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EPSTEIN-BARR VIRUS IMMORTALIZATION AND LATENCY

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1. ABSTRACT

Epstein-Barr virus infects human B lymphocytes. The interaction between the virus and these cells has been the subject of investigation for over three decades. Recent in vitro and in vivo studies, reviewed here, are revealing the mechanisms by which EBV induces and controls proliferation through the expression of six viral nuclear proteins and two plasma membrane proteins. This genetic program is referred to as immortalization and it is suggested that the purpose of immortalization is to use the innate proliferative potential of B cells to inflate the numbers of infected cells prior to virus production and cell lysis. Latency, on the other hand, has only been detected in situ in latently infected humans. It is characterized by the presence of very low numbers of viral episomes that appear to express the RNA for only one protein (Latent Membrane Protein 2) in B cells that bear the markers of a non-activated resting memory subset. Two models are proposed for the mechanism that establishes this state. The differences between immortalization and latency are highlighted in this review and it is suggested that many of the functions currently attributed to latency are actually features of immortalization. An appreciation of this distinction may assist the discussion of the nature of the interaction between the virus and the host in EBV-associated lymphoproliferative diseases and tumors.

2. INTRODUCTION

Upon resolution of a replicative infection, all herpesviruses can reside in the host in an inactive state termed latency. After receiving an appropriate stimulus, production of virions resumes in a process referred to as reactivation (189). Members of the three subfamilies of the Herpesviridae can be distinguished by the types of tissues within which lytic replication occurs and the types of tissues within which latent viral genomes persist. These pathogenic tropisms are considered to be the consequence of an evolutionary process that adapts the lytic/latent strategy to the demands of different tissue environments. This review focuses on the interaction that has evolved between a member of the gamma herpesvirinae subfamily (Epstein-Barr Virus). and human B lymphocytes. Recent evidence suggests that the majority of the viral genes that have been implicated in establishing immortalization mayin fact have no direct role in establishing latency. The term immortalization is used in this review to describe the execution of a deliberate genetic program encoded in the virus that operates B lymphocyte developmental controls to cause differentiation of infected cells into proliferating B blasts. Latency is used in this review exclusively to describe the interaction of virus with resting B lymphocytes as detected *in situ* in the peripheral blood of latently infected individuals and at other anatomical sites where latently infected B cells may await reactivation stimuli. This review provides an update on recent studies of immortalization and the new findings on the latent state. The information is used to develop two possible models, the parallel pathway and the serial pathway model, that distinguish immortalization from latency and provide a basis for understanding EBV latency in the context of a virus-host organism interaction.

3. IMMORTALIZATION

The ability of Epstein-Barr Virus to immortalize B lymphocytes *in vit*ro was discovered thirty-one years ago. (77, 163). From the beginning it was evident that the process of immortalization was extremely efficient, as the percentages of infected B lymphocytes that emerged as immortalized cell lines approached 100% in some experiments. (75). In the absence of a plaque assay, virus stocks have been routinely measured for biological activity by limiting dilution of B cells after infection and using outgrowth of immortalized cells as the endpoint. (135). Research has focused on the immortalizing phenotype of EBV principally because this activity has been proposed as the mechanism underlying the viral pathogenesis of lymphoproliferative diseases. (105, 135, 178).

EBV immortalized B cells are described as lymphoblastoid in appearance. (99). Relative to the quiescent precursor B lymphocyte, the immortalized cells have an enlarged appearance due to the increased cytoplasmic volume required to support high rates of RNA and protein synthesis. They are typically ovoid or slightly

elongated and frequently have a cluster of short villipodia projecting from the surface. Generation time is between 20 and 30 hours and undisturbed cultures grow into macroscopic clumps of cells that adhere to each other but not the substratum of plastic culture bottles. Cell growth is serum and density dependent and cultured cell lines grow most rapidly when maintained between concentrations of 10^5 and 10^6 cells per ml. Fibroblast feeder layers are required for growth at densities below 10^4 or for cloning sublines from a population of immortalized cells. (135). In many respects EBV immortalized cells look and behave like B cells that are stimulated to proliferate by the crosslinking of cell surface CD40 and the addition of IL4 to the culture medium. (12). In addition to CD19 and CD20 cell surface markers, immortalized cells express CD23, CD30, CD39, CD70, B7, CD58, and CD54. (111, 128, 157, 198, 199, 239, 240, 257). The density and serum dependence are suggestive of autocrine feedback loops operating to sustain and enhance growth. (67). IL5, IL6, IL10, and TNF beta (Tumor Necrosis Factor beta). have all been implicated as autocrine growth factors for EBV immortalized cells. (13, 142, 203, 212, 225, 236).

Despite the obvious alterations that accompany virus immortalization of B cells, and the potential disease implications, this virus-cell interaction has been described as 'latent' because most of the cells in a lymphoblastoid cell line produce no detectable virus. However, all cells in a cell line contain viral DNA and express viral proteins. The 170 Kb EBV genome is maintained as a multicopy episome. (214, 215). Cloned cell lines derived after immortalization by infection with less than one virion per cell contain between 10 and several hundred episomes per cell. (214). Episomes initiate replication during S phase of the cell cycle at a 1700 bp cis-acting plasmid maintenance origin (ori P). and replication is carried out by host cell enzymes. (175, 252, 254, 255). An unresolved issue regarding episome maintenance in proliferating cells is how the multicopy carrier state is achieved since detailed analyses of the replication of oriP containing plasmids have shown that there is only one initiation event per origin per S phase. (254). Early work in this area has already established that in *in vitro* infections the multicopy state is achieved by genome amplification only after several days post-infection and subsequent to the alterations associated with immortalization.(85).

Immortalized cells were initially observed to express a viral antigen EBNA (Epstein Barr Nuclear Antigen). by indirect anti-complement immunofluorescence with human sera. (173). To varying degrees, depending upon the cell line and the stimulus used, the cells could be made to produce EA, (Early Antigen)., VCA (Viral Capsid Antigen). and a modest number of virions. (135). The EBNA, EA and VCA 'antigen' designations represent not individual proteins but classes of proteins derived from the more than 85 open reading frames present in the viral genome. (10). At a low frequency (<0.1%). some of the cells in the culture spontaneously express EA and VCA and make virus. The transition from the immortalized state to the virus productive state is accompanied by lymphoblastoid to plasmcytoid phenotypic changes in the infected cell and by the sequential appearance of viral gene products and finally virions. (99).The activation pathway for viral gene expression leading to virus production is largely analogous to the classical transcription cascade described for herpes simplex lytic infection. At the apex of the cascade is the product of the BZLF1 ORF (ZEBRA, or Zta). which acts as EBV's immediate early transcriptional activator. (17, 70, 137, 190, 191). Derepression of the BZLF1 gene probably underlies the action of most chemical and physical inducers of lytic replication and the mechanism is commonly referred to as the latent/lytic switch. (15, 51-57, 122, 262, 263). According to the definitions being used in this review this transition might better be described as the proliferative/productive switch since the change is really from virus-driven lymphoproliferation to cell cycle arrest and virion production rather than the emergence of virus following re-activation from latency.

Virus driven lymphoproliferation is characterized by the expression of at least ten viral genes. Six EBNAs have been identified (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP)., along with two membrane proteins (LMP1 and LMP2). and two short RNAs (EBER1 and EBER2). with extensive putative secondary structure. (99). Of these genes, six (EBNA1, EBNA2, EBNA3A, EBNA3C, EBNA-LP and LMP1). have been implicated directly in the process of immortalization by inhibition with antisense oligonucleotides (193). or by knockout recombinational analyses. (37, 72, 96, 98, 230, 231, 232). Other studies, which eliminated large segments of the viral genome from immortalizing plasmids, have reduced the size of an immortalizing episome from 170 Kb to 64Kb and are consistent with these findings. (182, 232). All of the biological and molecular analyses of events following virus infection of primary B lymphocytes *in vitro* suggest that the virus genome is predisposed to initiate the immortalization program.

The sequence of events very likely begins with attachment of the gp 350/220 viral membrane glycoprotein to the CD21 molecule on the B cell. (146). Crosslinking of CD21 serves to trigger an initial activating signal that may be deliberately preparing the cell for what is about to happen next. Some of the immediate effects that occur simply from binding to CD21 include lck activation and Ca++ mobilization. (29, 31). This is followed by increased mRNA synthesis, blast transformation, homotypic cell adhesion, surface CD23 expression, and IL-6 production. (68, 223, 224). Once the viral genome has been uncoated and delivered to the nucleus, circularization and Wp promoter expression launch a cascade of events that lead to the expression of all of the EBNA proteins and the two membrane proteins. The EBNA-LP and EBNA2 proteins, whose coding sequences are promoter proximal in the EBNA transcription unit, are the first proteins detected. (3, 6). (figure 1). Although the Wp promoter can be used to drive EBNA expression in immortalized cells, a shift to the use of the upstream Cp promoter usually occurs 24 to 48 hrs post-infection. An initial hypothesis regarding control over EBNA gene expression had the switch from the initial Wp promoter to the Cp promoter usage concomitant with the switch to an expanded pattern of splicing that allowed

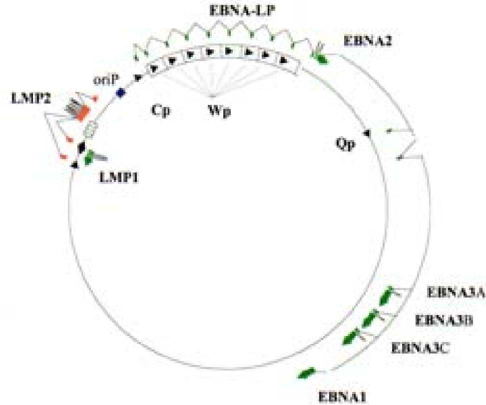

Figure 1. A simplified ciruclar map of the 172 kb EBV episome showing the relative location of the major internal repeat (top of the circle). the replication origin (OriP). and the fused termini repeats (TR). of the genome. The locations of the Cp, Wp and Qp promoters (dark triangles). for the EBNA tanscription unit and the LMP1 and LMP2 promoters are also indicated. Outsie the cirle, the locations of the leader exones and the coding exons for the immortalization proteins are shown and linked by carets to indicated the splicing patterns. Individual EBNA messages contain exons (arrowheads). The intergenic and intron spaces are filled with the genes for the early and late proteins.

expression of EBNA3A, 3B, 3C and EBNA1. (219, 244, 220). It is now clear that these events are more contemporaneous than concomitant and that the expanded pattern of splicing likely precedes the promoter switching. (205). This is consistent with the data that suggests that the downstream EBNAs are in fact regulating Cp activation. (62, 89, 166, 167, 171, 174, 216, 238, 256). Recent evidence from transient transfection assays using linked Cp and Wp reporter constructs in immortalized cells supports a model in which the Wp to Cp switch is driven by transcriptional interference after activation of the upstream Cp promoter. (166). The mechanisms regulating alternative splice and polyA site usage within the EBNA transcription unit remain largely unknown. There are no known transcriptional arrest sites within the EBV genome and there is some evidence to suggest that circumgenomic transcription is occurring in most if not all immortalized B cells. (169).

