# EBNA2 and Its Coactivator EBNA-LP

**Bettina Kempkes and Paul D. Ling**

**Abstract** While all herpesviruses can switch between lytic and latent life cycle, which are both driven by specific transcription programs, a unique feature of latent EBV infection is the expression of several distinct and well-defined viral latent transcription programs called latency I, II, and III. Growth transformation of B-cells by EBV in vitro is based on the concerted action of Epstein-Barr virus nuclear antigens (EBNAs) and latent membrane proteins(LMPs). EBV growth-transformed B-cells express a viral transcriptional program, termed latency III, which is characterized by the coexpression of EBNA2 and EBNA-LP with EBNA1, EBNA3A, -3B, and -3C as well as LMP1, LMP2A, and LMP2B. The focus of this review will be to discuss the current understanding of how two of these proteins, EBNA2 and EBNA-LP, contribute to EBV-mediated B-cell growth transformation.

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## Abbreviation

| | |
|---|---|
| CBF1 | C-promoter binding factor |
| EBNA | Epstein-Barr virus nuclear antigen |
| EBNA-LP | Epstein-Barr virus leader protein |
| LMP | Latent membrane protein |
| LCL | Lymphoblastoid cell line |
| LCV | Lymphocryptovirus |
| TAD | Transactivation domain |
| PML-NB | Promyelocytic leukemia nuclear body |

## 1 EBNA2 and EBNA-LP Expression

In vivo, the latency III transcription program is expressed during a short-time window immediately after the infection of tonsillar B-cells of healthy individuals as well as in tonsillar EBV-infected B-cells in patients suffering from infectious mononucleosis (Kurth et al. 2003; Thorley-Lawson 2001). Expression of EBNA2 and EBNA-LP in EBV-related malignant diseases is confined to immunodeficient patients, who lack efficient T-cell immunosurveillance, a state that may be caused by immunosuppressive drug treatment after transplantation. Since the viral expression program in immunodeficient patients closely resembles the pattern seen in immortalized B-cells in vitro, EBNA2 and EBNA-LP are likely to drive the proliferation of these highly malignant cells. EBNA2 and EBNA-LP are not expressed in latently infected memory B-cells of healthy individuals or in EBV-associated malignancies of immunocompetent people as exemplified by patients suffering from Burkitt's lymphoma or Hodgkin's disease (for review see: Bornkamm and Hammerschmidt 2001; Macsween and Crawford 2003). Both EBNA2 and EBNA-LP are expressed from transcripts initiating from either of two promoters, Wp or Cp (Alfieri et al. 1991; Bodescot et al. 1987; Woisetschlaeger et al. 1990, 1991). While the EBNA2 protein is encoded by a single exon, transcription of EBNA-LP is more complex. For transcripts initiating from Wp, the first exon, W0, is joined to either the W1 exon, which does not contain an initiation codon for EBNA-LP, or via an alternate splice acceptor site to W1′ that does (Fig. 1) (Sample et al. 1986; Speck et al. 1986). W1 is then joined with the W2 exon and additional

