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Dynamic Epstein-Barr Virus Gene Expression on the Path to B-Cell Transformation

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Abstract

Epstein-Barr Virus is an oncogenic human herpesvirus in the γ -herpesvirinae sub-family that contains a 170–180 kb double stranded DNA genome. *In vivo*, EBV commonly infects B and epithelial cells and persists for the life of the host in a latent state in the memory B cell compartment of the peripheral blood. EBV can be reactivated from its latent state leading to increased expression of lytic genes that primarily encode for enzymes necessary to replicate the viral genome as well as structural components of the virion. Lytic cycle proteins also aid in immune evasion, inhibition of apoptosis, and the modulation of other host responses to infection. *In vitro*, EBV has the potential to infect primary human B cells and induce cellular proliferation to yield effectively immortalized lymphoblastoid cell lines, or LCLs. EBV immortalization of B cells *in vitro* serves as a model system for studying EBV-mediated lymphomagenesis.

While much is known about the steady state viral gene expression within EBV immortalized LCLs and other EBV-positive cell lines, relatively little is known about the early events after primary B-cell infection. It was previously thought that upon latent infection EBV only expressed the well-characterized latency associated transcripts found in LCLs. However, recent work has characterized the early, but transient, expression of lytic genes necessary for efficient transformation as well as delayed responses in the known latency genes. This review summarizes these recent findings that show how dynamic and controlled expression of multiple EBV genes can control the activation of B cells, entry into the cell cycle, inhibition of apoptosis, and control of innate and adaptive immune responses.

Keywords

Epstein-Barr virus; latency; viral transformation; EBNA; LMP; lytic; herpesvirus; viral gene expression

I. Background

Epstein-Barr virus (EBV) is a large, double-stranded DNA-containing gammaherpesvirus. EBV is one of the most ubiquitous infectious agents known where nearly 90% of adults are infected worldwide. In most individuals, EBV infection occurs in the early years of life and does not cause disease as a consequence of a robust adaptive immune response to the virus (Rickinson and Kieff, 2007). However, infection in adolescence can trigger infectious

mononucleosis and in the setting of immune suppression, such as following organ transplant or during HIV infection, EBV drives B-cell lymphomas. EBV is also associated with epithelial-derived cancers including nearly all nasopharyngeal carcinomas (NPC) and approximately 10% of gastric carcinomas (GC) worldwide. Importantly, EBV was discovered in the context of African endemic Burkitt lymphoma (BL) where it is nearly uniformly clonally present in these tumors. And finally, approximately 30–40% of Hodgkin's lymphomas are EBV-positive. Therefore EBV infection represents a major clinical entity that has many diverse pathological manifestations.

EBV infection of primary human B cells *in vitro* drives their proliferation and long-term immortalization (Henle et al., 1967). The viral gene expression program associated with B-cell immortalization is called latency III in which all six EBV nuclear antigens (EBNAs) and three latent membrane proteins (LMPs) are expressed as well as the viral non-coding RNAs (EBERs and miRNAs) (Table 1 and Figure 1). The viral EBNA proteins include EBNA1, 2, 3A, 3B, 3C, and LP. EBNA1 facilitates latent viral DNA replication through targeting episomes to host chromosomes and recruiting cellular DNA replication machinery each S phase (Yates, Warren and Sugden, 1985). EBNA1 also serves as a transcriptional activator of other viral EBNA genes and cellular genes (Altmann et al., 2006; Reisman and Sugden, 1986). EBNA2 is the major viral transcriptional trans-activator with an acidic activation domain that associates with p300/CBP histone acetyltransferase activity (Wang, Grossman and Kieff, 2000) and a domain that accesses promoters and enhancers through binding to cellular sequence-specific DNA binding proteins including RBP-J κ /CBF1/CSL and PU.1 (Grossman et al., 1994; Henkel et al., 1994; Johannsen et al., 1995; Yalamanchili et al., 1994). EBNA-LP (leader protein) is a critical co-activator of gene expression with EBNA2. EBNA-LP negatively regulates histone deacetylase (HDAC) function thereby promoting transcriptional activation (Portal et al., 2011). EBNA3A, 3B, and 3C are transcriptional repressors that associate with polycomb group complex (PRC) proteins, HDACs, and the SMRT/NCOR complex (Hickabottom et al., 2002; Knight et al., 2003; Radkov et al., 1999). EBNA3A and 3C are critical for B-cell immortalization (Tomkinson, Robertson and Kieff, 1993), while EBNA-3B has been shown to have a regulatory function in tumorigenesis *in vivo* (White et al., 2012). EBNA3s target host and viral chromatin sites through similar DNA binding proteins as EBNA2 (e.g. RBP-J κ) (Cooper et al., 2003; Robertson et al., 1995) and lead to repression through epigenetic silencing of a subset of EBNA2 targets (Radkov et al., 1997) and other genes including the cyclin-dependent kinase inhibitor, p16INK4A, and the apoptosis-inducing protein, Bim, thereby promoting cell proliferation and survival (Maruo et al., 2011; Paschos et al., 2009; Skalska et al., 2010). The coordinated activities of the EBNA proteins serve to control viral and host gene expression through direct interactions with cellular control circuits in the nucleus.

The three latent membrane proteins, LMP1, 2A, and 2B are mimics of cellular signaling proteins responsible for B-cell activation and survival. LMP1 mimics a constitutively activate CD40 receptor, which is the B-cell protein that normally receives T-cell help through CD40L signaling in the germinal center (Gires et al., 1997). LMP1 strongly activates the pro-survival NF κ B, p38, and JNK signaling pathways (Soni, Cahir-McFarland and Kieff, 2007). The activation of NF κ B by LMP1 is required for B-cell immortalization (Cahir-McFarland et al., 2004; Cahir-McFarland et al., 2000; Kaye, Izumi and Kieff, 1993).

LMP2A, on the other hand, mimics a constitutively active B-cell receptor through aggregating downstream SH2-domain containing tyrosine kinases including Lyn and Syk to promote PI3K activity (Longnecker et al., 1991). LMP2B is identical to LMP2A except that it lacks the N-terminal domain responsible for Lyn and Syk recruitment and therefore acts to regulate LMP2A activity (Longnecker et al., 1992). While LMP2A is not critical for B-cell transformation *in vitro*, it likely has an important role *in vivo* as a modulator of endogenous B cell receptor signaling important to promote survival of EBV-infected cells and possibly tumors (Caldwell et al., 1998; Miller et al., 1995).