All of the products of the EBNA transcription unit are involved in transcriptional control and participate in the activation of the expression of the viral membrane protein genes (LMP1 and LMP2). and several cellular genes. The combined action of these viral and cellular products drives infected cells into S phase 24 - 48 hrs post infection. A brief description of the viral products expressed in immortalized cells and what is currently known about their involvement in immortalization is presented below.

3.1. Functions of Immortalization Proteins

3.1.1. EBNA1

When researchers talk about human subjects and their seroconversion to an EBV positive status, they are usually referring to the persistent low titer IgG anti-EBNA1 response that nearly all individuals develop after infection. (77). Because human sera were initially used as a tool to probe for gene expression in virus immortalized cells, EBNA1 became the first EBV nuclear antigen detected. (173). DNA fragment transfection studies quickly identified that the coding sequence was in the BKRF1 ORF. (74, 79, 218). EBNA1 plays a crucial role in virus immortalization. It is a sequence specific DNA binding protein that binds to the origin of plasmid replication (oriP)., and is required not only for initiation of DNA replication but also for efficient segregation of viral episomes at mitosis. (7, 92, 130, 172, 252, 253). The BKRF 1 ORF from the prototype B95-8 virus encodes 641 amino acids. There is a short N terminal domain (89 residues). that is separated from the rest of the molecule by a long 239 residue glycine-alanine copolymer. (figure 2). The copolymer varies in length amongst strains and has been used, along with variations in the repeating unit of other nuclear antigen genes, to 'ebnotype' viruses in epidemiological studies. (48). The glycine-alanine repeat has also been shown to interfere with the proteolytic processing of EBNA1 by the antigen presenting machinery and is therefore responsible for the absence of a CD8 T cell mediated response to immortalized cells directed at this target. (109).

The sequences downstream of the glycine-alanine repeat at the COOH end of the molecule are hydrophylic and contain the DNA binding/dimerization domain. The crystal structure of a fragment of EBNA1 (residues 470-607). encompassing the core of this domain was solved at 2.5Å resolution. (19). The crystallographic asymmetric unit contained one dimer with a central eight stranded antiparallel beta barrel comprised of four strands from each monomer. Beta strands are connected by alpha helices on the outside of the barrel. The first structure that was reported did not include bound nucleic acid, however, much could be inferred from the striking similarity of the EBNA1-DNA binding domain to the DNA binding domain of the bovine papillomarvirus transcriptional activator E2. In particular the DNA recognition helix in E2 had a structural counterpart in EBNA1 (residues 514-527). The similarities were used to model EBNA1 sequence specific docking to DNA and to infer both the mechanism of cooperative binding of dimers to two adjacent sites and the degree of DNA bending caused by EBNA1 interacting with its recognition sequences. Subsequent crystallographic studies of the EBNA1 dimer bound to DNA largely confirmed the inferences made from the structural homology with E2 and refined the origin unwinding model of EBNA1 activity at the oriP replication origin. (18).

The origin of plasmid replication (oriP). is composed of two distinct EBNA1 binding elements spaced approximately 1 Kb apart. (175). The FR (family of repeats). and the DS (dyad symmetry). elements each contain multiple 18 bp EBNA1 binding sites. (246). There are 20 sites in the FR element and 4 in the DS element. Without the FR the origin will still work albeit poorly. At least seven repeats in the FR are needed for the origin to function optimally. (43, 63, 161, 252, 253). The DS element contains four EBNA1 binding sites arranged in two

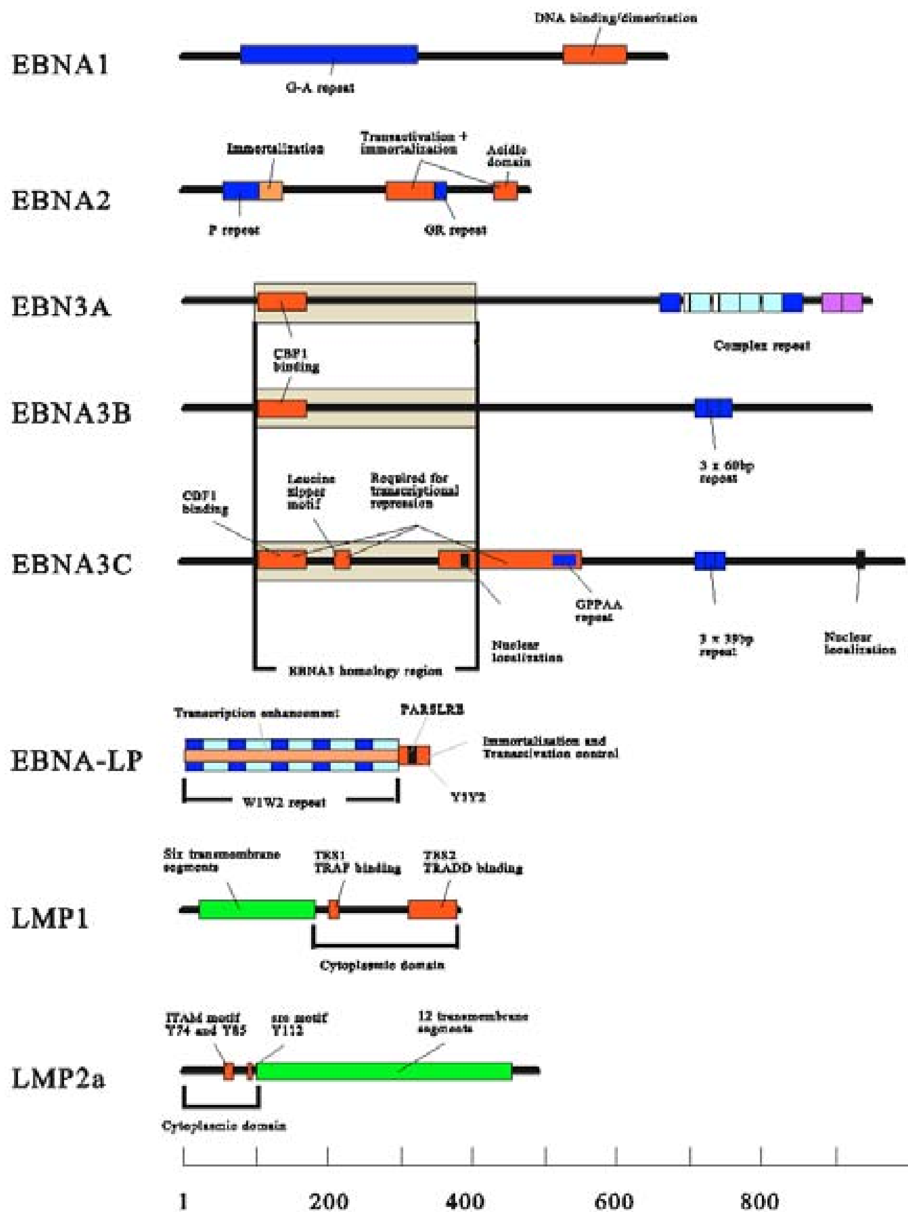

Figure 2. Stuctural features of the immortalization genes. The positions of repeating amino acid sequences (blue boxes)., regions known to be essential for function or containing recognized motifs critical to function (red boxes)., and membrane spanning domains (green boxes). are depcited on the primary sequence of the proteins. See text for details.

palindromic sets. Binding of EBNA1 dimers to the DS element is cooperative and it is from here that bidirectional replication forks emerge during plasmid replication. (217). Two interesting features of oriP/EBNA1 mediated replication are that all replication events subsequent to origin recognition are mediated by host enzymes at S phase and that the FR element aborts the movement of the leftward replication fork forcing the rightward propagating fork to replicate the entire episome. Replication thus begins and ends in oriP. (43, 63).

Several reports have implicated EBNA1 in transcriptional activation. (151, 167, 200, 205, 216, 246, 252). oriP lies 2200 bp upstream of the Cp promoter used by the virus in immortalized B cells to drive expression of the entire EBNA transcription unit. The importance of EBNA1 to Cp activation has been demonstrated by a series of studies that include analyses of events following infection with EBNA2 deleted viruses, (205). RNAase protection analyses of B cells immortalized by viruses with the EBNA2 enhancer deleted from the Cp upstream region, (47, 220). and transient transfection assays of reporter constructs identifying upstream regions essential for Cp transactivation in virus immortalized cells. (167). These

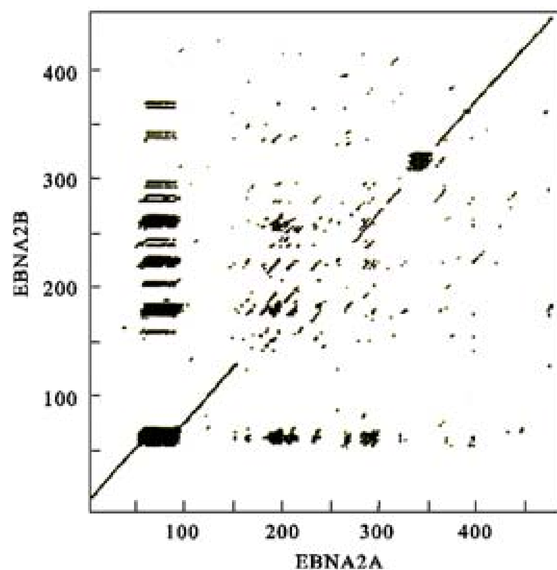

Figure 3. A plot of the homology between the EBNA2 protein sequences of the type 2A and type 2B strains of EBV. This display highlights the repeating sequences (the polyP near the N terminal end and the GR repeat near the C terminus). as bubbles on the diagonal. The discontinuous part of the diagonal line where maximum divergence occurs is in the middle of the protein sequence.

studies clearly demonstrate a positive regulatory effect of oriP and EBNA1 on Cp transcription initiation.