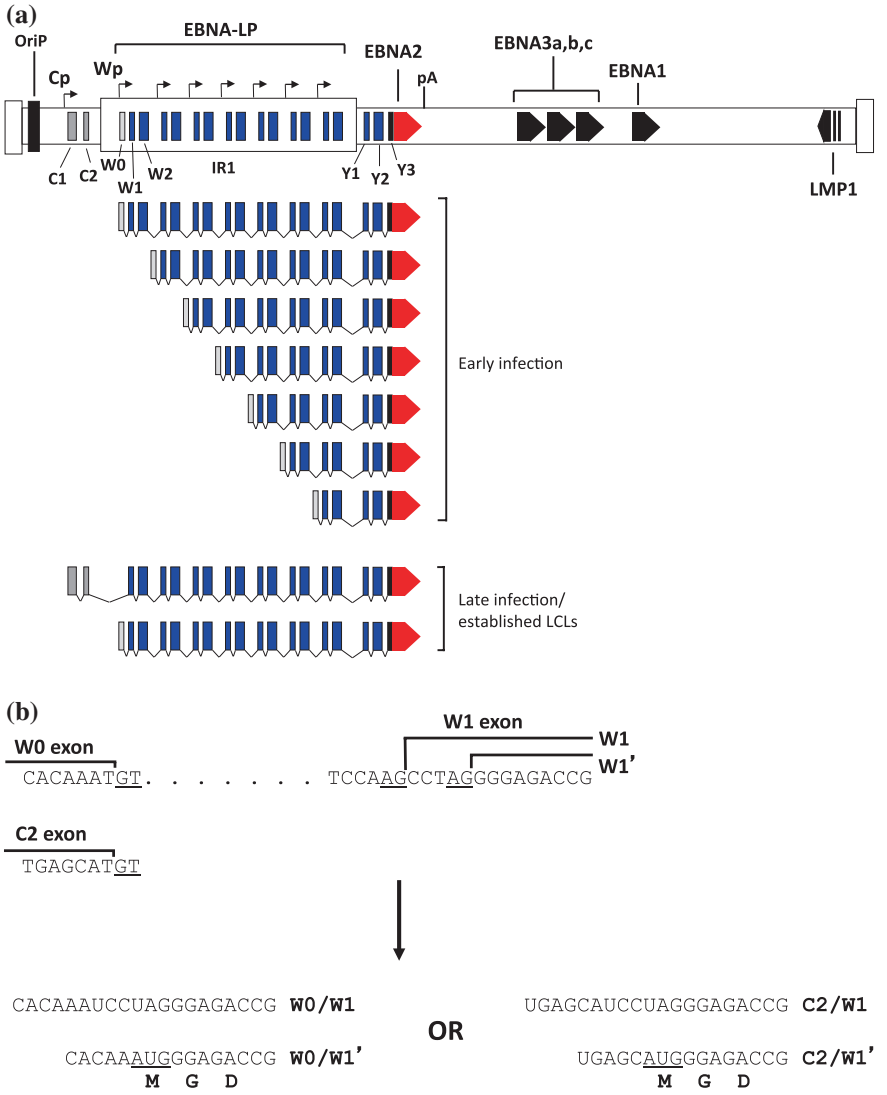


**Fig. 1** Exon organization of EBNA-LP and EBNA2 gene transcripts. **a** Transcription of the EBNA-LP gene initiates from either the W promoter (Wp) or C promoter (Cp). The different noncoding (C1, C2, W0) and coding exons for EBNA-LP (W1, W2, Y1, and Y2 in *blue*) and EBNA2 (Y3 in *red*) are indicated. During early stages of infection, transcription initiates from available Wp residing in each IR1 repeat, which results in the production of multiple EBNA-LP protein isoforms. During later stages of infection or in established LCLs, transcription from Cp is stimulated and there is a bias toward the OriP-proximal Wp. The level of Cp versus Wp-initiated transcription varies depending on several circumstances. The viral latent origin of replication (OriP), polyadenylation site (pA) for EBNA-LP/EBNA2 transcripts, and location of other EBNA genes and latent membrane proteins (LMP) are shown. **b** Alternative splicing generates an initiation codon for EBNA-LP. Splicing from W0 or C2 to a slightly shorter W1′ exon generates an AUG initiation codon for EBNA-LP, while splicing to the longer W1 exon does not. Splice donor and acceptor sites are *underlined*, and the resulting transcripts are shown below. The first three amino acid residues for EBNA-LP are shown below the transcripts that can translate EBNA-LP (adapted from Dr. S. Speck, with permission)

W1/W2 exons from each downstream repeat within major internal repeats (IR1). A complete transcript encoding EBNA-LP also joins unique exons Y1 and Y2 and the exon coding for EBNA2 (Bodescot et al. 1984; Sample et al. 1986; Speck et al. 1986). The final transcripts are bicistronic, encoding both EBNA-LP and EBNA2, or monocistronic for EBNA2 and containing a long 5′ untranslated leader sequence. Following initial infection of primary B-cells, transcription initiates first from Wp, but generally switches to Cp during later stages of infection or in established lymphoblastoid cell lines (LCLs), correlating with the detectable expression of EBNA2, which is also known to positively regulate Cp (see section on EBNA2 genetic and biochemical analysis). For transcripts initiating from Cp, the first two exons C1 and C2 can join either with W1 or W1′ to produce mRNAs encoding EBNA-LP or EBNA2 only, like those expressed from Wp (Rogers et al. 1990). EBNA-LP and EBNA2 are the first two latent proteins detectable following infection of primary B-cells (Alfieri et al. 1991; Allday et al. 1989).

The number of IR1 repeats determines the size of EBNA-LP, although during early stages of infection, multiple isoforms of EBNA-LP are detectable (Allan and Rowe 1989; Dillner et al. 1986; Finke et al. 1987). This phenomenon has not yet been explained, but one hypothesis is that because each IR1 repeat contains a Wp, there may be multiple sites for transcription initiation from different Wps. On the other hand, alternative splicing may occur across the IR1 repeats, generating transcripts encoding EBNA-LP proteins with varying repeat sequences. A methodical study of the number of IR1 repeats needed for optimal EBV-mediated B-cell immortalization has shown that at least two repeats are needed, but that 5 or more are associated with viruses that have optimal transforming activities (Tierney et al. 2011). This effect is apparently due to greater transcriptional activity from Wp rather than the size of EBNA-LP isoforms that are made. Sequencing of nonhuman primate lymphocryptoviruses (LCVs) confirms that these viruses encode the predicted EBNA2 and EBNA-LP proteins, and at least one study has shown that the complex transcriptional unit encoding EBNA-LP is well conserved (Peng et al. 2000a).