In addition to protein-coding genes, EBV is the current champion of human viruses with regard to generating non-coding RNAs including miRNAs (Cullen, 2011b). EBV encodes two short, polIII-derived non-polyadenylated RNAs called EBER1 and EBER2 that both activate and suppress aspects of the interferon response (Jochum et al., 2012b; Nanbo et al., 2002). In addition, EBV produces 25 precursor miRNAs that generate 44 mature miRNA species (Cullen, 2011a; Skalsky et al., 2012). The EBV miRNAs are expressed from two transcriptional clusters, 22 miR-BARTs and 3 miR-BHRF1s. Deletion of the BHRF1 miRNAs reduces B-cell immortalization efficiency by approximately 10-fold (Feederle et al., 2011; Seto et al., 2010), while loss of the EBERs has no impact on B-cell growth *in vitro* (Swaminathan, Tomkinson and Kieff, 1991), although this remains controversial. The targets of the viral miRNAs and the EBERs and their role in the pathophysiology of EBV infection remain poorly understood.

This review will focus on the key events in viral gene expression that occur following primary B-cell infection and through long-term outgrowth in culture in the absence of a T-cell response. These events likely mimic critical temporal changes that occur during B-cell infection *in vivo*. However, additional pressures *in vivo* including the adaptive immune response and spatial constraints will not be efficiently modeled. Initially upon primary B-cell infection *in vivo*, it is thought that the latency III growth program drives naïve cells to proliferate (Joseph, Babcock and Thorley-Lawson, 2000) and ultimately into a germinal center (GC) reaction in lymphoid tissue (Thorley-Lawson and Allday, 2008). The GC environment leads to a restriction of viral gene expression to latency II in which only the EBNA1, LMP1 and LMP2 proteins are expressed (Table 1 and (Roughan and Thorley-Lawson, 2009)). Finally, as infected cells transition to the memory cell compartment this further restricts viral gene expression to either latency '0' in which no viral protein-coding genes are expressed or latency I where only EBNA1 is expressed during S phase (Babcock et al., 1998). The viral miRNAs and EBERs may be expressed in these tightly restricted, antigenically inert resting memory B cells as well. If the EBV-infected B cell differentiates towards the plasma cell lineage, then the virus activates the lytic cycle genes and generates new progeny to exit the cell (Sun and Thorley-Lawson, 2007). Lytic virus replication also occurs spontaneously in approximately 1–5% of latency III expressing LCLs in culture and the route to immortalization may include a transit through a “prelatent”, abortive lytic phase of infection in which a subset of lytic genes are expressed, though no viral DNA replication or particle formation occurs. Lytic viral gene expression will be discussed in detail below in the context of the temporal stages of viral gene expression during B-cell immortalization.

Most of what we have learned about EBV latency over the past nearly fifty years has come from the study of the genes expressed in immortalized LCLs or more tightly restricted BL cell lines. A few seminal studies characterized the temporal expression pattern of the canonical EBV latency genes following primary B-cell infection giving rise to our understanding of the early kinetics of viral gene expression (Alfieri, Birkenbach and Kieff, 1991; Hurley and Thorley-Lawson, 1988; Sinclair et al., 1994; Woisetschlaeger, Strominger and Speck, 1989; Woisetschlaeger et al., 1990). However, in recent years, several new studies have highlighted the more complex dynamics of early events in EBV transformation of B cells *in vitro* and are beginning to shed light on distinct phases of latency that may have particular relevance with respect to pathophysiology of EBV-related diseases. Here we focus on these distinct phases highlighting recent advances and discussing how these studies will impact future work.

II. Virion-Associated RNAs and Very Early Events in Infection

Many diverse herpesviruses have been shown to contain RNA molecules within their virions (Bechtel, Grundhoff and Ganem, 2005; Bresnahan and Shenk, 2000). While the presence and role of “virion-associated RNAs” remain controversial (Marcinowski et al., 2012; Sarcinella et al., 2004), these RNAs appear to be packaged selectively over host RNAs (Cliffe, Nash and Dutia, 2009; Greijer, Dekkers and Middeldorp, 2000; Sciortino et al., 2001) and virion-associated RNAs are incorporated in the proportions they are found in the host cell (Terhune, Schroer and Shenk, 2004). Virion-associated RNAs have been identified in the related herpesviruses: herpes simplex virus type 1 (HSV1), cytomegalovirus (CMV), murine gamma-herpesvirus (MHV68), and Kaposi’s sarcoma-associated herpesvirus (KSHV) and most recently in EBV (Jochum et al., 2012b).

Zeidler and colleagues explored the role of RNAs associated with the EBV virion during early infection. Within two hours after infection of primary B cells with the prototype EBV strain B95-8, many EBV transcripts can be detected by quantitative reverse-transcription PCR (qRT-PCR) (Jochum et al., 2012b). These include the latency-associated transcripts LMP1, LMP2A/B, EBNA2, and the noncoding RNAs EBER1 and EBER2; as well as the lytic immediate early genes BZLF1, BRLF1, and BMRF1; the EBV-encoded immune evasion proteins BNLF2a, BGLF5, and BCRF1; and the anti-apoptotic viral homolog of BCL-2 BHRF1. These virion-associated RNAs were resistant to external RNase treatment suggesting that they were indeed packaged within the viral particle and transduced to newly infected cells. Lending credence to this hypothesis, the BRLF1 protein was detected by immuno-precipitation and Western blot after infection of a permissive B-cell line in the presence of the RNA polymerase inhibitor Actinomycin D (Jochum et al., 2012b).

Another viral mRNA detected in the EBV virion encodes the immunoevasin BNLF2a, which functions by inhibiting the proper loading of antigenic epitopes on HLA class I molecules (Hislop et al., 2007). It is expected that early expression of BNLF2a in B cells could dampen their recognition by EBV-reactive CD8⁺ T cells. B cells infected with a BNLF2a-deleted virus elicited a modest increase in response from EBV-reactive CD8⁺ T cells co-cultured with the infected B cells as compared to wild type virus-infected B cells. Importantly, this

phenotype was preserved in the presence of Actinomycin D suggesting that RNAs inside the virion rather than those newly transcribed upon infection were responsible for the effect.

In the provocative study described above, the levels of virion-associated RNAs and the phenotypes observed after B-cell infection were extremely modest. In addition, all of the RNAs detected were also found to increase within hours after infection, indicating that irrespective of the relevance that these virion-associated RNAs may have to transformation, *de novo* transcription still appears to be the critical step in B-cell proliferation. Until further studies are performed to verify these effects, the physiological relevance of these findings remain unclear.

III. Viral Gene Expression During the Prelatent Phase Early After Infection

Epstein-Barr Virus infects and immortalizes naïve, resting B cells. The resting G0 state of these cells provides a unique challenge for a DNA virus in that it must initiate entry into the cell cycle to replicate its own genome. While it was previously thought that the latency associated Epstein-Barr Nuclear Antigens (EBNAs) were entirely responsible for cellular activation (Kieff and Rickinson, 2007), it has recently been shown that a number of genes normally associated with lytic reactivation are expressed at early times after infection with the virus called the prelatent phase (Figure 2). In this section we will discuss the potential roles for these genes in the context of EBV transformation.