OriP is not the only location within the EBV genome where specific EBNA1 binding sites are located. There are two low affinity binding sites located at positions +10 and +34 downstream of Qp promoter. (200). Qp is an alternative TATA-less promoter located between Cp and the EBNA1-ORF. (figure 1). In transient transfection assays, Qp driven reporter expression inversely correlated with the amount of EBNA1 expressing plasmid included in the transfection experiment. (200). Evidence of this type supports a model in which Qp operates constitutively in response to ubiquitous transcription factors to ensure EBNA1 levels but is subject to feedback regulation by excess EBNA1. (153). A predication of this hypothesis is that the amount of EBNA1 made should directly correlate with the affinity of the binding sites in the Qp promoter region. Recombinant viruses with altered Qp binding affinity for EBNA1 would be one way to test the predicted dynamics of EBNA1 repression as a function of pool size. Such experiments are needed to lend support for this model since other evidence (the lack of correlation between the number of EBNA1 molecules and the number of viral genomes in cell lines carrying different numbers of genomes). does not suggest EBNA1 concentration is involved in feedback control. (211).

3.1.2. EBNA2

The observation that the P3-HR1 subclone of the Jijoye Burkitt's Lymphoma cell line produced a virus which was incapable of immortalizing B lymphocytes was the first clue that EBNA2 had an essential role in immortalization. (137). P3-HR1 virus had a 6.8 Kb deletion that had, in addition to removing the COOH end of EBNA-LP, excised the entire EBNA2 ORF from the viral genome. (25, 170). An immortalizing phenotype was restored to the virus when the missing DNA segment was transfected into P3HR1 cells and recombined into the viral genome. (72). The EBNA2 protein was first detected with human antisera, and analyses of numerous virus isolates with different human sera provided the first indication that there were two distinct serological types of Epstein Barr virus. (80, 195, 196). Genetic analyses have since confirmed this and provided evidence that the biological differences in efficiency of immortalization (type 1 is better than type 2). are attributable to differences in the EBNA2 gene. (38, 180). The ORF for EBNA2 from the prototype type 1 B95-8 virus encodes 484 residues and contains two sequence reiterations, a 40 residue polyproline stretch (aa 58-98). and an 18 residue arg-gly repeat (aa 341-359). (10). The protein is acidic overall and has a highly acidic domain at the C-terminus. A homology plot between the B95-8 (type 1). and the Jijoye (type 2). EBNA2 sequences highlights the repeating units (bubbles on the diagonal). and the central area of maximal divergence (disappearance of a diagonal track). (figure 3). Interestingly, neither the serological epitopes (located near the COOH end). nor the regions essential to biological function (see below). are encompassed by this region. (196).

EBNA2 is a transcriptional activator that upregulates expression of viral and cellular genes. Extensive *in vitro* molecular analyses (footprinting and gel shift assays). along with transient reporter gene transactivation studies have produced a comprehensive picture of how EBNA2 functions. EBNA2-responsive elements in the promoters of the cellular CD23 gene, the viral EBNA Cp promoter and the viral promoter for the LMP1 gene have been mapped. (71, 76, 89, 129, 241, 260). EBNA2 does not, however, directly interact with DNA. In the promoters and enhancers that EBNA2 has been shown to transactivate is a conserved sequence motif for a ubiquitous cellular Cp binding factor (CBF1). originally misidentified as a recombination mediating protein RBP-Jk (Recombination site Binding Protein-Jk). (71, 76). CBF1 binds EBNA2. (114, 115, 247). CBF1 bound to the CBF1 binding site at position -375 in the Cp promoter has been used in gel supershift assays to map the EBNA2 sequences responsible for the CBF1-EBNA2 protein-protein interaction. A 28 amino acid core domain bounded by residues 310-337 is sufficient to mediate the interaction. (244). (figure 2). By luring EBNA2 to a promoter, CBF1 brings the EBNA2 transactivating domain into play. Sequence 425 to 470 near the COOH end of EBNA2 contains 15 acidic residues that constitute an acidic binding domain similar to that of other transcriptional activators. (36, 115). In *in vitro* studies GST fusion proteins containing the EBNA2 acidic domain depleted nuclear extracts of basal transcription activity and were also shown to directly interact with TFIIB, TFIIE, TFIIH, TAF40 and RPA70 basal transcription factors. (233, 234, 235). By molecular genetic analysis, the CBF1 binding domain and the acidic domain of EBNA2 were both required for rescue

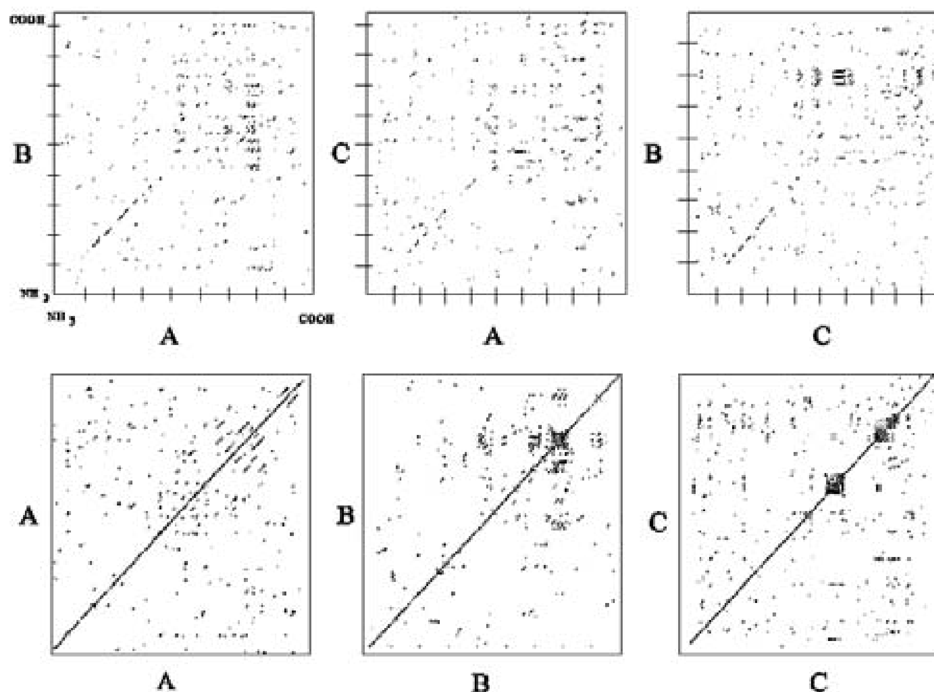

Figure 4. Homology plot comparisons of the 3 EBNA3 proteins. Upper panels are comparisons of each protein with another member of the EBNA3 family highlighting divergence. Lower panels show each protein compared with itself., highlighting the unique repetitive units within each molecule.

of the immortalizing phenotype back into P3HR1 virus. (35, 247). It is therefore the transactivating activity of EBNA2 that is responsible for its immortalizing phenotype.

Many issues remain to be resolved before the transactivating function is completely understood. For instance, a third region of EBNA2 (aa 95-110). is required for immortalization but has an undefined role in transactivation. (figure 2). Also, some deletion mutations in EBNA2 (eg 463-483). increase transactivation activity as well as immortalization efficiency while other deletions (eg 337-357)., that also increase transactivation activity, have an opposite effect and reduce immortalization efficiency. (247). Most of the promoters upon which EBNA2 acts are neither completely inactivated nor totally unresponsive when the CBF1 binding site is eliminated. (47, 62, 97, 106, 129, 166, 167, 220, 256). Both the LMP1 promoter and the Cp promoter remain on and responsive to EBNA2 transactivation but at a reduced efficiency. Indeed analyses of mRNA production by RNAase protection assays in cell lines immortalized by viruses with Cp deletions affecting the CBF1 binding site showed the Cp promoter usage was essentially unaffected. (47). It is, therefore, likely that the transcriptional effects of EBNA2 are not solely mediated by an interaction with CBF1. There is already evidence *in vitro* that a second binding activity (CBF2). within the Cp promoter increases the ability of low levels of EBNA2 to transactivation Cp. (62). Other factors are likely to play similar roles in other EBNA2 responsive promoters.