## 2 The EBNA2 Protein

EBNA2 forms granules (also called speckles) and localizes to the nucleoplasm, the chromatin fraction, and the nuclear matrix but excludes the nucleoli (Petti et al. 1990). Most studies on EBNA2 used the laboratory EBV strain B95-8 which encodes a 487 amino acid EBNA2 protein[1] (Baer et al. 1984; Skare et al. 1982) (Fig. 2). EBNA2, isolated from total cellular extracts, has an apparent molecular weight of approximately 84 kDa as judged by SDS-PAGE. Phosphorylation of

[1]In this review, we will refer to the primary structure of EBNA2 using the Swiss-Prot data entry of EBV laboratory strain 95-8:P12978.

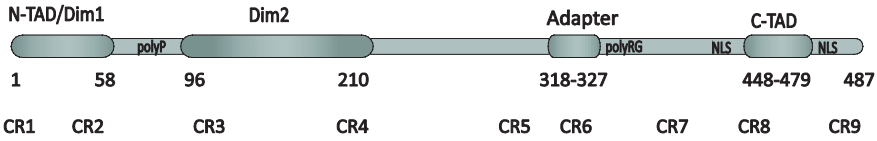


**Fig. 2** Schematic illustration showing the primary structure of EBNA2. Characteristic features of EBNA2 are a poly-proline (polyP) and a poly-arginine-glycine (RG) stretch and conserved regions (CR1–9), which have been defined by comparison of EBV strain types 1 and 2, baboon, and rhesus macaque lymphocryptoviruses. Regions of EBNA2, which mediate self-association, are labeled Dim1 and Dim2. CR5 and CR6 lie within the region, which mediates promoter targeting (adapter) by association with CBF1. Two transactivation domains (TAD) map to the amino (N-TAD) and carboxyl termini C-TAD, while the two nuclear localization signals (NLS) reside at the carboxyl terminus. Amino acid numbering refers to the B95-8 primary structure of EBNA2

EBNA2 may partially account for the discrepancy between the expected molecular weight and the electrophoretic mobility of the protein (Grasser et al. 1991). Notably, EBNA2 proteins from distinct nuclear compartments exhibit differential phosphorylation patterns, and these phosphorylation patterns vary during the cell cycle (Petti et al. 1990; Yue et al. 2004). A further modification of EBNA2 is the arginine methylation of the RG repeat (Barth et al. 2003), which is considered to be a modulator of EBNA2 activity. The RG repeat binds to poly G and histone H1 in vitro (Tong et al. 1994) and also serves as a substrate for arginine methyltransferase 5 (Liu et al. 2013). A poly-proline stretch (poly-P) separates two N-terminal dimerization domains, Dim1 (1–58) and Dim2 (96–210), which both mediate homotypic adhesion. In addition, Dim1 serves as an N-terminal transactivation domain (N-TAD) that may interact with EBNA-LP (Gordadze et al. 2004; Harada et al. 2001; Peng et al. 2004b). A second transactivation domain (C-TAD) resides in the C-terminus of EBNA2 (448–479) (Cohen and Kieff 1991). The core fragment of the transactivation domain (AA: 453–466) can be replaced by an acidic fragment of the herpesviral VP16 transactivation domain indicating that EBNA2 and VP16 share functional similarities (Cohen 1992; Cohen and Kieff 1991). In fact, both transactivation domains bind to TFIIB and TAF40, components of the transcription initiation complex, and TFIIH, a factor involved in promoter clearance. In addition, both bind to RPA70, the replication protein A (Tong et al. 1995a, b). In contrast to VP16, EBNA2 does not bind to TBP (Tong et al. 1995c). Both transactivation domains also recruit histone acetyltransferase activity by interacting with CBP, p300, and PCAF (Wang et al. 2000). The structure of C-TAD in complex with CBP/p300 or the TFB1/p62 subunit of the TFIIH complex has recently been solved by nuclear magnetic resonance (NMR) spectroscopy. C-TAD is an intrinsically unstructured region which folds into a 9-residue alpha helix upon complexation (Chabot et al. 2014). The structure of the entire EBNA2 protein has not been solved. The high proline content, the poly-P regions, and the RG repeats most likely prevent globular folding of the entire protein in the absence of cognate binding partners.