To escape from latency and produce infectious virus, lytic stimuli induce the expression of the lytic master regulator gene BZLF1 (Sinclair, 2003). BZLF1 protein induces its own expression and leads to a cascade of viral lytic gene induction that ultimately results in the production of virions and the lysis of the host cell. As such, it was surprising when multiple groups reported BZLF1 transcripts at early times after infection, when lytic reactivation would be detrimental to the establishment of latency (Halder et al., 2009; Kalla et al., 2010; Wen et al., 2007). It is important to note that while one group observed the production of infectious virions due to this early expression of BZLF1 (Halder et al., 2009), the other two groups did not observe productive infection as evidenced by the lack of viral DNA replication and expression of viral structural genes (Kalla et al., 2010; Wen et al., 2007). Hammerschmidt and colleagues did observe spontaneous lytic reactivation and productive virus formation, but only after two weeks post infection when promoters of late lytic genes had become methylated at key CpG sites important for full BZLF1 activation (Bergbauer et al., 2010; Kalla et al., 2010).

III.a. BZLF1 Regulates Initial B Cell Cycle Entry

BZLF1 is related to the activating protein 1 (AP1) family of transcription factors in humans (Farrell et al., 1989). It has also been shown that BZLF1 can functionally interact with many different cellular proteins to influence growth and survival (Adamson and Kenney, 1999; Lieberman and Berk, 1991; Sinclair, 2003; Zerby et al., 1999; Zhang, Gutsch and Kenney, 1994). Thus, it has been postulated that early expression of BZLF1 in resting primary B cells could act as a cellular transcription factor that induces the cells to enter the cell cycle. While BZLF1-knockout viruses (ZKO) are competent to immortalize B cells (Feederle et al., 2000; Katsumura et al., 2009), recent work indicates that ZKO-infected

resting naïve and memory B cells isolated from adenoids were impaired for initial B-cell proliferation (Kalla et al., 2010). The reduced transformation efficiency of the ZKO virus was rescued by infecting either naïve B cells driven to cycle by CD40 ligand/IL-4 pre-treatment or GC-derived B cells (Kalla et al., 2010). Thus, these new experiments highlight the possible role of early BZLF1 expression in B-cell activation and initial cell cycle entry. It is also worth noting that other canonically lytic genes expressed during this pre-latent phase might have a more pronounced effect on B-cell transformation.

III.b. vBcl2 Proteins Inhibit Apoptosis Early After Infection

Herpesviruses are known to encode anti-apoptotic homologs of human BCL2 genes that protect their host cells from death (Boya et al., 2004). EBV is the only member of the herpesvirus family to encode two of these so called vBcl-2s, the genes BHRF1 and BALF1 (Figure 2). While BHRF1 and BALF1 were thought to be primarily lytic genes with their own promoters (Austin et al., 1988; Pearson et al., 1987), it has recently been shown that BHRF1 and BALF1 are expressed early after primary B-cell infection (Altmann and Hammerschmidt, 2005). Especially interesting was the observation that while both single knock outs of BHRF1 and BALF1 individually produced viruses that were transformation competent, the BHRF1⁻/BALF1⁻ double knockout virus was completely transformation incompetent. Despite the two vBcl-2s being originally characterized as lytic genes the producer cell lines containing both the single and the double knockout viruses produced equal titers of virus as compared to a wild type B95-8 producer cell line (Altmann and Hammerschmidt, 2005), indicating there was no defect in lytic reactivation and implying that the redundant EBV vBcl-2s were critical for transformation into latency.

Cell cycle analysis of the vBcl-2 double knockout revealed that the phenotypic effects were observed immediately after infection; the double knockout infected primary B cells never entered the cell cycle (Altmann and Hammerschmidt, 2005). The double knockout-infected cells also displayed increased apoptotic markers and after a week in culture most of the cells had died. In comparison to an EBNA2 deleted virus, which is also transformation incompetent and does not enter the cell cycle upon infection, the vBCL double knockout-infected cells died much faster with higher levels of apoptotic markers. Interestingly, the vBCL double knockout virus could transform B cells induced into cycle by CD40L/IL-4, albeit at reduced efficiency. However, the LCLs that grow out of those infections are phenotypically normal and no longer require exogenous signaling for survival (Altmann and Hammerschmidt, 2005). Therefore, the EBV vBcl2 proteins are only required to prevent apoptosis at an early point during B-cell transformation.

While their role in B-cell survival is clear, the mechanism for BHRF1 and BALF1 expression early after infection is not well understood. Since BHRF1 is transcriptionally regulated by BZLF1 during lytic reactivation it was assumed that the presence of BZLF1 at early times during infection might also activate the vBcl-2s. Surprisingly, primary B cells infected with a ZKO virus expressed the same levels of BHRF1 and BALF1 over the same time course as WT B95-8-infected cells, implying that they were transcribed independent of BZLF1 (Altmann and Hammerschmidt, 2005). One explanation for the existence of BHRF1 and BALF1 mRNA (as well as other early lytic genes) at early times is that the EBV genome

is transduced into the cell in a naked, chromatin-free state (Booy et al., 1991) permitting free access to transcription factors (Altmann and Hammerschmidt, 2005; Kalla et al., 2010). However, other groups have published that it is possible to detect BHRF1 transcripts spliced from the latency associated W promoter (Wp), and that these transcripts are detected at early times and in mature LCLs (Arvey et al., 2012; Kelly et al., 2009) (Figures 3 and 4).

III.c. Prelatent Genes and Immune Evasion

In an immunocompetent host EBV infection is held in check by a strong CD8⁺ T cell response (Rickinson and Kieff, 2007). This response is biased towards epitopes encoded by the lytic genes of EBV, but certain T cells also recognize latent epitopes (Merlo et al., 2010). Importantly, EBV deploys a number of immune evasion molecules during lytic reactivation to counteract the T cell response. These genes include BCRF1, a viral homolog of IL-10 (Miyazaki, Cheung and Dosch, 1993; Zeidler et al., 1997), BGLF5, a DNase and exoribonuclease that shuts off host protein synthesis and down-regulates Toll-like Receptor 9 (van Gent et al., 2011; Zuo et al., 2008), BNLF2a, an inhibitor of the TAP antigen processor pathway (Hislop et al., 2007), and BILF1, a G-protein coupled receptor that degrades MHC class I molecules (Zuo et al., 2011). Since it has come to light that there are a number of lytic EBV genes expressed early after infection the question was asked whether or not EBV also expresses these immune evasion genes to protect the early infected cells from immune system discovery.