3.1.3. EBNA3A, EBNA3B, EBNA3C

EBNA3A, 3B and 3C are terms used to describe 3 proteins migrating in the region of 110 to 130kd on denaturing polyacrylamide gels that were initially detected with human antisera on Western immunoblots. (100, 197). The genes for the three proteins lie in a tandem arrangement within a 10Kb region between map coordinates 92,000 and 102,000. (10). The open reading frame for each protein is created by removal of a short intron that separates a short N terminal sequence from a long C terminal ORF. Actual ORF lengths for EBNA3A, 3B and 3C are 944, 937 and 992 residues respectively. Each protein contains a different and complex reiteration of sequences near the COOH end. (21, 22, 23, 24, 81, 90, 100, 159, 160, 181, 206). Homology plots reveal three related proteins whose relationship has become almost totally obscured. (figure 4). Conserved sequences are confined to the N terminal third of the molecules while overall homology is less than 28%. Divergence can also be seen to be operating when each gene of EBV type1 is compared to its counterpart in EBV type 2. Primary sequences are only 84% 80% and 72% homologous. (94). It has been suggested that the pressure that drives the divergence in this region may be due to the necessity to vary the immunodominant epitopes being recognized by cytotoxic T cells. (179). For reasons that are not understood, the EBNA3 proteins are the primary source of peptides for recognition of virus immortalized B cells by effector T cells. For at least the EBNA3B protein, immune response pressure for epitope variation based upon an HLA A11 epitope and the frequency of the HLA A11 allele in geographically isolated populations seems to have occurred. (39,40). However, detailed molecular evolutionary analyses of virus isolates from different

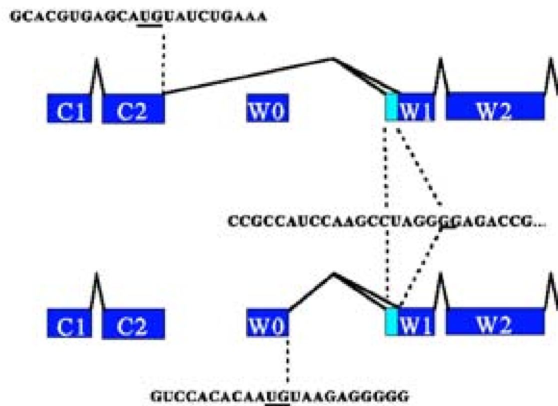

Figure 5. The alternative splicing mechanism that generates the AUG initiator codon for the EBNA-LP ORF. The first two bases are encoded by either the C2 exon for Cp promoter initiated messages or the W0 exon for Wp promoter initiated messages. The ratio of EBNA-LP coding to non-coding messages varies in lymphoblastoid cell lines but usually favors the non-coding splice by a factor of approximately 5:1.

regions of the globe does not support an immune evasion model for sequence divergence.(98). Indeed, an inverse correlation between epitope variation and HLA frequency distribution has been observed.. Not only does such a result point to an immune-pressure-independent mechanism driving sequence divergence within EBNA3 proteins, it implies that evolutionary pressure is being exerted to conserve CTL epitopes rather than eliminate them. This is a striking observation. A model of the virus-host relationship would need to incorporate a mechanism whereby some advantage is gained from the deliberate exposure of the immortalized cells to elimination by immune surveillance (179).

The genetic analyses have demonstrated that both EBNA3A and EBNA3C are essential to immortalization which implies that their roles are different (i.e., they cannot substitute for each other). (230, 231). Since EBNA3B is not essential to the immortalization process, the role of this protein in the virus life cycle is unknown. The conserved regions in the amino terminal domains of the EBNA3 proteins correspond to functionally significant domains. (183). Transient transactivation studies demonstrate that all 3 gene products can interfere with EBNA2 activation of the LMP2 promoter. (108, 126, 183, 238). All three EBNA3s physically interact with the cellular tranactivator CBF1 via their amino terminal conserved domains. CBF1 lies at the nuclear end of the signal transduction pathway that emanates from the notch receptor and overexpression of the notch protein has been observed in human T cell malignancies. This connection has been used to infer that CBF1 responsive genes play important roles in cell proliferation control. (83, 183, 184, 258). Consistent with this notion is the recent demonstration of the immortalization potential of EBNA3C in a ras cooperation assay. (156). How the effects of each of the EBNA3 proteins differ with respect to regulation of CBF1 mediated gene expression is not yet clear. The EBNA3C interaction with CBF1 inhibits CBF1 binding to DNA *in vitro* and *in vivo*, and repression of the Cp promoter depends on the presence of the CBF1 binding site in the promoter. (171, 184, 238). Repression may not simply be titration of limiting CBF1 since it also requires the basic DNA binding/leucine zipper-like motif (aa 207-368). within EBNA3C and another region (aa 346-543). downstream. Also, other promoters with CBF1 binding sites that are EBNA2 responsive (eg. the cellular CD21 gene). are not repressed by EBNA3C. (171). These data imply that repression may involve DNA binding by EBNA3C within the repressed promoter and that repression is context specific, requiring the participation of other regulatory molecules. Adding to the complexity of the function of the EBNA3 proteins are the reports that transcriptional activating functions within EBNA3A and EBNA3C can be unmasked by mutagenesis. (126, 171). This may mean that the wild type proteins under some circumstances may act as gene activators, not repressors, and that an experimental system defining these circumstances could reveal a great deal more about how these proteins actually function. Recently, EBNA-LP (which cooperates with EBNA2 in transactivation). has been shown to cause a redistribution of EBNA3A within the nucleus. (171). This would indicate that the effects of the EBNA3 proteins on EBNA2 transactivation can be modulated by EBNA-LP and seems to be leading towards a model that would have EBNA3 proteins participating in a regulatory network consisting of all the EBNA transcription factors, each affecting the others' behavior.

3.1.4. EBNA-LP

As early as 1984 cDNA sequencing had revealed that there was an open reading frame for a repetitive protein buried within the short W1W2 leader exons of the EBNA messages. (21). Around the same time, improved Western blotting techniques were showing that some human sera detected a ladder of proteins made in immortalized cells. The ladders had steps of around 6Kd in a range between 30 to 60Kd which correlated well with the cDNA sequence prediction of a repeat unit of 7Kd. (197). Proof of the correlation came in the form of a monoclonal antibody (JF186). to a peptide sequence within the W1 exon of the repeating unit that detected the same protein ladder. EBNA-LP (EBNA4 or EBNA5 in some early publications). was thus confirmed as the fifth nuclear antigen to be detected in EBV immortalized cells. (44).

cDNA sequences have revealed several interesting aspects of EBNA-LP expression. (20, 21, 22, 23, 24, 210). The AUG initiator codon for the ORF is created by an alternative splice between a promoter proximal exon and the first exon of the repeat. (figure 5). Both Cp and Wp, by choosing between two 3' splice sites 5 bp apart, can produce bicistronic, monocistronic, or non-coding mRNAs. It is important to realize that the EBNA transcription unit can be completely shut down, from the perspective of protein production, by RNA processing alone. This level of control over gene expression is seldom appreciated and poorly understood yet may be crucial to understanding the patterns of viral gene expression detected in lymphoproliferative diseases and tumors. The EBNA-LP

ORF is derived from repeating W1 and W2 exons of the major internal repeat unit (IR1). and the unique Y1 and Y2 exons just downstream of IR1. (figure 2). Variation in the size of EBNA-LP is due mainly to the variation in the size of IR1 in different viruses (5). and the ladder is attributed to exon-skipping during assembly of leader exons from primary transcripts. The only apparent rule for exon-skipping is that W1 and W2 always seem to be skipped in pairs. At the 3' end of the reiteration virtually any exon may be spliced on, creating a huge variety of potential COOH ends for EBNA-LP. Only the proteins with Y1 and Y2 as a COOH end have been investigated in any depth, and these are considered to be the major repeat containing protein products.

EBNA-LP mutant viruses with deletions or point mutations preventing expression of Y1 and Y2 have a defective immortalization phenotype. (72, 124). Efficiency of immortalization is reduced at least 10 fold and requires the presence of a fibroblast feeder layer. Immortalized cells grow slowly with an atypical small rounded appearance. With passage in culture cells eventually achieve lymphoblastoid appearance but most of the initial colonies do not give rise to long-term cell lines. Exactly how EBNA-LP functions during the immortalization process is unclear. The protein is expressed at very high levels shortly after infection, but few established lymphoblastoid cell lines (LCLs). make such large amounts. EBNA-LP is a nuclear phosphoprotein whose phosphorylation on serine residues is cell cycle stage dependent. (103, 158). Biochemical fractionation experiments have shown an association between EBNA-LP and HSP70 family heat shock proteins. (104, 125). An earlier report based on *in vitro* protein-protein interactions suggested an association with the retinoblastoma protein (pRB). could occur but this report remains unconfirmed. (222). By immunofluoresence, EBNA-LP is located at discrete sites within the nucleus co-localizing with the promyelocytic leukemia (PML). protein in nuclear bodies (NB). (221). The PML protein has growth suppressive properties and was originally identified in the context of the PML-retinoic acid receptor alpha fusion protein (PML-RARα).of acute promyelocytic leukemia. PML co-localizes with the non-phosphorylated form of the retinoblastoma protein (pRB). within nuclear bodies (NB) and can abolish pRB mediated transcriptional activation. (2). PML therefore is considered to be part of a transcriptional regulatory complex sequestering active transcription factors to NB sites. Disrupting its regulatory function as occurs in the PML-RAR alpha fusions of promyelocytic leukemia exposes its oncogenic potential.

EBNA-LP localization to NBs might therefore be expected to have an impact on transcriptional regulation. Two recent reports suggest that this is in fact the case. (73, 152). In transient transfection assays, EBNA2 transactivation of viral promoters was enhanced 10- to 100-fold by co-transfection with EBNA-LP. The effect was mediated by the W1W2 repeating domain and at least two repeating units were required to mediate the enhancing effect. Enhancement also works on cellular promoters responsive to EBNA2 stimulation and this may be the mechanism underlying the ability of these two proteins to co-operate in causing a G_0-G_1 transition. (207). Since the COOH terminus is not required, the role of the 45 unique residues encoded by Y1 and Y2 (which were implicated by genetic analyses of immortalization functions). needs to be determined. Deletion analysis of EBNA-LP in the transcription assay suggests that progressive removal of C-terminal sequences unmasks negative regulatory effects encoded in the Y1 exon within the unique C terminus. (73). Within the unique COOH end, and partially encoded by Y1, is the sequence motif PARSLRE. This is a highly conserved motif present in cyclin-dependent kinases and used by these enzymes to interact with their regulatory cyclins. It may be that the regulatory role for the COOH end of EBNA-LP in modulating the transcriptional enhancement is mediated through interactions with cyclins. This would fit nicely with the cell cycle related effects that have been described for EBNA-LP. (4). So while it remains to be determined exactly how EBNA-LP co-ordinates its activity with that of EBNA2 (or EBNA3A, 3B, and 3C for that matter)., the key would seem to be understanding the regulatory effects of the COOH terminus. EBNA-LP is apparently one more contributing factor involved in the regulation of viral and cellular genes by the EBNA transactivators. Together, the temporal sequence of EBNA gene expression, the regulation of product pools from the EBNA transcription unit, and the overlapping positive and negative regulatory effects of the different EBNA proteins suggest that activation of the B lymphocyte's proliferation machinery by the EBNAs is much better orchestrated than one would have guessed only a few years ago.

