNA-LP was reported to co-localize with Rb and p53, evidence for a functional consequence of EBNA-LP binding to Rb and p53 is still missing (Allday *et al.* 1995; Inman & Farrell 1995). The failure to link EBNA-2 and EBNA-LP to cell-cycle control led to the proposal that EBV mimics physiological B-cell activation brought about by antigen and T-cell help rather than that it bypasses checkpoints in the cell cycle. This is indeed suggested by the fact that EBV upregulates all kinds of cell-surface molecules which might be important in signalling and growth regulation. When the phenotype of resting B cells is compared with that of EBV-immortalized LCLs, it becomes evident that EBV induces dramatic changes in the gene-expression programme of the cell. Among the cellular genes induced by EBV are growth factors such as IL-5 (Baumann & Paul 1992), IL-6 (Tosato *et al.* 1990), IL-10 (Burdin *et al.* 1993; Nakagomi *et al.* 1994), TNF-α and lymphotoxin (Estrov *et al.* 1993; Gibbons *et al.* 1994), thioredoxin (Wakasugi *et al.* 1990), receptors such as the transferrin receptor, CD21, CD23 (Calender *et al.* 1987; Wade & Allday 2000; Wang *et al.* 1987), the TNF-α receptor (Gibbons *et al.* 1994), TRAF1 (Mosialos *et al.* 1995), a protein related to the p40 subunit of the IL-12 receptor (Devergne *et al.* 1996*b*), the G-protein-coupled chemokine receptor CCR7 (Birkenbach *et al.* 1993; Burgstahler *et al.* 1995), activation markers (CD39), adhesion molecules (CD44, CD48, CD54 and CD58), and molecules involved in cytoskeleton formation such as vimentin and actin-bundling protein (Birkenbach *et al.* 1993; Mosialos *et al.* 1994). It seems likely that these molecules participate in the induction and maintenance of proliferation by EBV by establishing autocrine loops or cell–cell-mediated cross-stimulatory pathways. It should be kept in mind, however, that there is no simple way of testing such hypotheses. A conditional knock-out system for cellular genes (e.g. conditional induction of antisense RNA or ribozymes) is urgently required to elucidate the role of individual cellular genes for the process of B-cell immortalization.

4. EPSTEIN–BARR VIRUS LIFE CYCLE *IN VIVO*

The fact that EBV-transformed cell lines can be established spontaneously from the peripheral blood of EBV-sero-positive healthy individuals with high frequency indicated that EBV persists lifelong in the body in a latent state. Spontaneous outgrowth of EBV-infected cells is usually a two-step process; EBV is first released from latently infected cells and subsequently it infects and immortalizes resting B cells (Rickinson *et al.* 1974, 1977). The state of the virus in latently infected cells *in vivo* must therefore be different from the state of the virus in cells immortalized by EBV *in vitro*. The issue of *in vivo* latency has been addressed experimentally using a combination of different techniques that allow study of the phenotype and gene-expression pattern of single latently infected cells *in situ* or *ex vivo*. EBER RNAs are expressed at a high level in all EBV-carrying cells *in vitro* regardless of the viral gene-expression programme, except during productive infection (Gilligan *et al.* 1990; Howe & Steitz 1986). Since detection of EBER RNAs by *in situ* hybridization is extremely sensitive and fast and can be applied to formalin-fixed sections, this technique has been widely used by pathologists to trace the EBV-carrying cells *in vivo*. It is, however, an unanswered question whether EBER RNAs are indeed expressed in all states of viral latency *in vivo*.

(a) *Infectious mononucleosis*

During acute infectious mononucleosis, EBV-infected lymphoblasts that resemble EBV-immortalized cells *in vitro* are present in large amounts in tonsils and lymph nodes. These cells are localized mainly in extrafollicular areas and were shown to express, to various degrees, EBNA-2, LMP-1 and LMP-2 in addition to the EBER RNAs (Niedobitek *et al.* 1992, 1997*a*,*b*; Pallesen *et al.* 1991). Staining for antigens of the lytic cycle (BZLF1, BHLF1 or EA-D (early antigen, diffuse type)) revealed small lymphoid cells with signs of plasmacytoid differentiation frequently located in epithelial crypts that were present at a frequency lower than that of lymphoblastoid cells expressing EBNA-2, LMP-1 or LMP-2 (Anagnostopoulos *et al.* 1995; Karajannis *et al.* 1997; Niedobitek *et al.* 1992, 1997*a*).