Two of the EBV immunoevasins have recently been detected early after infection, BCRF1 (vIL-10) and BNLF2a (Jochum et al., 2012a). Infection with mutant EBV lacking either BCRF1, BNLF2a, or both immunoevasins made B cells more susceptible to killing in co-culture with EBV-specific CD8⁺ T cells (Jochum et al., 2012a; Lautscham et al., 2001). BCRF1 (vIL-10) was specifically implicated in suppressing pro-inflammatory cytokine release and mitigating natural killer cell killing of EBV-infected B cells, while BNLF2a was responsible for reducing CD8⁺ T cell recognition (Jochum et al., 2012a).

It has long been known that latent infection by EBV can modulate the immune response due to up regulation of cellular IL-10 (Zeidler et al., 1997). In these most recent experiments, the viral immunoevasins have been implicated in immune control early after infection. It is thus possible that these factors play an important role in dampening immune responses during a pre-latent phase *in vivo*, but further experiments will need to be performed to characterize this. It is worth noting that EBV encoded miRNAs and other noncoding RNAs have also been implicated in down-regulating factors important for immune clearance of EBV (Nachmani et al., 2009), and these RNAs are also induced early after infection.

IV. miRNAs and Other Non-Coding RNAs Involved in Transformation

Micro RNAs (miRNAs) are small 21–25 nucleotide non-coding RNAs that negatively regulate mRNAs by targeting complementary sequences within their transcripts (Bartel, 2009). EBV was the first human virus shown to express miRNAs (Pfeffer et al., 2004), and to date EBV expresses more pre-miRNAs (25) than any other human virus (Forte and Luftig, 2011; Skalsky and Cullen, 2010). EBV's miRNAs fall into two main clusters, the BHRF1

cluster found near the BHRF1 gene, and the BART miRNAs located within the BART transcripts near oriLyt (Figure 2).

EBV miRNAs are differentially expressed depending on the infected cell type or tumor tissue and based upon the latency gene expression program (Table 1 and (Forte and Luftig, 2011)). The BHRF1 cluster of miRNAs is only expressed in latency III-infected cells as well as certain W promoter restricted Burkitt Lymphomas that also express the BHRF1 protein (Amoroso et al., 2011;Xia et al., 2008). The BHRF1 miRNAs come on very early after primary B-cell infection and are expressed at relatively high levels through outgrowth (Figure 4). The BART miRNAs, on the other hand, are expressed mostly in latency II-infected epithelial cells including EBV-induced nasopharyngeal and gastric carcinomas (Cai et al., 2006;Cosmopoulos et al., 2009;Kim et al., 2007). Despite the preference for epithelial cells, BART miRNAs are also expressed at reduced levels in lymphoblastoid cell lines and DLBCLs (Amoroso et al., 2011;Cai et al., 2006;Edwards, Marquitz and Raab-Traub, 2008). It is also important to note that the B95-8 prototype EBV strain, often used as a wild-type control in labs, contains a deletion ablating the majority of the BART miRNAs (Cai et al., 2006;Grundhoff, Sullivan and Ganem, 2006). A recent study added the missing BART miRNAs back into the B95-8 background and showed that they did not increase the transformation efficiency of the virus (Seto et al., 2010), indicating that the BART miRNA functions are primarily seen in other cell types or *in vivo*.

The role of the EBV miRNAs in B-cell transformation was characterized by two groups using genetic approaches (Feederle et al., 2011;Seto et al., 2010). Removal of the BHRF1 cluster severely compromised EBV-induced early B-cell proliferation and suppression of apoptosis (Feederle et al., 2011;Seto et al., 2010). However, BHRF1 miRNA-deficient LCLs could be generated. These LCLs were markedly deficient in S-phase progression indicating a persistent role for these miRNAs in B-cell proliferation. In very recent studies using humanized mouse models it was discovered that the presence of an intact BHRF1 miRNA locus was required for high-level systemic virus load, but both wild-type and BHRF1 mutant viruses had the same oncogenic potential (Wahl et al., 2013).

The role of miRNAs in gene repression depends on interactions between the miRNA and mRNA targets through the 5' miRNA seed sequence (Bartel, 2009). Recently, technology has emerged to identify all miRNA/mRNA targets in cells and this was used to identify EBV miRNA targets in LCLs (Skalsky et al., 2012). EBV miRNAs targeted hundreds of mostly cellular mRNAs. Together with earlier studies using computational predictions of miRNAs binding sites, the EBV miRNA targetome is now emerging. For example, miR-BHRF1-3 can target the interferon-inducible T cell attracting chemokine CXCL11 (Xia et al., 2008). Among the BART miRNAs, miR-BART5 has been shown to target PUMA, the p53-regulated modulator of apoptosis (Choy et al., 2008b) and miR-BART3-5p has been shown to target MICB, a Natural Killer cell ligand important in immune surveillance (Nachmani et al., 2009). BART miRNAs have also been shown to target viral mRNAs, including the lytic viral DNA polymerase BALF5 by miR-BART2 (Barth et al., 2008), LMP2A by miR-BART22 (Lung et al., 2009), and LMP1 by miR-BART1-5p, miR-BART16-3p, and miR-BART17-5p (Lo et al., 2007). While miRNA repression of the canonically pro-growth LMP1 gene is interesting, none of the above miRNAs were validated in a detailed analysis

of miRNA targeting in LCLs (Skalsky et al., 2012). It is of note, however, that LMP1 was targeted strongly by a set of host miRNAs belonging to the miR-17/20/106 seed family of c-Myc-induced miRNAs (Skalsky et al., 2012). Tight regulation of LMP1 through miRNAs is potentially critical because overexpression of LMP1 can be cytostatic and also can sensitize cells to pro-apoptotic stresses (Liu et al., 2002; Lu et al., 1996).

EBV also encodes two small non-polyadenylated RNAs termed Epstein-Barr Encoded RNAs (EBERs), EBER1 and EBER2 (Lerner et al., 1981). These RNA polymerase III driven genes are the most abundant viral transcripts in latently infected cells (Rosa et al., 1981; Rymo, 1979). While EBERs are not essential for the transforming capability of EBV B95-8 (Swaminathan, Tomkinson and Kieff, 1991), an EBER deletion mutant generated in the Akata strain background is approximately 100-fold less efficient in B-cell transformation than wild type Akata-strain EBV (Yajima, Kanda and Takada, 2005). EBERs have been shown to bind RIG-I to activate type I interferon gene expression (Jochum et al., 2012b) and also to directly bind to and inhibit the downstream RNA-activated Protein Kinase (PKR) thereby preventing interferon- α induced apoptosis (Nanbo et al., 2002). EBERs have also been shown to induce IL-10 (Kitagawa et al., 2000), a cytokine that has proven to be very important to immune modulation in the EBV life cycle.