3.1.5. LMP1

The most abundant mRNA in immortalized B cells is encoded at the right end of the linear genome by the LMP1 gene. (50, 202). The initial transcript is made in the direction opposite from the EBNA transcription unit. At the time that the cDNA sequence of LMP1 RNA was determined, a concerted effort was being made to understand the nature of the lymphocyte-detected membrane antigen (LYDMA)., a structure on the surface of immortalized B cells recognized by cytotoxic T cells. (102, 145, 226). The structure of LMP1 predicted from the ORF in the cDNA was a 386 residue integral membrane protein with six transmembrane segments. A short 20 residue amino terminus and a long 200 residue carboxyl terminus were both predicted to be located on the cytoplasmic side of the membrane. (figure 2 and figure 6). For a while LYDMA and LMP1 became synonymous. It is now clear that although LMP1 can serve as a source of peptides for T cell recognition, it is not LYDMA and that LYDMA as a unique viral gene product does not exist. (179).

LMP1 is a transforming protein that seems to exert most, if not all, of its transforming activities by acting as a constitutively-active receptor. (65, 143). Localization studies place approximately half of the LMP1 molecules in an immortalized B cell in the plasma membrane. (123). Within the plasma membrane LMP1 is aggregated in patches reminiscent of patches formed by ligand engaged growth factor receptors. (34). LMP1 is concentrated in glycosphringolipid-rich membrane regions, which also

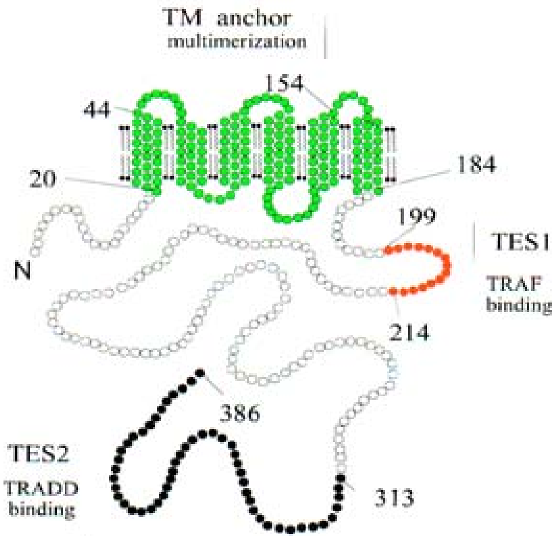

Figure 6. Diagrammatic representation of the major features and orientation of the LMP1 molecule in the plasma membrane. The three short loops connecting the six membrane-spanning segments on one side of the membrane are located outside the cells. The sequences bounding the transformation effector sequences TES1(red). and TES (black). are indicated.

mark the clustering sites for G protein-associated receptors. Proteolytic cleavage studies performed on live cells have confirmed the proposed model orienting both the N and C-terminus to the cytoplasmic side of the plasma membrane. (113). LMP1 clustering is not ligand dependent and no external ligand has been identified that interacts with the three short loops exposed on the surface of immortalized cells. Mutational analyses demonstrate that the amino terminus and the transmembrane segments of LMP1 mediate the aggregation of LMP1 into patches and that clustering of LMP1 in the membrane of infected cells is an essential feature of the immortalizing phenotype of EBV.

How LMP1 clustering mediates signal transduction within the cell was revealed by a yeast two-hybrid screen for proteins that interacted with the large C terminal cytoplasmic domain. (143). This and subsequent studies have revealed that the C terminal domain interacts with Tumor Necrosis Factor Receptor-associated Factors (TRAFs)., in particular TRAF1 and TRAF3 (formerly LAP1, CD4Gp, CRAF1, or CAP1). and with the Tumor Necrosis Factor Receptor-associated death domain protein (TRADD). (42, 59, 88, 138, 143). The interactions of TRAFs and TRADD with LMP1 are mediated by separate regions within the C terminal domain termed Transformation Effector Sites (TES). or C Terminal Activation Regions (CTAR). (59, 88). TES1 (LMP1 residues 199-214). binds TRAFs and TES2 (LMP1 residues 333-386). binds TRADD. (figure 5). As suggested by the names, these protein-protein interaction regions are required for both immortalizing activities and signal transduction of LMP1. One of the principal effects of LMP1 clustering with TRAFs and TRADD is the activation of NFκB and the expression of NFκB regulated genes. (88).

The consequences of stimulating the TNFR signaling pathway by LMP1 have been most dramatically demonstrated by transfection of the LMP1 gene into EBV negative lymphoblasts. Morphological changes include clumping, appearance of villipodia, enhanced expression of B cell activation markers (CD23, CD39, CD40, CD44, and HLA Class II)., and expression of adhesion molecules (LFA1, CD54, CD58). (111, 112, 128, 157, 200, 239, 240, 257). These effects are similar to what occurs when virus infects resting primary B lymphocytes and are also similar to the effects of stimulation through CD40, a B cell receptor that also signals through association with the same TRAFs as LMP1. (46, 259). These changes in infected B cells are, however, not sufficient to sustain proliferation. B cells immortalized by viruses which carried a conditional EBNA2 allele but which had constitutive LMP1 expression (ie: on and not regulated by EBNA2). ceased proliferating when EBNA2 expression was shut off. (259). Further, when LMP1 was conditionally repressed in B cell lines (DG75, BJAB, AKATA). by controlling expression from a tetracycline responsive promoter, withdrawal of tetracycline and upregulation of LMP1 expression led not to enhanced proliferation but to growth arrest and accumulation of cells in the G2 phase of the cell cycle. (59). Nevertheless, it is clear that LMP1 functions are essential to immortalization as LMP1 mutant viruses with deletions affecting TES1 and/or TES2 are defective. Cells infected by viruses with TES2 mutations will proliferate temporarily, but functional TES2 appears to be required to achieve sustained *in vitro* outgrowth of virus-infected B cell lines. (88). It appears that the LMP1 activated signaling pathways have to be integrated with other events to cause proliferation.

One interesting feature of LMP-1 expression is that while ectopic expression may be cytostatic, the LMP1 expressing cells are protected from apoptosis. (60, 95, 154, 242). Several studies have shown that in T cells, B cells, and epithelial cells, LMP1 expression protects cells from p53 mediated apoptosis. Upregulation of Bcl-2 and A20 anti-apoptotic genes appears to be the principle underlying mechanism. (60, 242). It has been suggested that these anti-apoptotic effects may have profound consequences in the pathogenesis of primary EBV infection and in the course of EBV associated diseases where LMP1 expression has been detected.

3.1.6. EBERS

Two small EBV encoded ribonucleic acids (RNAs)., EBER 1 and EBER 2, are both transcribed by the RNA pol III from the most active region of the EBV genome. (45, 48, 144, 192, 229). Accumulation of these RNAs has been estimated to exceed 10^7 copies per cell. The EBERs coding region is within the EcoRI J restriction fragment near the left end of the EBV genome. EBER 1 and EBER 2 are 166 nucleotides and 172 nucleotides long, respectively. They are separated by 161 bp and both contain two 9bp sequences preceding the 5' termini. Both EBERs contain RNA pol III intragenic transcription control

regions and can be transcribed by RNA pol III *in vitro*. The EBERs are neither capped nor polyadentylated but are protected from rapid degradation by adopting self-complementary secondary structure and forming higher order complexes with cellular proteins. The latter has been demonstrated in immunoprecipitation experiments with systemic lupus erythematosus antisera that recognize La antigen and by immunofluorescence with anti- L22. (192). EBER 1 has been shown to form complexes with the cellular ribosomal protein L22, a ligand for human 28S ribosomal RNA. Complex formation results in a relocalization of L22 from the nucleolus to the nucleoplasm. (45, 232). Recombinant virus analyses have shown that viruses without EBER genes are fully competent to produce immortalized B cells that are not in any detectable way different from wt immortalized cells. EBERS have been suggested to have roles in RNA processing and blocking interferon mediated inhibition of early steps in the immortalization process but no convincing evidence for this or any other functional role in EBV infection has been forthcoming. (66, 68).

Because of the huge numbers of copies of EBERs made in infected cells *in situ* hybridization (ISH). with probes for EBERs has been successfully used for detection of EBV. (10, 84, 99). This method has been relied upon for many years, but more recently polymerase chain reaction (PCR). for EBV DNA has provided another means of reliable detection. (48, 147). The great advantage of ISH for EBERs is that it provides localization of EBV within tissue sections and can be performed in less than two hours. On the other hand, PCR analysis can provide a more sensitive test for detecting a low copy number of EBV-DNA and can be used for distinguishing virus types and strains in various isolates using restriction fragment length polymorphism.

3.2. Perspective on immortalization

What is the advantage to having a battery of genes that usurps control of a B lymphocyte's proliferation and differentiation machinery? Most likely and primarily, access to more B cells. The innate proliferative potential of B cells is probably being used to inflate the numbers of infected cells prior to virus production and sacrifice of the infected cells. If a re-evaluation of the virus-host relationship does in fact find that there is a strict lymphotropism to primary infection and no replicative epithelial cell intermediate, then immortalization of B cells almost becomes a necessity. The chance encounter between a virus particle and a B cell becomes less likely and, when it occurs, a strategy that delays virus production until the infected cell has circulated within the lymphoid compartment makes even more sense. Regional lymph nodes not only provide the right environment for infected cell proliferation, but also contain a rich source of susceptible cells to continue and expand the infection process. This notion is not new. It needs to be re-iterated here to emphasize that immortalization comes under the overall productive strategy. Lymphoproliferation is a tool used to make more virus infected cells and more virus. A prediction of this model is that viruses incapable of immortalization would be less virulent and perhaps even avirulent and incapable of establishing an infection in a host. The recently described rhesus monkey model for EBV infection will undoubtedly be used to test this hypothesis. (141). The classical view of this process maintains that exposure to the immune system is the penalty for causing lymphoproliferation. Recent studies, suggesting evolutionary pressure acts to conserve CTL epitopes within EBNA3 proteins, imply that the immortalized cells seem to deliberately betray themselves by processing the virus proteins responsible for immortalization and presenting them to T cells on HLA class I molecules.(98).