(b) *In EBV-sero-positive healthy individuals circulating B cells are latently infected*

In situ hybridization has revealed EBER-positive B cells at low frequency localized to interfollicular areas of lymphoid tissues of virtually all EBV-sero-positive individuals (Niedobitek *et al.* 1992). Adult sero-positive individuals also carry latently infected cells in the peripheral blood and most of them shed virus in the saliva (Yao *et al.* 1985*a*). The virus load in the peripheral blood can be quantified either in an *in vitro* immortalization assay measuring the frequency of cells releasing immortalizing virus or directly by polymerase chain reaction (PCR) analysis. About 1–50 per 10^6 B cells in peripheral blood are latently infected (Bird *et al.* 1981; Chen *et al.* 1995; Crawford *et al.* 1981; Von Knebel Doeberitz 1983; Miyashita *et al.* 1995; Qu & Rowe 1992; Tierney *et al.* 1994; Yao *et al.* 1985*a*). In one individual these numbers are remarkably constant over time (Khan *et al.* 1996; Miyashita *et al.* 1995; Yao *et al.* 1985*a*). Fluorescence-activated cell sorting of peripheral blood cells using various surface markers followed by PCR analysis of the various cell fractions revealed that the cells carrying the viral genome are contained in the compartment of resting $CD19^+$, $CD23^-$, $CD80^-$ B cells (Miyashita *et al.* 1995, 1997). In these cells the viral genome is maintained exclusively as an episome indicating that viral replication and infection do not take place in the latency compartment (Decker *et al.* 1996). Since circularization requires B-cell activation and entry into the cell cycle (Hurley & Thorley-Lawson 1988), this implies that a latently infected cell must have been activated at an earlier step during its life cycle.

In the peripheral blood, EBV resides in the IgD^-, $CD27^+$, $CD5^-$ memory B-cell subset (Miyashita *et al.* 1997; Joseph *et al.* 2000*a*), whereas in tonsils there is no such restriction and naive B cells carry the virus as well as memory B cells (Babcock *et al.* 1998, 2000; Joseph *et al.* 2000*b*). The pattern of viral gene expression has been studied by several authors in latently infected peripheral B cells by reverse transcription PCR (RT–PCR). There is

agreement that EBNA-2 is not detectable in these cells and that the LMP-2 message is consistently found (Chen *et al.* 1995; Miyashita *et al.* 1997; Qu & Rowe 1992; Tierney *et al.* 1994). The data regarding EBNA-1 expression are less consistent. Remarkably, BZLF1 transcripts have been found in the peripheral lymphocytes of *ca.* 70% of healthy individuals (Prang *et al.* 1997). Spontaneous induction of the lytic cycle, however, appears to be abortive and to be tightly controlled by cytotoxic T cells with specificity for immediate early or early antigens (Benninger-Doring *et al.* 1999; Bogedain *et al.* 1995; Steven *et al.* 1997).