EBV-encoded noncoding RNAs have been implicated in suppression of the EBV-directed immune response, innate immunity, the maintenance of latency, and the establishment of B-cell growth transformation. However, none of these RNAs are absolutely essential for EBV transformation of primary B cells. Instead it appears that these RNAs work by fine-tuning the expression of host and viral proteins to aid in the efficiency of EBV-induced outgrowth.

V. Dynamic Control of Viral Promoters Leads to Specificity of Latent Gene Expression

During latent infection, EBV encodes nine latency-associated proteins. Six of these, the Epstein-Barr Nuclear Antigens (EBNAs), are produced from one extensively spliced latency transcript that initiates downstream of one of two promoters: the W promoter (Wp), which is present multiple times within the BamHI W fragment repeats, or the C promoter (Cp), which is further upstream within the BamHI C fragment (Figures 2 and 3). Transcription initiated from either of these promoters (W0 or C1/C2 exons) splice downstream to multiple repeats of tandem W fragment exons (W1/W2). The transcript encoding EBNA-LP is unique in that the first W1 splice acceptor is not used, but rather a 5 nt downstream acceptor called W1' (prime) (Rogers, Woisetschlaeger and Speck, 1990). This W0–W1' splicing event generates the ATG start codon to initiate the EBNA-LP coding sequence and is shown in greater detail in the inset of Figure 3. Downstream splicing of the EBNA-LP transcript is then similar to the other EBNAs until the final exons contained within the Y and H BamHI fragments such that the protein is encoded by W1–W2 repeats and the first two Y exons (Figure 3). EBNA2 has a similar transcript structure to EBNA-LP except that it lacks the EBNA-LP specific W1' splice and ends in a unique ORF-containing exon that spans the BamHI Y and H fragments (Figure 3). All other EBNA transcripts contain the W fragment repeats in their 5' UTRs, yet transcripts can skip the EBNA2 exon and splice from the second or third unique Y fragment

exon far downstream to splice acceptors within the U fragment and then alternatively splice even further downstream to the EBNA3s or EBNA1. Therefore, the EBNA transcripts rely heavily on alternative splicing and can extend to over half the length of the full EBV genome. While all of the EBNAs are capable of being initiated by either promoter (Woisetschlaeger, Strominger and Speck, 1989), EBV controls the specificity and timing of expression early after infection by differential use of the two promoters.

The W promoter is activated by B-cell host factors concurrent with the pre-latent expression of viral lytic genes immediately upon infection. These Wp-activating factors include YY1 and CREB/ATF (Bell et al., 1998; Kirby, Rickinson and Bell, 2000), and the B-cell specific transcription factor BSAP/Pax5 (Tierney et al., 2000). As the W promoter is located once per W fragment repeat, transcription of the EBNAs can and does start from any of these fragments early after infection (Rooney et al., 1989). Furthermore, alternative splicing to W1–W2 repeats can occur. This leads to EBNA transcripts with differing 5'UTR sizes and also different sized EBNA-LP protein isoforms. The W promoter has evolved to be efficiently activated by the milieu of transcription factors present in resting B cells and EBNA transcription initiated at Wp starts as early as twelve hours post infection (Alfieri, Birkenbach and Kieff, 1991).

Within the first twenty-four hours post infection Wp only initiates expression of EBNA-LP, EBNA2, and the vBCL2 homolog BHRF1 despite the potential for Wp to drive expression of all of the EBNA latency transcripts (Alfieri, Birkenbach and Kieff, 1991; Kelly et al., 2009). It is hypothesized that this is due largely to Wp not being a strong activator of transcription elongation and that the EBNA transcripts farther downstream (EBNA1 and the EBNA3s) are not efficiently produced at this time. During this time EBNA2 interacts with host DNA-binding factors including RBP-J κ and together with its co-activator, EBNA-LP, up-regulates many host genes such as the proto-oncogenes c-fgr and c-myc (Zimmer-Strobl and Strobl, 2001) as well as cyclin D2 promoting the G0/G1 cell cycle transition (Sinclair et al., 1994). This early state of EBNA2/EBNA-LP driven growth with minimal expression of the EBNA3s has also been associated with a period of hyper-proliferation and a concomitant growth-suppressive DNA damage response (DDR) (Nikitin et al., 2010). It is thought that during the transition from Wp to Cp usage, cells with high EBNA2/LP activity, but low EBNA3s are unable to attenuate c-Myc levels and therefore arrest due to oncogene-induced senescence. Despite apparently poor transcriptional elongation downstream of Wp, EBNA1 is expressed at sufficient levels to promote latent viral DNA replication.

EBNA1 has also been implicated in the transition from Wp to Cp through its role as a transcriptional enhancer. To facilitate proper episome segregation EBNA1 binds to *oriP*, the origin of plasmid replication, located upstream of both Wp and Cp (Figure 2). In addition to its crucial role in episome maintenance, the EBNA1/*oriP* interaction has been described as a potent enhancer of Cp (Puglielli, Woisetschlaeger and Speck, 1996; Sugden and Warren, 1989; Woisetschlaeger, Strominger and Speck, 1989). Canonical Cp activation is through an EBNA2 responsive element bound by RBP-J κ and other transcription factors (Jin and Speck, 1992; Sung et al., 1991; Woisetschlaeger et al., 1991). Ultimately, Cp activity is strongly up-regulated through viral EBNA gene products after they accumulate two to three days post infection (Figure 3).

As the activity of Cp rises, the activity of Wp wanes. While LCLs still maintain some level of Wp-initiated transcripts, the number of Cp-initiated transcripts is generally three to four fold higher (Yoo et al., 1997). This Wp/Cp ratio is reduced after infection and also correlates with the number of cell divisions the infected B cell has undergone (Nikitin et al., 2010). The reduced Wp usage is likely to due to the relative strength of Cp as well as to transcriptional interference due to Cp being upstream of Wp (Puglielli, Desai and Speck, 1997; Puglielli, Woisetschlaeger and Speck, 1996). Ultimately, all EBNA transcripts are produced from Cp including EBNA1 and EBNA3s -3A, -3B, and -3C (Figure 3). The rise in expression of the EBNA3s after three to four divisions results in the down-regulation of EBNA2 host targets such as c-Myc, as well as Cp itself due to competition for the host factor RBP-J κ (Krauer et al., 1996; Marshall and Sample, 1995; Robertson et al., 1995). Expression of the EBNA3s at this time also attenuates the hyper-proliferative and growth-suppressive DDR phenotype observed early after infection (Nikitin et al., 2010). EBNA3C also specifically down-regulates both p16INK4A (Maruo et al., 2011; Skalska et al., 2010), as well as the pro-apoptotic protein BIM (Paschos et al., 2012) to facilitate outgrowth.