Once immunosurveillance takes hold it should eliminate all the immortalized cells, leaving a nest of truly latently infected B cells. The consequence is the paradigm for all herpesviruses. Hosts are rarely lost and every host becomes a carrier. The advantage derived from cooperating with the host in the elimination of immortalized cells is establishment of the latent phase. The virus survives, along with the host, to be reactivated and transmitted later. As discussed below the latently infected cells are memory B cells. The study of these cells is beginning to change our understanding of the virus-host interaction and may require a change in EBV nomenclature. Most of the proteins involved in immortalization do not appear to contribute to latency and therefore labeling them as 'latent' proteins and immortalized cells as 'latently infected' may be misleading. Immortalization and latency are two very different virus-host cell interactions, and as discussed below could even be mutually exclusive. A change of definition now will also serve to clarify the discussion on the meaning of the various patterns of viral gene expression that are observed in lymphoproliferative diseases, lymphomas and other tumors.

4. LATENCY

Most infections with Epstein-Barr virus occur in childhood and are clinically unapparent. (135, 178). Symptomatic infection in adolescents and adults is infrequent but can cause a recognizable clinical entity (infectious mononucleosis). Whether infection is apparent or not, the consequence is a life-long carrier state characterized by episodic shedding of virus into saliva, persistent low antibody titers to EBNA1, a high frequency of circulating CTL precursors to EBV infected B cells and a corps of latently infected B cells that escape immunosurveillence. (135, 178).

The existence of latently infected cells in the blood and lymphoid tissues has been known for decades principally because explanted tissues rich in lymphocytes spontaneously produced lymphoblastoid cell lines (LCLs). with the features of EBV immortalized B cells. (110, 150, 163, 164, 188, 249). That latent infection occurs in the lymphoid compartment was originally demonstrated by two key pieces of evidence: (i). During prolonged acyclovir treatment, virus shedding in the oropharynx ceases; however the frequency of establishment of spontaneous immortalized B cell lines from the peripheral blood remains unchanged. (ii). Analyses of EBV strains recovered from

bone marrow transplant recipients indicates that existing latent infection can be eradicated from seropositive recipients during replacement of their heamopoetic cell lineages with bone marrow from seronegative donors. (69, 250).

Early studies on the nature of EBV latency relied on the spontaneous outgrowth phenomenon to characterize the peripheral blood lymphocyte compartment from which cell lines would emerge. The findings have suggested that (i). virus emerged only from fractions containing B cells, (ii). that cells of both high and low buoyant density contained LCL precursors and (iii). that LCLs were being generated predominantly by a 2-step mechanism. (110, 248, 249, 251). This last finding implies that after explantation latently infected cells do not directly convert into proliferating lymphoblastoid cells but instead produce virus which subsequently infects bystander B cells that are susceptible to the immortalization program. Spontaneous outgrowth of LCLs can be inhibited by acyclovir which blocks virus production. (250). This finding defines an important property of latently infected cells that distinguishes latency from other virus-cell interactions which resemble latency and are encountered in lymphoproliferative diseases and lymphomas (including Burkitt's lymphoma).

Measuring frequency of spontaneous outgrowth is a rather cumbersome and indirect method for examining latency and does not allow for much insight into the state of the latently infected cells. More direct observations have recently been made with *in situ* hybridization and polymerase chain reaction techniques. The presence and distribution of EBV positive cells in lymphoid tissues has been examined by *in situ* hybridization with EBER probes. (8, 82, 94, 148, 149, 177, 213, 243). The implicit assumption is that latently infected cells make EBERs. The most extensive study showed that 4 of 12 normal lymph nodes contained small EBER positive cells localized to extrafollicular areas at a frequency of approximately 1-10/0.5 cm^2. (148). This was contrasted with acute stage mononucleosis lymph nodes where the distribution was similar but the frequency was >1000/0.5 cm^2 and large positive cells were mixed in with the small positive cells. Lymphadenopathies were often associated with increased frequency of EBV positive cells (38/60 cases). and one remarkable case showed a diffuse expansion of EBER positive cells with centroblast-like or centrocyte-like morphology in a germinal center. The implication of this observation is that these EBV positive cells, which accounted for approximately 40% of the germinal center cells, were an integral part of the germinal center process and behaving essentially like normal B cells.

EBER staining and PCR have also been used in recent investigations of the replicative processes occurring in acute phase infectious mononucleosis. (93). Analyses of histological sections, throat washings, exfoliated oropharyngeal cells and peripheral blood mononuclear cells revealed that only lymphocytes, and not epithelial cells, became infected. This is in stark contrast to the prevailing view based largely on earlier work with less sensitive techniques that posited oropharyngeal epithelial cells as the initial targets of EBV infection and replication and as the source of the virus that infected B cells and was shed in the saliva. (178). A reappraisal of the role of epithelial cells in the pathogenesis of EBV infection in immunocompetent individuals is needed.

The development of the polymerase chain reaction technique has allowed the detection of viral DNA from latently infected cells directly and sensitively. Pioneering work in this area used PCR to detect viral DNA in tissue specimens obtained at autopsy from patients with no indication of EBV related disease. (31). Viral genomes were readily detectable in the parotid gland (7/15)., submandibular gland (8/10). nasopharynx (8/10)., tonsil (8/10). larynx (5/6)., lung (5/9). cervical lymph node (7/10)., mediastinal lymph node (7/10)., abdominal lymph node (4/10)., spleen (6/10)., and kidney (4/10). Less frequent detection was reported for the liver (1/10)., pancreas (1/4)., ovary (1/5)., uterine cervix (1/4). and testis (1/3). The frequency of positive cells in these tissues was not investigated. Detection and quantitation of latently infected B cells in the peripheral blood of healthy carriers has been reported. (139, 140, 226, 236). The more recent and comprehensive study of Miyashita *et al*. showed that the frequency of EBV positive cells in nine carriers ranged from 23 to 625/10^7 B cells with a mean of 125. (139). For a given individual the viral load was relatively stable over a period of at least 2 years. The number of genomes per cell was estimated to be less than 5. By pre-sorting peripheral blood lymphocytes based upon cell surface markers, infected cells were detected amongst the $CD19^+$, IgD^-, $CD23^-$, $CD80^-$, and $Ki67^-$ population, consistent with the view that latency was in resting non-activated memory B cells. (9,139). The initial estimates of the numbers of latent B cells detected by PCR agreed with the previous estimates made on the basis of spontaneous immortalization frequency. This could mean that the majority of the population of B cells harboring viral genomic DNA are competent to reactivate and produce virus, at least after explantation and culture *in vitro*. This conclusion favors the idea that the circulating latently infected cells are the product of a latency-producing program as opposed to just one of many imaginable consequences of a dead-end infection. However, recent estimates comparing PCR measured frequency of latently infected cells directly to the spontaneous outgrowth frequency revealed that spontaneous reactivation was 5 fold less than expected. (9). This suggests that *in vitro* reactivation from latency may not be such an efficient process and/or that the latently infected population may be heterogeneous containing a large fraction of cells incapable of producing virus.

Another application of the PCR techniques is for the detection of RNA expression (RT-PCR). Several groups have reported results of RT-PCR assays on RNA taken directly from peripheral blood lymphocytes of healthy carriers. (30, 139, 168, 228). These studies have all depended upon the assumption that the products of mRNA splicing in latently infected cells will resemble the mRNA products of the immortalization program. Neither

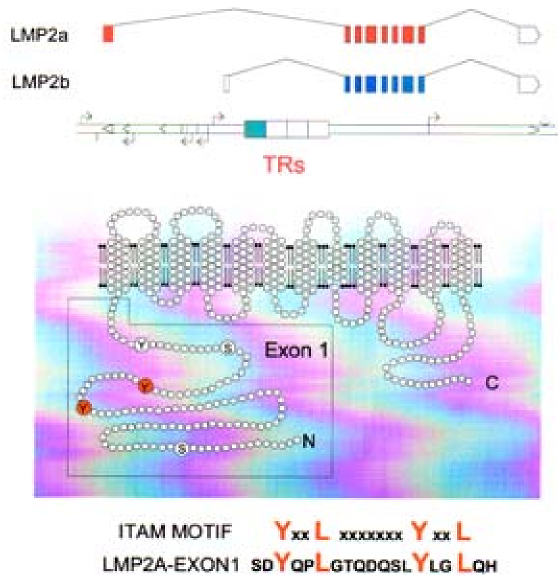

Figure 7. Structure of the LMP2 gene. A region of the genome spanning approximately 13 kb is shown, including the terminal repeats (TR)., promoters (bent arrows). and poly A sites (arrows). The structure of the LMP2 mRNAs is shown above with coding exons colored in red and blue A diagrammatic depiction of the protein structure is presented below. Both LMP2 and LMP2b have 12 membrane spanning segments and a short COOH tail. LMP2a exon1 has several tyrosine and serine residues (highlighted as enlarged balls). that are important phosphorylation sites. The two tyrosines of the ITAM motif are in red and the actual sequence of the motif is shown below.

alternative RNA structures for expression of these genes nor the RNA products of genes not expressed in immortalized cells have been investigated. The first report of mRNA in latently infected cells detected Cp promoter activity (3/4). and LMP2a expression (4/4). (168). A second study also detected LMP2a (4/6). but in addition showed that by using nested sets of primers EBNA1 messages initiated by the Qp promoter were also present (3/7). (228). In this study Cp activity was not detected. A third study also detected LMP2a mRNA 6/7 and EBNA transcripts (7/7). (30). This report presented evidence for Cp activity 6/6 and not Qp activity. The most recent study using sorted resting CD19+ CD80$^-$ CD23$^-$ B cells detected LMP2a RNA and 'sporadic' EBNA1 RNAs derived from Qp promoter transcripts. (139). The different studies agree on some important points. First, the messages for key proteins involved in immortalization are not detected (eg. LMP1, EBNA2). Second, all of the studies detect LMP2a expression (16/18). and third, all of the studies detected EBNA transcription unit promoter activity, although there is no consensus on which promoter is active or whether any EBNA genes are expressed. In the most recent reports LMP2a RNA was readily detectable, while EBNA1 messages were only occasionally detected. (9, 139).