(c) *Passage of EBV-infected cells through the germinal centre*

EBV infects any kind of B cell in the mucosal epithelium of the tonsils, yet it is confined to memory B cells in the circulation after primary infection. This implies that virus-infected naive B cells must enter the memory cell pool in a rather specific fashion. The question arises whether EBV is dependent upon, or bypasses, the physiological process of antigen-driven memory B-cell selection. During a primary T-cell-dependent immune response, antigen-loaded naive B cells move together with simultaneously primed T cells into the zones of the lymph node separating T- and B-cell areas and form extrafollicular foci called periarteriolar lymphatic sheaths where B cells, supported by appropriate T-cell help, proliferate and differentiate into plasma cells secreting non-hypermutated IgM or IgG. While this transient extrafollicular response is ongoing, antigen-specific $CD4^+$ cells are recruited and germinal centres are formed leading to rapid expansion of antigen-specific B cells within germinal centres. This dramatic proliferation of antigen-stimulated B cells is associated with diversification of the antibody response by hypermutation, selection of high-affinity antibodies, elimination of cells producing self-reactive or low-affinity antibodies, and class switching to other Ig isotypes. Passage of antigen-stimulated B cells through germinal centres results in the formation of either antibody-secreting plasma cells or memory cells. In order to gain access to the memory compartment, EBV-infected B cells may either pass the germinal centre in a fashion that is similar or identical to that of non-infected B cells, or alternatively, viral gene products like LMP-1 and LMP-2A may divert the physiological process. An answer to this question is not yet available. Niedobitek *et al.* (1992) presented histological sections of two cases in which EBV-infected B cells were involved in germinal-centre reactions. In both cases, a single germinal centre within the section displayed diffuse expansion of EBER-positive cells. In a recent study comparing hyperplastic tonsils from children from Brazil and Germany, EBER-positive cells were detected more frequently and at higher numbers in the germinal centres of children from Brazil than those from Germany, and a few of these expressed LMP-1 (Araujo *et al.* 1999). A picture as to the expression of viral genes in the different subsets of tonsillar B cells is now emerging from studies combining cell separation with RT–PCR. Naive tonsillar B cells appear to be continuously infected and express the full set of genes as expressed in *in vitro*-immortalized cells including EBNA-2 (Joseph *et al.* 2000*b*), whereas centroblasts and centrocytes during their passage through germinal centres give rise to a more restricted pattern of viral gene expression that includes LMP-1, LMP-2, and EBNA-1 transcribed from Q*p*, but not EBNA-2 (Babcock & Thorley-Lawson 2000). This pattern of viral gene expression has previously only been described for EBV-associated tumours like Hodgkin's disease and NPC. It will be important to see whether the viral antigens are transcribed and expressed in a minority or majority of EBV-carrying B cells in the germinal centres.

(d) *Virus production* in vivo

As already mentioned, EBV is continuously produced and shed into the saliva of the majority of healthy EBV-sero-positive individuals. The virus-producing cells are small lymphocytes in the lymphoepithelial tissue of the oropharynx (Tao *et al.* 1995*a*,*b*). Remarkably, the amount of virus shed into the saliva correlates with the number of latently infected cells in the peripheral blood that release virus that is detected in an *in vitro* immortalization assay, indicating that there must be a dynamic equilibrium between the different compartments (Yao *et al.* 1985*a*,*b*). Virus production appears to correlate with plasmacytoid differentiation of the infected cells even though this correlation does not seem to be complete (Altmeyer *et al.* 1997; Crawford & Ando 1986). Virtually nothing is known about the signals that interrupt viral latency and induce the lytic cycle *in vivo*. From *in vitro* studies we know that many different pathways including B-cell-receptor signalling converge on the regulation of the lytic-cycle switch (Bauer *et al.* 1982; Goldfeld *et al.* 1995; Mellinghoff *et al.* 1991; Tovey *et al.* 1978; Zur Hausen *et al.* 1978).

Continuous virus production in the oropharynx not only gives rise to transmission of the virus to another individual via infected saliva, but also leads to infection of B cells within the host. In the tonsillar lymphoepithelium of a healthy individual, the infection is not restricted to a given B-cell subpopulation. In the tonsils, naive B cells are continuously infected *in vivo* and transcribe the complete set of viral genes involved in growth transformation *in vitro* (Joseph *et al.* 2000*b*). This raises the critical question as to the fate of these potentially dangerous cells *in vivo*. Theoretically, there are two options: EBV-infected proliferating cells may either be totally eliminated by cytotoxic T cells, or part of the infected cells may be silenced by an unknown mechanism and thus they may get access to germinal centres and the memory compartment.

(e) In vivo *latency in immunocompromised individuals*

It has long been known that the number of latently infected cells in the peripheral blood is markedly increased during immunosuppression (Crawford *et al.* 1981; Ragona *et al.* 1986; Strauch *et al.* 1974; Yao *et al.* 1985*b*) and that this increase is associated with a concomitant increase in the amount of virus produced in the oropharynx (Lam *et al.* 1991; Yao *et al.* 1985*b*). Cell sorting and PCR analysis of peripheral blood cells revealed that EBV-infected resting memory B cells, and not proliferating lymphoblasts, accumulate in the peripheral blood of