Once the EBNA proteins are expressed at full levels and infected cells begin cycling, EBNA2 and EBNA-LP activate the promoters of the viral latent membrane proteins. Specifically, EBNA2 and EBNA-3C have been shown to co-activate the promoter of the essential latent membrane protein 1 (LMP1) (Johannsen et al., 1995; Lin et al., 2002). Previously it was thought that this activity and LMP1 levels rose within two days to that observed in LCLs. However, recent studies have challenged this dogma and suggest mechanisms and hypotheses for why a delay in LMP1 may be relevant to EBV biology and pathogenesis.

VI. Delayed Expression of Latent Membrane Protein 1 (LMP1) Ultimately Required for Transformation

Latent Membrane Proteins 1 and 2A/2B (LMPs) are latency associated transcripts found in both latency II and latency III EBV infected cells (Table 1). LMP1 and LMP2B are expressed from an EBNA2-responsive bi-directional promoter while LMP2A, which is not essential *in vitro* for transformation, is expressed from a unique promoter (Laux et al., 1994; Longnecker et al., 1993; Zimmer-Strobl et al., 1991). Recent studies indicate that LMP1 and LMP2A transcripts do not accumulate to LCL levels at early times post infection (Nikitin et al., 2010). Moreover, LMP1 does not reach LCL mRNA or protein levels until approximately two weeks post infection (Price et al., 2012) (Figure 4).

LMP1 acts as a constitutively active homolog to the human CD40 membrane protein (Mosialos et al., 1995) and is essential to the transforming capability of EBV (Kaye, Izumi and Kieff, 1993). Both CD40 and LMP1 signal through the NF κ B pathway in a similar fashion (Luftig et al., 2003; Luftig et al., 2004), and inhibition of the NF κ B pathway in LCLs results in spontaneous apoptosis (Cahir-McFarland et al., 2000). In addition, the majority of genes that change at the expression level when an EBV-negative Burkitt's lymphoma cell line (BL41) was infected and converted to latency III gene expression overlap with the gene expression changes induced by simply expressing LMP1 in the same cell line (Cahir-

McFarland et al., 2004). Collectively, these data point to LMP1 being a critical modulator of gene expression and essential for the survival and establishment of EBV transformed B cells. As such, it was unexpected when recent results indicated that LMP1 expression and its characteristic NF κ B activity levels were delayed until approximately two weeks post infection (Price et al., 2012).

The relevance of the delay in LMP1 expression was assessed by modulating NF κ B activity within the first two weeks after infection. Heterologous activation of NF κ B through CD40 ligation within the first week after infection significantly increased transformation efficiency, while activating NF κ B once LMP1 was expressed had no impact on transformation (Price et al., 2012). Conversely, when EBV-infected primary B cells were treated with an I κ B Kinase β (IKK β) inhibitor at early times after infection there was no decrease in transformation efficiency, yet IKK β inhibition at late times, when LMP1 was expressed, significantly decreased transformation and outgrowth consistent with the effect of IKK inhibitors on LCLs (Cahir-McFarland et al., 2000; Keller, Schattner and Cesarman, 2000).

The increase in LMP1 expression levels between early and late times of infection is regulated at the level of transcription. The LMP1 promoter is activated by EBNA2 through its interactions with host DNA binding factors RBP-J κ and PU.1 (Johannsen et al., 1995). Similarly, other host factors have been implicated in the activation of the LMP1 promoter including ATF4 (Pratt, Zhang and Sugden, 2012; Sjoblom et al., 1998), IRF7 (Ning et al., 2003), and even NF κ B itself (Demetriades and Mosialos, 2009). Recent work indicates that many of these factors display attenuated activity in early proliferating B cells as evidenced by genome-wide transcriptional target levels (Price et al., 2012). Thus, LMP1 promoter activity may be low in the first two weeks after infection due to the lack of activity or expression of these critical transcription factors.

LMP1 mRNA levels may also be controlled post-transcriptionally. Recent studies indicate that the c-Myc controlled miRNA family miR17/20/106 binds to the LMP1 3'UTR to negatively regulate its expression (Skalsky et al., 2012). As c-Myc levels are high in early EBV-infected hyper-proliferating B cells, the concomitant rise in miR-17 family miRNAs may prevent LMP1 accumulation (Forte et al., 2012). A complementary hypothesis to this postulates that c-Myc directly antagonizes NF κ B activity as has been shown in other B-cell lymphomas (Faumont et al., 2009; Klapproth et al., 2009) thereby preventing NF κ B-mediated feed-forward signaling on the LMP1 promoter (Demetriades and Mosialos, 2009; Johansson et al., 2009).

Irrespective of the method by which LMP1 expression is delayed during outgrowth, a critical question remains as to how proliferating B cells early after infection survive without NF κ B anti-apoptotic signaling. One hypothesis is that EBNA2 targets may be sufficient for survival in the B-cell environment early after infection (Lee et al., 2004; Lee et al., 2002). A second, and potentially complementary, hypothesis was described above and involves the expression of two viral Bcl-2 homologs, BHRF1 and BALF1, early after infection (Altmann and Hammerschmidt, 2005). The BHRF1 protein is also anti-apoptotic in the context of a subset of Burkitt's lymphomas where it is expressed as a latency transcript initiated from the W promoter (Table 1). This is the same viral promoter that has maximal activity during the

early, LMP1 low phase of the EBV life cycle (Kelly et al., 2009) (Figures 3 and 4). Defining how EBV infected B cells survive without NF κ B induced growth and survival signals early after infection will lead to a much better understanding of how EBV can prevent apoptosis in B cells that are already primed for death.

VII. Heterogeneity in Steady-State EBV Gene Expression in Lymphoblastoid Cell Lines

Recent studies of a large set of lymphoblastoid cell lines have illuminated our understanding of the heterogeneity in viral gene expression that exists between LCLs independent of normal donor variation (Arvey et al., 2012). One major finding from this work is that the level of spontaneous lytic reactivation varies extensively between LCLs. This was found by characterizing the number of lytic and latent mRNA transcripts per cell across a set of over 300 different LCLs (International HapMap et al., 2010; Montgomery et al., 2010; Pickrell et al., 2010). It was clear that many LCLs had both latent and lytic genes expressed at high levels while other lines were tightly latent. Single cell analysis such as immunofluorescence for latent and lytic proteins clearly indicates that most (e.g. >95%) of cells within an LCL culture are latent (Kieff and Rickinson, 2006). However, it was not previously appreciated how much a change from ~0.1% to ~1% to ~5% lytic cells could alter the overall transcriptome of a LCL culture until now. These studies strongly suggest that investigators using the LCL lines from large-scale projects such as the HapMap and ENCODE consortium to study human genetic variation should be aware that these lines not only vary due to underlying genetic changes between individuals, but also in the extent that EBV lytic replication, even in a subset of cells, may skew the cellular mRNA transcriptome (Arvey et al., 2012; Choy et al., 2008a).