Latency, therefore, most likely occurs in resting B cells, where very few copies (<5). of the viral episome express the LMP2a message. (226). The actual number of genomes present per cell is not known with certainty. It is an important point because genome amplification after infection occurs within the immortalization program. If latently infected cells are found to contain 1 copy per cell or dozens of copies per cell it will indirectly infer whether latency was established before or after initiation of the immortalization program. It is important to understanding EBV pathogenesis to know whether immortalized cells retain the capacity to become latently infected resting cells.

The answer to this question would also assist the development of a model to explain the regulation of viral gene expression in latently infected cells. The LMP2a promoter which lies directly upstream of exon 1 has been studied but only in the context of immortalization. (107, 260, 261). Activation is considered to occur late in the process and in an EBNA2 responsive manner with kinetics similar to LMP1 expression. It is possible that this promoter is not used in latently infected cells. There is a consensus splice acceptor site between the TBP binding site and the start of the LMP2a ORF that can and does accept splices from leader exons of the EBNA transcription unit. (our unpublished observation). It is therefore conceivable that LMP2a expression (and EBNA1 expression). in latently infected cells is controlled by alternative splicing of long Cp or Qp initiated transcripts. The constitutively active Qp promoter would seem to be the logical choice for this sort of regulation since a similar (though not identical). restriction of viral gene expression occurs in Burkitt's lymphoma cells where the Qp promoter is responsible for maintaining EBNA1 levels in these proliferating cells. If in the future it is determined that infected cells destined to become latent reservoirs routinely participate in normal B cell processes, specifically germinal center expansion, then BL cells might be a model for (or might actually be). EBV infected centroblasts. Repression of viral gene expression in these cells involves methylation of viral DNA sequences in methylation hypersensitive sites upstream of the EBNA transcription unit and the LMP1 promoter. (549, 182, 185, 186, 204). Examining the state of the viral genome and the pattern of viral gene expression in latently infected cells is providing clues to the mechanism by which latency is established. The obvious candidate for a viral arbiter between latency and reactivation is the LMP2a protein, since it is the only protein whose message is consistently detected in latently infected cells.

4.1. Function of the Latency Protein

4.1.1. LMP2

In 1985, two messages 1.7 and 2.0 Kb in length were described as being expressed from rightward transcribed sequences at the left end of the EBV genome in B95-8 cells. (84). The structure of the cDNA clone of the larger message subsequently revealed that the transcript actually originated across the fused terminal repeats in sequences at the other end of the genome. (107). (figure 7). The two mRNAs are produced by alternative promoter usage and differ only in the sequences of the first exon sharing 8 common 3' exons. (106, 1107). Both messages are expressed in most if not all LCLs but the ratio of the two messages varies widely and unpredictably amongst

the cell lines suggesting little co-ordinate regulation of promoter activity or mRNA abundance. (107, 201). The two messages encode proteins of 497 (LMP2a). and 378 (LMP2b). residues that differ only in that LMP2a carries an extra 118 residue N terminal domain encoded in LMP2a exon1. (figure 7). LMP2b exon 1 is noncoding and translation initiation is presumed to occur at the first available in-frame methionine in exon 2. The 378 residues common to both proteins are predicted to form twelve membrane spanning segments linked by short loops on both sides of the membrane and ending with a short 28 residue COOH tail. (201). The N terminal domain of LMP2a is cytoplasmic and contains an ITAM (Immunoreceptor Tyrosine-based Activation Motif). (176). ITAMs are present in the Fc receptor and the T and B cell antigen receptor associated molecules (Cd3 gamma, CD3 delta, CD3 epsilon, zeta, Ig alpha, Ig beta). and are used to dock the receptors with molecules that contain SH2 domains, principally cytoplasmic tyrosine kinases. (16, 176, 208, 209).

Genetic analyses of the LMP2 gene have shown that the protein is dispensable for B cell immortalization by virus. Three different virus recombinants [(i). a stop codon is place of residue 19 in the LMP2a a sequence allowing LMP2b expression only, (ii). a stop codon at residue 260 truncating both LMP2 proteins after the fifth membrane spanning segment, and (iii). a deletion between residues 120 and 260 that allows only LMP2a cytoplasmic domain expression] all gave rise to essentially normal LCLs. (119, 120, 121). While these and other studies using production of immortalizing recombinant viruses as a phenotypic readout have agreed on the dispensability of LMP2 to immortalization, a recent report reached a different conclusion. (26, 101). This study used packagable mini-episomes and an insertion mutant that replaced the second, third and part of the fourth exon of LMP2 with the hyg gene. It was found that the mutant mini-episomes showed a reduced clonogenic efficiency and a bias towards the presence of *wt* complementary helper virus episomes in the cell lines that were produced. It was suggested that although LMP2 expression is not required it may provide an advantage for initiation and/or maintenance of B cell immortalization.(26).

Because of their hydrophobic character the LMP2 proteins have been difficult to study in lymphoblastoid cells. Several rabbit polyclonal sera, three rat monoclonals and recently two murine monoclonals have been produced with specificities against the LMP2a N terminal cytoplasmic domain. (61, 118, 148, 194). LMP2a migrates as a 55Kd phosphoprotein on denaturing polyacrylamide gels, is associated with membrane fractions and has a high turnover rate in LCLs. (118, 194). In transiently transfected BJAB cells and in LCLs, $^{32}PO_4$ metabolic phosphorylation occurred on tyrosines and serines. (117). By immunofluorescence on LCLs the protein displayed a patchy plasma membrane fluorescence, that at least partially overlapped the localization of LMP1. (118). By *in situ* immunofluorescence of lymphoid tissues from patients with acute mononucleosis LMP2a expression was detected in diffusely distributed interfollicular immunoblasts. (148). This last result is best interpreted as evidence that LCL-like cells *in vivo* express LMP2a rather than as direct confirmation that truly latently infected cells actually make the LMP2a protein.

The function of LMP2 may be similar in many respects to LMP1. Plasma membrane clustering, lack of evidence for an external ligand interaction, and a cytoplasmic domain containing motifs for protein-protein interaction with signaling transducers all point towards a role in constitutive activation of a B cell signaling pathway. The big difference between LMP1 and LMP2, of course, is in the pathways that are affected. By inspection LMP2a contains three tyrosine based SH2 domain interaction sites. Two motifs centered on Y74 and Y85 are spaced exactly 7 residues apart as in receptor associated ITAMs, while a third is centered on Y112 and lies further downstream. In general, docking of a particular SH2 domain containing protein to a tyrosine motif requires phosphorylation of the tyrosine and is governed by the primary sequence around the phosphotyrosine, particularly the +1, +2 and +3 residue. (208). A prediction can thus be made that the LMP2a ITAM motif should direct interaction with a dual SH2 domain containing cytoplasmic signal transducer. If LMP2a is a mimic for the Ig alpha and Ig beta ITAMs, then the tranducer is probably the syk tyrosine kinase. By analogy the Y112 motif is an excellent candidate for an interaction with src family tyrosine kinases. Because of the hydrophobic nature of LMP2a, direct biochemical proof of these interactions has been difficult to obtain. The best available evidence comes from co-immunoprecipitation studies. Detection of interactions was after immunoprecipitation followed by *in vitro* kinase assays followed by re-immunoprecipitation or immunoprecipitations followed by Western blot probing with sera for the specific interacting species. (27, 117). LMP2a immunoprecipitates from transiently transfected BJAB cells contain tyrosine kinase activity and the kinases are most likely src family members since lyn and to a lesser extent fyn tyrosine kinases are present. Syk has been detected in LMP2a immunoprecipitates from mild detergent lysates of LCLs after *in vitro* kinase reactions followed by re-immunoprecipitation with anti-syk antisera and identification of a 70Kd $^{32}PO_4$ labeled band on an autoradiograph. (132). It seems likely that these studies are detecting LMP2a interactions however the methods used are indirect. LMP2a immunoprecipitates from mild detergent lysates of ^{35}S met labeled or $^{32}PO_4$-labelled cells contain dozens of proteins. Amplification of signals by *in vitro* kinase reactions and/or re-immunoprecipitations may lead to detection of signals that are not the result of direct protein-protein interactions.

The effects on the signaling pathways affected by LMP2a expression in lymphoblastoid cell lines have been more readily amendable to investigation. Chimeric receptors replacing the cytoplasmic portion of CD8 alpha with the cytoplasmic domain of LMP2a have been used in signal transduction studies in both B cells and T cells. (1, 14). Crosslinking these receptors with anti-CD8 antibodies triggers calcium release and cytokine production showing that the LMP2a cytoplasmic domain can transduce

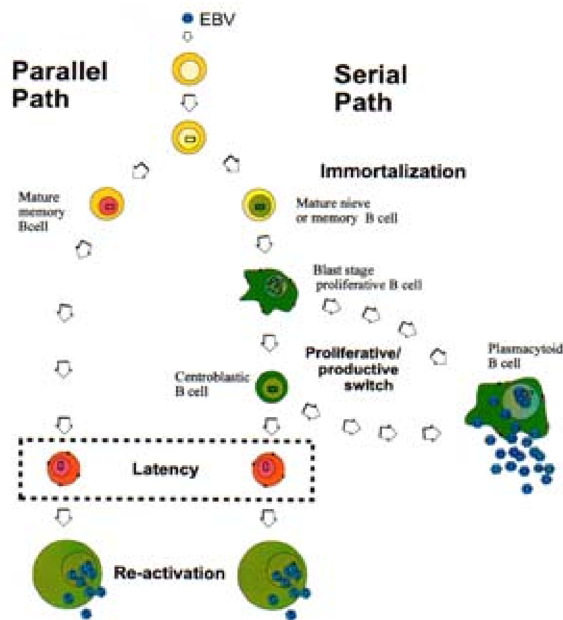

Figure 8. The parallel or serial pathway from infection to latency.

receptor-ligand signaling. Converting either Y74 or Y85 to alanine completely abrogated the response. (1, 14). Transfected BJAB cell lines expressing LMP2a displayed reduced calcium release in response to crosslinking class II or CD19 molecules. (134). LMP2a expression caused IgM receptor expression on the cell surface to be reduced by 4 fold on average and also caused calcium mobilization to be reduced to about the same degree. The (reduced signaling). effect of LMP2a expression on the IgM receptor was not as great as on the other receptors. This does not mean that the impairment of IgM receptor signaling is not significant. Surface IgM crosslinking can induce immortalized cells to produce virus, and other studies have shown that there was an appreciable increase in virus production after crosslinking IgM on cells that had been immortalized by LMP2a deficient virus. (131, 133, 134).