immunosuppressed individuals (Babcock *et al.* 1999; Qu *et al.* 2000; Rose *et al.* 2000). The only difference to the normal situation is that lytic viral replication can be observed in the peripheral blood of immunocompromised individuals and that the number of peripheral lymphocytes carrying the viral genome by far exceed the number of cells expressing LMP-2A (Babcock *et al.* 1999). The fact that the number of EBV-infected resting memory cells increases during immunodeficiency and not the number of proliferating cells indicates that a dynamic equilibrium can be maintained that is stable over virus loads differing by several orders of magnitude before the system collapses. This illustrates the extraordinary evolutionary adaptation of the virus and its host. A recent study showed that the pattern of viral gene expression appeared to be unchanged (LMP-2 expression only) up to a virus load of 1000 copies per 10^6 lymphocytes (Qu *et al.* 2000). In patients with copy numbers exceeding 1000 per 10^6 lymphocytes, both LMP-1 and LMP-2 transcripts were detected by RT–PCR. It is important to develop diagnostic criteria in longitudinal studies to judge which viral load and which associated transcription pattern is likely to be dangerous and develop into a post-transplant lymphoproliferative disease.

(f) *Is there a role for epithelial cells in the viral life cycle* in vivo?

Epithelial cells of the oropharynx have long been suspected to play an important role in the productive cycle of the virus. This was based on (i) the presence of the viral genome in epithelial cells of undifferentiated NPC (Desgranges *et al.* 1975*a*,*b*; Trumper *et al.* 1976; Wolf *et al.* 1973), (ii) early studies searching for the viral genome in normal tissue by *in situ* hybridization (Sixbey *et al.* 1984; Wolf *et al.* 1984), (iii) the analogy to other lymphotropic herpesviruses, and (iv) last but not least, replication of EBV in the lateral tongue epithelium of acquired immune deficiency syndrome (AIDS) patients with oral hairy leucoplakia (Greenspan *et al.* 1985). The latter finding showed clearly that EBV is able to replicate very efficiently in epithelial cells under certain conditions and suggested that massive viral replication at the lateral part of the tongue of AIDS patients is just an amplification of the normal situation. Although this is a plausible assumption that may well be true, the actual evidence for an involvement of epithelial cells in the natural viral life cycle in healthy individuals is poor. This concept has been challenged by pathologists who did not find any evidence for infection of epithelial cells, either in healthy individuals or in the course of infectious mononucleosis (Anagnostopoulos *et al.* 1995; Karajannis *et al.* 1997; Niedobitek *et al.* 1992, 1997*a*; Tao *et al.* 1995*a*; Weiss & Movahed 1989). Double-staining experiments provided evidence that at the site of virus shedding in the lymphoepithelium of the oropharynx, lymphocytes, and not epithelial cells, are harbouring the virus (Karajannis *et al.* 1997).

The notion that long-term viral latency *in vivo* is restricted to the haemopoietic system and does not involve epithelial cells has been convincingly demonstrated by the fact that allogeneic bone marrow transplantation from an EBV-sero-negative donor to an EBV-sero-positive recipient can wipe out viral latency *in vivo* (Gratama *et al.* 1988). The pivotal importance of the B lymphoid system for the natural life cycle of the virus is underlined by the fact that patients with X-linked agammaglobulinaemia lacking mature B cells cannot be infected with EBV (Faulkner *et al.* 1999, 2000).

(g) *Open questions*

Over the past years we have experienced a rapid accumulation of knowledge regarding the interaction of EBV with its host *in vivo*, but many questions still remain unresolved. The central question is, of course, whether EBV-infected proliferating B lymphoblasts can be converted into resting B cells *in vivo* and if so, how this is achieved. Viral genes expressed in proliferating cells *in vivo* would have to be silenced at some stage of B-cell differentiation by an unknown mechanism in order to establish *in vivo* latency. However, there is an alternative model that proposes that a switch from proliferation to *in vivo* latency does not necessarily occur *in vivo*. EBV-infected proliferating cells would be completely eliminated by cytotoxic T cells and *in vivo* latency would be established by infection of resting cells not involving proliferation prior to the establishment of *in vivo* latency. According to this model, EBV would be able to discriminate between naive and memory cells. The virus would initiate the proliferation programme only in naive but not in memory cells, and inversely would establish *in vivo* latency only in memory cells but not in naive B cells. Several points argue against this model. First, the viral genome is circularized in latently infected memory cells and circularization of the viral genome requires activation of the cells and entry into the cell cycle prior to establishment of latency (Decker *et al.* 1996; Hurley & Thorley-Lawson 1988). Second, there is no evidence from *in vitro* infection and immortalization studies that the virus is able to discriminate between naive and memory cells.