The high resolution characterization of EBV mRNAs across many LCLs also identified new viral mRNA isoforms in both lytic and latent genes. For example, new splice junctions were identified in the lytic BHLF1 and BZLF1 genes suggesting novel regulation of the encoded proteins. Additionally, the Wp-initiated BHRF1 transcript was detected in LCLs as previously described (Austin et al., 1988; Kelly et al., 2009; Lin et al., 2010), but included BHRF1 splice variants from multiple upstream W or Y exons and downstream to two different splice acceptors. Thus, high resolution mapping and precise quantitation of transcripts by next generation deep mRNA sequencing likely holds much power in deciphering many of the questions posited in this review regarding how the EBV transcriptome is regulated at different stages during B-cell transformation.

A final new piece of the EBV gene regulatory puzzle emerged from the recent Arvey, *et al.* study. The underlying chromatin and transcription factor binding site architecture on the EBV genome was assessed from Chip-Seq experiments as part of the ENCODE project. Compilation of these experiments corroborated much previous work including PU.1 regulation of the LMP1 promoter (Johannsen et al., 1995) and CTCF chromatin domain boundaries (Tempera, Klichinsky and Lieberman, 2011). Additionally, many new factors were found to bind to the promoters of the BART miRNAs and the LMP1 promoter. Intriguingly, a new role was identified for the transcription factor Pax5 in binding to the

EBV terminal repeats suggesting a potential role in circularization, the earliest stage in viral gene regulation. These new studies highlight the impact that the ENCODE project will have on EBV biology simply by having chosen a LCL as one of the main model cell lines for study.

VIII. Conclusions and Future Directions

The recent in-depth characterization of EBV gene expression after primary B-cell infection has challenged many of the original paradigms in EBV biology as well as corroborated others. New roles have been postulated for “virion-associated” mRNAs delivered with the viral particle, an early “prelatent” stage of gene expression associated with canonically lytic mRNAs detected within the first 48h of infection, and, finally, new data have emerged on the kinetics of latent membrane protein expression indicating a delay of nearly two weeks post infection. Together, these findings along with the discovery of many virally-encoded miRNAs and next generation mRNA deep sequencing identifying many sub-dominant latent mRNA isoforms in LCLs harken a new era in our understanding of how EBV persists in and controls human B lymphocytes.

The identification of EBV-encoded virion-associated RNAs was not surprising as this has been reported for other herpesviruses (Bechtel, Grundhoff and Ganem, 2005; Bresnahan and Shenk, 2000). However, it remains to be demonstrated that these RNAs are relevant for EBV pathogenesis. The translation of two of these mRNAs was observed in the absence of *de novo* transcription after B-cell infection (Jochum et al., 2012b). However, the extent to which the low level of virally transduced RNAs impact B-cell proliferation or survival in the background of increasing viral gene expression remains to be determined. It has been suggested that such virally-transduced RNAs are simply passengers in the virion tegument and likely play no role during infection (Dölken et al., 2010). Although, an unintended consequence of viral RNA delivery early in infection may be the activation of interferon responses as indicated by Zeidler and colleagues (Jochum et al., 2012b). The impact of type I interferon induction may be mitigated by the EBERs through antagonism of PKR (Nanbo et al., 2002). Therefore, the interplay between these virion-associated RNAs and events early in infection will be important to clarify in the future.

The prelatent expression of canonically lytic mRNAs during the first 24–48 hours following B-cell infection has been observed by several groups. Primary among these genes is the main viral lytic trans-activator, BZLF1. Expression of BZLF1 appears to be leaky and the lytic cycle is abortive in the first day after infection due to the lack of Z promoter methylation and lack of repressive chromatin structure on the early incoming EBV genome (Kalla et al., 2010). Therefore, a set of early lytic mRNAs including BZLF1, BRLF1, and BMRF1 are expressed and may play a role in B-cell activation. The Hammerschmidt group has identified a genetic role for early (B95-8 strain) Z expression in B-cell proliferation of naïve or memory, but not GC centroblasts (Kalla et al., 2010). However, the Takada group, using an Akata EBV strain lacking the BZLF1 gene did not observe a phenotype in B-cell immortalization (Katsumura et al., 2009). In experiments using humanized mice, Shannon Kenney’s group found that Z expression appeared to be critical for tumorigenesis (Ma et al., 2011). Other genes expressed during the pre-latent phase may also have an impact on B-cell

immortalization *in vitro* or *in vivo* including those regulating the adaptive and innate immune system. The role of the pre-latent phase is an emerging and important area of study in EBV biology. However, new findings in the latent phase have also recently questioned our understanding of the early phase of EBV infection.

The well-described switch from Wp to Cp usage was confirmed by the study of Nikitin, *et al.* (Nikitin et al., 2010). However, in their experiments, B cells were sorted based on B-cell population doublings rather than time post infection. While this approach removed the heterogeneity based on whether infected cells had proliferated, additional heterogeneity was observed with respect to activation of a growth-suppressive DNA damage response (DDR) due to B-cell hyper-proliferation (Nikitin et al., 2010). In approximately 50% of the infected, hyper-proliferating cells, activation of the DDR was observed. This cell population expressed a greater amount of the EBNA-LP protein relative to the EBNA3s as compared to later divisions or LCLs. Therefore, it is quite possible that within the hyper-proliferating pool of cells, a subset of cells express LCL-level EBNA3s and lower EBNA-LP and consequently do not activate the DDR. These cells are likely to be those successfully transformed by the virus. What drives the underlying heterogeneity in viral gene expression in these cells? Is it expression of factors regulating Cp such as RBP-J κ ? Or is there heterogeneity regarding expression of the factors regulating the splicing efficiency between the EBNA-LP specific W1' splice site versus the other EBNA3s W1 splice site? These questions will be important to address in the future as the answers will define how EBV overcomes the growth suppressive DDR in order to transform B cells. It is expected that such factors may be deregulated in EBV-associated cancers and will provide important clues to the early stages of B-cell lymphomagenesis.