The impairment of signal transduction by LMP2a might serve to stabilize the proliferating phenotype of virus infected cells by reducing or delaying the onset of lytic replication triggered by environmental signals. This would provide the selective advantage needed to account for LMP2a expression in immortalized LCLs. The impairment of receptor signaling in *wt* virus immortalized B cells appears to be due to the chronic stimulation of the tyrosine kinase pathways causing them to become desensitized. (132). Lyn, syk, PI3-K, PLC gamma2 and vav are all constitutively phosphorylated on tyrosines and baseline and activatable levels of lyn kinase are markedly reduced. Interestingly, LMP2a expression does not cause constitutive MAPK activation which might explain why LMP2a does not cause B cell proliferation. Although the Shc to grb2/SOS to ras pathway appears to be unaffected, crosslinking IgM receptors does not lead to MAPK activation in LMP2a expressing cells. (132). Protein kinase C activation by phorbol esters will bypass this apparent block and trigger the ras cascade leading to MAPK activity. (155). Thus, other than being somehow blocked at an early step, the pathway is intact in wt virus immortalized cells. (132, 155).

The effect of LMP2a expression on signaling in immortalized B cells and lymphomas is unlikely to accurately mimic the role of LMP2a in latency. LCLs and lymphoma cells are proliferating and there is a low level of active MAPK in these cells and LMP2 is phosphorylated on serines. Recent evidence suggests that the serine phosphorylation of S102 on LMP2a may be due to MAPK phosphorylation. The erk 2 form of MAPK binds to the LMP2a cytoplasmic domain *in vitro* and MAPK co-immunoprecipitates with LMP2a from LCLs. Of the two sites on the LMP2a cytoplasmic domain phosphorylated by MAPK (S15 and S102). one, the S102 site, lies within a highly conserved proline rich motif that is situated between the ITAM and the Y112 src kinase interaction motif. There is a possibility that serine phosphorylation modulates LMP2a protein-protein interactions with tyrosine kinases and other molecules. Resting, latently infected cells probably have no phosphorylated MAPK or tyrosine kinases and LMP2a may not be phosphorylated at all. The LMP2a mediated activation of tyrosine kinases pathways might occur in a manner analogous to the crosslinking of the CD8 alpha-LMP2 chimeras but depend upon an encounter with a stimulus that turns on the LMP2a associated erk2 MAPK enzyme. Regardless of the detailed mechanism of the activation, such a model predicts that LMP2a tranduces signals received by latently infected cells, rather than blocks them as it appears to do in immortalized cells.

4.2. Perspective on Latency

The recent studies on the state of the latently infected B cell *in situ* must for the most part be confirmation of long held suspicions. The cells are resting memory B cells and the virus is virtually silent. (9). It was unexpected that EBNA1 messages were not readily detected and that LMP2a messages were relatively easy to find. The data on the function of these two molecules is not inconsistent with the requirements of latency. There is no need for an origin of replication binding protein in cells that are in G_0 and there could easily be a role for a signal transducer that mediates either the descent into latency or the re-emergence into virus production. The question before us now is how the latent state is achieved and maintained, and ultimately how reactivation is triggered. There are, in essence, two models that can account for the latest information available on the initiation of latent infection *in vivo*. One model (the parallel pathway model). delineates latency as a separate pathway from immortalization, while the other (the serial pathway model). places latency as one possible downstream consequence of immortalization. (figure 8). Favoring the parallel pathway model are the observations of immortalized cells *in vitro* that suggest that proliferation is relentless. Under stress, cells do not drop out of cycle in an orderly G_1/G_0 transition. Cell cycle controls appear to have been deactivated and, as a consequence, *in vitro*

immortalized cells cannot be coaxed into a state resembling *in situ* latency. This dysregulation of the cell cycle in immortalized cells is inconsistent with them being the precursors for latently infected resting cells. The parallel model therefore implies that cells entering latency remain at rest after infection. These cells are the already differentiated products of normal B cell ontogeny (mature memory B cells). and the infectious virions produced by immortalized lymphoblastoid cells would encounter them in the lymphoid tissues. This direct entry into latency obviates the need for a mechanism that de-activates and arrests all the genes and pathways triggered during immortalization. It also fits nicely with the evidence suggesting that reactivation from latency also bypasses immortalization and goes directly to virus production, as though the decision to enter latency had something to do with a permanently pre-empted immortalization program in the latently infected cell. One key difficulty appears to be that the LMP2a expression that has been detected in latently infected cells requires circularization of the viral genome (and circular genomes are exclusively detected in latently infected cells). (9). Circularization of the Herpes simplex genome appears to be a rapid consequence of release of viral DNA into the nucleus.(162). The only report on this essential step in EBV infection concluded that circularization was relatively slow in EBV infected B cells and required activation (up-regulation of CD23). and cell cycle progression (G0 to G1, but not S phase transit). (85). The current view of events following EBV infection of B cells *in vitro* holds that expression of EBNA-LP and EBNA2 from the Wp promoter occurs first. Circularization awaits the entry of the cells into cycle that these virus gene products promote. The expression of the other viral genes including LMP2a is delayed. (3, 6, 85, 219, 244, 245). These events are hallmarks of immortalization and imply that commitment to this process precedes any expression of the LMP2 gene. There is only one study that has actually examined LMP2 expression relative to EBNA2 and this study reported rapid and similar kinetics for these two gene products. (32). It therefore remains a possibility that some infected cells could circularize their genomes prior to cell cycle advance, express LMP2 exclusively and enter latency directly, without launching the immortalization program. Before this direct entry model is discarded, these issues need to be addressed.

The serial pathway model of latency proposes that EBV infected cells participate in all aspects of B cell immune development within lymph nodes. (9). Viral genomes enter memory B cells through the differentiation of virus-activated infected cells within lymphoid follicles. In order for this differentiation to occur the infected cells would need to proceed through germinal center reactions. Direct evidence that this can occur has been obtained from analyses of lymph node biopsies. (1`47, 48, Nalesnick, personal communication). There is even some evidence to suggest that the phenotype of one EBV-associated tumor (Burkitt's Lymphoma). is frozen at the centroblast stage. BL cells are small round rapidly dividing cells which do not express B cell activation markers or high levels of HLA molecules. They provide a window into how viral gene expression might be down-regulated during this stage of development. BL cells maintain the viral genome during their rapid proliferation by replicating it in an EBNA1 dependent manner along with their own genome at S phase. Viral gene expression is restricted to EBNA1 which is expressed from a unique Qp promoter. At least one problem with this model lies in the frequency with which virus-infected cells become associated with GC reactions. Studies on lymph node biopsies from mononucleosis patients suggest the incidence is very rare. (148). It could mean that the association is transient and/or that not many GC events are required to establish the B cell latency that has been observed. (147).

Immortalization might be viewed as required to position virus-infected cells for entry into germinal center (GC). reactions from which plasmacytoid (virus productive). or memory (latently infected). cells emerge. (figure 8). The role of LMP2a in the process comes at the end. Virus activated B cells which entered GC reactions through antigen-independent mechanisms would require a mock-antigenic stimulus to survive. LMP2a, which has an ITAM motif similar to the immunoglobulin receptor signaling molecules Igα and Igβ, supplies the missing signal. Support for this notion has been provided by studies on LMP2a transgenic mice. (28). Immunoglobulin-negative B lineage cells expressing LMP2a survive development in the bone marrow and colonize peripheral lymphoid organs. The implication is that LMP2a signaling replaced the Ig receptor signaling and allowed development to occur. The same function would conceivably allow EBV infected GC cells to survive differentiating into memory cells.

Many questions about the mechanism that diverts some immortalized cells into virus production and others into latency need to be addressed before a serial pathway model can be fully accepted. Are all GC reaction products programmed to become latently infected memory cells? After immortalization is established, how is the down-regulation of virus gene expression achieved? How is immediate reactivation prevented in the memory-differentiated B cells? The mechanisms that repress all viral gene expression but allow LMP2a RNA synthesis are not understood. The structure of the latent LMP2a message (and thus the protein product). needs to be better defined. The activity of the EBNA promoters needs to be resolved. Which are active? What RNA products are produced? In addition, what is the role of DNA methylation in silencing promoters in latency? Methylation of viral promoters could be responsible for the observation that latently infected cells can produce virus following reactivation but do not seem able to run the immortalization program. Finally, none of the preceding discussion precludes the possibility that parallel and serial pathways for entry into latency are operating simultaneously. The real question could be: To what extent does a successful latency rely on one or other pathway to seed a sufficient number of memory B cells to ensure the future propagation of the virus?

Latency is not latency unless it can be followed by re-emergence. What triggers activation of the virus productive program is not known. *in vitro* cultured PBLs

from healthy carriers spontaneously produce immortalized cell lines in a two-step process consisting of a direct burst of virion production followed by infection and immortalization of bystander B cells. Using RNA-PCR we found that explanted PBLs made no detectable viral RNA (besides LMP2a). for at least two to three weeks in culture. Then, between two three-day time points, all the viral RNAs appeared at once.(our unpublished findings). Therefore re-emergence of virus *in vitro* does not appear to be a slow progressive process or result in proliferating cells *per se*. I t is a sudden, quick event that apparently bypasses immortalization. It is not difficult to imagine that some aspect of the LMP2 signaling function underlies the lytic program activation in latently infected cells explanted from the blood of healthy carriers. As we move forward it will be necessary to develop systems to study some of the predictions about LMP2a function under conditions that more closely resemble the latent state.

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