The next important question is how EBV-infected B cells are recruited to the memory compartment. Is the virus dependent upon the physiological process of antigen-driven B-cell memory selection or is this pathway diverted by viral gene products that interfere with B-cell receptor- and T-cell signalling? Does antigenic stimulation and selection play a role or is the B-cell-receptor signal substituted by the viral constitutively active surrogate receptor LMP-2A? What is the role of CD40 and more generally, of T-cell signals? LMP-1 can partially substitute for CD40 thus allowing B-cell differentiation to proceed in the absence of a CD40 signal. Importantly, LMP-1 also inhibits germinal-centre formation and downregulates *Bcl-6*, a gene essentially required for the formation of germinal centres (Carbone *et al.* 1998; Dent *et al.* 1997; Ye *et al.* 1997), but does not seem to interfere with the production of antibodies with high affinity (Uchida *et al.* 1999). This raises the question whether the germinal centre is the (only) site where EBV-infected B cells may enter the pool of memory B cells. Is there an extrafollicular B-cell differentiation pathway, eventually engaged by LMP-1, that may also lead to the formation of memory B cells? Remarkably, another route to the memory compartment has been described in *lymphotoxin-*$\alpha^{-/-}$ and *lymphotoxin-*$\beta^{-/-}$ mice, and in *lyn*$^{-/-}$ mice lacking germinal centres, and yet, this alternative

pathway allows class switching and affinity maturation of antibodies, although at lesser efficiency (Futterer *et al.* 1998; Kato *et al.* 1998; Matsumoto *et al.* 1996). Is EBV able to use this route? The following observations are of particular interest in this respect: (i) as mentioned, LMP-1 downregulates *Bcl-6*, (ii) LMP-2A inhibits signalling from the B-cell receptor through *Lyn*, another gene that plays an important role in germinal-centre-dependent B-cell differentiation (Kato *et al.* 1998), and finally (iii) EBV induces the chemokine CCR7 receptor that directs B and T cells to extrafollicular areas of lymph nodes (Birkenbach *et al.* 1993; Burgstahler *et al.* 1995; Forster *et al.* 1999). The latter finding may explain why EBER-positive cells have been regularly found in extrafollicular areas and only rarely in germinal centres (Niedobitek *et al.* 1992).

Another important question is whether EBV-infected cells on their way into the memory compartment are more vulnerable to secondary genetic changes than non-infected cells. Once the virus has established latency in memory cells, how is the virus reactivated? Is reactivation linked to differentiation signals, and if so, how? How does the virus enter into epithelial cells? Is this part of the physiological virus–host interaction or a special condition linked to severe immunosuppression? What determines the switch from quantitative to qualitative changes when the virus load is steadily increasing during immunosuppression? An answer to some of these questions will hopefully become available in the next decade.

5. CONCLUDING REMARKS

As mentioned in § 1, it is the peculiarity of EBV to interact with its host in three distinct ways and in a highly regulated fashion: (i) EBV induces on the one hand proliferation upon infection of B cells *in vitro* and *in vivo*, (ii) it enters into a latent phase *in vivo* after the proliferative phase *in vivo*, and (iii) finally it can be reactivated giving rise to synthesis of virus progeny for reinfection of cells of the same individual or virus transmission to another individual. It appears that in EBV-infected healthy individuals *in vivo* these three phases are not separated in time; rather they take place simultaneously and continuously at different sites and in different anatomical and functional compartments that are linked to each other in a dynamic steady-state equilibrium. We have learnt a lot in the past about each of the three specific modes of virus–host interaction. The development of a genetic system has particularly improved the tools and accelerated the learning process regarding B-cell transformation by EBV and the events occurring in the lytic cycle and during synthesis of viral progeny. For the latter, the journey has only started but the train runs at high speed. It is particularly exciting that the third mode of host–virus interaction, i.e. latency, which for many years had been technically impossible to approach, is now in the process of being unravelled. We have learnt important lessons from transgenic mice and many of the interesting crosses regarding the link to cellular signalling pathways are still to come. It is not possible to study the problem of latency in humans using the genetic approach, but it may be envisaged that the genome of primate LCVs will be cloned in infectious form in *E. coli* and may be used as a tool to study basic questions regarding latency of a virus in its natural host also at a genetic level.

The great challenge in the future will be not only to describe each of the three types of virus–host interaction at a molecular level, but it will also be mandatory to learn how the entry and exit into and out of the different compartments is regulated and how they are physiologically connected.

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