Following the early expression of the EBNA genes, the viral latent membrane proteins are activated. Another surprise that was recently published is the long delay in LMP1 activation after B-cell infection. Early studies suggested that LMP1 was expressed at LCL levels from 48h post-infection (Alfieri, Birkenbach and Kieff, 1991). However, it is clear from recent studies that, while detectable at 2 days post infection, the level of LMP1 (and LMP2) is approximately 50–100 fold less than that in LCLs (Nikitin et al., 2010;Price et al., 2012). In fact, the primary data in the early studies also indicated this temporal pattern of expression (Alfieri, Birkenbach and Kieff, 1991;Allday, Crawford and Griffin, 1989). Therefore, early proliferation by EBV is not likely to require the LMPs. In fact, consistent with this hypothesis, a recent study suggests that EBV infection in humanized mice could promote tumorigenesis in the absence of LMP1 (S. Kenney, unpublished results, 13th Annual International EBV meeting). Perhaps the low level of LMP1 and consequent lack of NF κ B activity during the first two weeks post infection is important to attenuate the processing of antigens for MHC class I presentation (Rowe et al., 1995). This hypothesis is supported by the data in humanized and LMP1-transgenic mice indicating that LMP1-expressing B cells are highly sensitive to CTL activity whereas in the absence of T cells, EBV-positive B-cell tumors arise and are LMP1 positive (Ma et al., 2012;Zhang et al., 2012). It remains to be determined, therefore, whether LMP1 expression early in B-cell infection would be tolerated in an immune-competent setting either *in vitro* or *in vivo*.

The role of the viral latent genes including EBNA_s, LMP_s, and non-coding RNAs are being intensely investigated in B cells and other model systems. The ability to use large scale data sets such as that derived from the HapMap and ENCODE projects to define EBV gene regulation is a true bonus from the original goals of those efforts. Specifically, the use of LCLs as a model system in much of the large scale ChIP Seq and also RNA-Seq studies has opened the door for the high resolution characterization of underlying chromatin regulatory elements, mRNA isoform differences, and understanding how lytic versus latent gene expression patterns relate to host gene expression (Arvey et al., 2012). These and other recent RNA-Seq studies (Lin et al., 2010; Skalsky et al., 2012) highlight how these genome- and transcriptome-wide approaches will be tremendously useful in the future to tease apart EBV gene expression changes in all cell types at high resolution.

A final important note regarding studies of EBV gene regulation relates to the fact that infection of primary human B cells *in vitro* is not the only model system to understand EBV biology. EBV infects epithelial cells *in vitro* and *in vivo* leading to dysplasia and carcinoma. Other cell types have also been shown to be EBV-positive in certain tumor settings, such as NK/T lymphomas. Therefore, EBV gene expression in each of these settings will have both common and also cell- and tumor-specific modes of regulation. In the future, developing systems to characterize such differences in gene regulation will be critically important towards defining legitimate therapeutic targets and understanding the molecular pathophysiology of EBV-associated diseases.

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Figure 1. Latency III gene expression in a Lymphoblastoid Cell Line

Schematic diagram of latency proteins and RNAs expressed at steady-state in EBV-transformed LCLs. The nucleus is depicted by the inner, gray-shaded dotted circle. The latent membrane proteins (LMPs) are depicted in the plasma membrane as monomers, but likely exist as multimers and signal from multiple cellular membranes. The EBNA proteins are all shown as nuclear, but may have functions in the cytoplasm as well (e.g. EBNA-LP).

Figure 2. Schematic of the Epstein-Barr Virus genome

Letters on the inner edge of the circular genome denote BamHI digestion fragments. Cis-acting elements within the genome, such as the origin of plasmid replication (*oriP*), the two origins of lytic replication (*oriLyt*) and the terminal repeats formed when the linear genome is circularized are denoted in blue squares. Lytic genes that appear to be active early after infection in the pre-latent phase are shown in orange boxes. Coding exons for the latency genes are shown in green boxes. EBV latent mRNAs can be initiated from different promoters depending on latency type and time after infection: the W promoter (Wp), the C Promoter (Cp), the Q promoter (Qp, only in Latency I), and the LMP promoters are labeled. The unspliced pre-mRNAs driven from these promoters is shown as a dotted line. EBV

encoded noncoding RNAs, such as the miR-BHRF1 cluster, the miR-BART cluster, and the EBERs are shown as red triangles.

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Figure 3. EBV latency mRNAs are expressed as alternative isoforms and distinct transcripts

At the top is a schematized EBV linear genome showing the positions of latency gene exons in black boxes and BamHI fragment names listed below (not to scale). Also shown on the genome are the terminal repeats (open boxes), the C promoter (Cp, green boxes), the W promoter (Wp, yellow box), the Q promoter (Qp, blue box), the bi-directional Latent Membrane Protein promoter (LMPp, purple boxes), the LMP2A-specific promoter (purple box), and the canonical EBNA poly-adenylation sites (pA, arrow). The ORF-containing exon of the lytic gene BHRF1 is shown as an orange box. All coding exons are shown as full height boxes while non-coding exons are half-height. Early after infection latency transcripts are initiated primarily from the W promoter, as shown in yellow. The special instance of alternative splicing between the upstream Wp or Cp splice donor and the W1 or W1' exon that leads to inclusion of an ATG start codon and EBNA-LP protein production is shown (Inset). After EBNA2 and EBNA-LP production reach a significant level early after infection, the C promoter is activated and transcribes the rest of the EBNAs, as shown in

green. Later in infection, the LMP promoters are active and LMP1, LMP2A, and LMP2B and transcribed and spliced as shown in purple. In Latency I only the Q promoter is active to transcribe EBNA1.

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Figure 4. Timing of latent and lytic gene expression after infection by EBV

Relative expression levels of the RNA species are shown as shaded bars. Dark shading is indicative of the relative maximum amount of expression of the given RNA over the course of B cell growth transformation by EBV. Lytic genes expressed during the pre-latent phase are shown in orange, latency genes are shown in green, and noncoding RNAs are shown in red.

Table 1

EBV Latency Types and Gene Expression

Latency Type	Viral Genes Expressed	Associated Diseases and Cell States	Normal Infected Cell Type
Latency 0	EBERs, miR-BARTs	Burkitt's Lymphoma	Peripheral memory
Latency I	EBERs, miR-BARTs, EBNA1	Burkitt's Lymphoma	Dividing peripheral memory
Latency II	EBERs, miR-BARTs, EBNA1, LMP1, LMP2A/B	Hodgkin's Lymphoma, NPC ¹ , Gastric Carcinoma	Germinal center cells
Latency III	EBERs, miR-BHRF1s, miR-BARTs, EBNA1, EBNA-LP, EBNA2, EBNA3s, LMP1, LMP2A/B	AIDS-Associated DLBCL ² , PTLD ³ , LCL ⁴	Activated, naïve cells
Wp-Restricted Latency	EBERs, miR-BARTs, EBNA1, truncated-EBNA-LP, EBNA3s, BHRF1	Burkitt's Lymphoma	

¹Nasopharyngeal Carcinoma

²Diffuse Large B-Cell Lymphoma

³Post-Transplant Lymphoproliferative Disease

⁴Lymphoblastoid Cell Line