Mainly based on the sequence diversity of the EBNA2 alleles, EBV can be categorized into two individual strains called type 1 and 2 (or type A and B respectively). Type 1 and 2 EBV strains differ in their capacity to immortalize primary B-cells (Adldinger et al. 1985; Dambaugh et al. 1984) a feature that is predominantly determined by sequence variation in the C-terminus of EBNA2 (Tzellos et al. 2014; Tzellos and Farrell 2012). Lymphocryptoviruses have also been isolated from baboon and macaque. While the EBNA2 orthologs of baboon and macaque LCV show significant sequence similarity with EBNA2 protein encoded by the B95-8 strain (Cho et al. 1999; Peng et al. 2000a), sequence similarity with the positional EBNA2 homologue of marmoset LCV is below 20 % (reviewed in Wang 2013).

## 3 The EBNA-LP Protein

The EBNA-LP protein is composed of several 22 and 44 amino acid segments encoded by the W1 and W2 exons, which are joined with unique 11 and 34 amino acid segments at the carboxyl-terminal end that are encoded by the Y1 and Y2 exons. Once expressed, EBNA-LP localizes predominantly in the nucleus, which is facilitated by a bipartite nuclear localization signal (Peng et al. 2000b). Nuclear staining is diffuse early on after infection but then localizes in punctate structures known as promyelocytic leukemia nuclear bodies (PML NBs) (Bandobashi et al. 2001; Dillner et al. 1986; Ling et al. 2005; Nitsche et al. 1997; Szekely et al. 1996; Wang et al. 1987b). Transient expression of EBNA-LP from eukaryotic expression vectors tends to mimic the diffuse nuclear localization observed at early points following virus infection. Consistent with its association with PML NBs, biochemical fractionation studies on LCLs indicate that EBNA-LP is associated with the nuclear matrix (Petti et al. 1990; Yokoyama et al. 2001a). PML NBs are organized by the PML protein, which contains SUMO interaction motifs (SIMS) and is also posttranslationally modified by sumoylation. Many proteins associated with PML NBs also are SUMO-modified and have SIM domains, which presumably help mediate PML NB assembly or localization. Curiously, EBNA-LP is devoid of any obvious SIM motifs or lysine residues that are needed for SUMO conjugation, but as discussed later, localization of EBNA-LP to PML NBs may be mediated by a cellular factor. Other studies have also reported that a proportion of EBNA-LP can localize in the cytoplasm and this can be influenced by the number of W repeats (Garibal et al. 2007). Consistent with these studies, Ling et al. (2009) used heterokaryon assays to evaluate whether EBNA-LP shuttles between cytoplasmic and nuclear compartments. Only smaller isoforms with 2W repeats were found to shuttle in these assays, while larger isoforms did not, leading to speculations that the observed shuttling of the smaller isoforms was due to diffusion rather than through an active process. In contrast, EBNA-LP proteins with only a single W repeat (W1 and W2 exons) localized exclusively in the cytoplasm. Whether shuttling or cytoplasmic localization contributes significantly to EBNA-LP-mediated coactivation remains unknown.

EBNA-LP is a phosphoprotein (Petti et al. 1990). Phosphorylation appears to occur predominantly on serine residues, and while this can be detected throughout the cell cycle, it is hyperphosphorylated during G2/M and hypophosphorylated during G1/S (Kitay and Rowe 1996). There are three serine residues that are well conserved among human and nonhuman primate lymphocryptovirus (LCV) EBNA-LP homologs, and one of them is within a cyclin-dependent p34cdc2 site (Peng et al. 2000a, b). This serine is located within the 44 amino acid segment encoded by W2 and is critical for EBNA-LP-mediated coactivation (McCann et al. 2001; Peng et al. 2000b; Yokoyama et al. 2001b).

## 4 EBNA2-Associated Cellular Proteins, Which Mediate Chromatin Targeting

Like all transcription factors, EBNA2 carries a transactivation domain and a region that mediates DNA contact. Since EBNA2 cannot bind to DNA directly, it uses adaptor proteins to bind to cis-regulatory regions of its target genes and indirectly confers sequence-specific DNA contact.

So far, the best studied cellular DNA adaptor protein of EBNA2 is the DNA binding protein CBF1, which was first identified as a downstream effector molecule of EBNA2 in the context of viral promoter activation. CBF1 is a ubiquitously expressed protein and belongs to the group of CSL proteins (CBF1 for C-promoter binding protein, Su(H) in *Drosophila melanogaster*, Lag1 in *Caenorhabditis elegans*) also known as recombination binding protein-J (RBPJ, RBP, or RBPJκ). The minimal domain of EBNA2 that mediates CBF1 binding has been mapped to the EBNA2 fragment aa 318–327 (Ling and Hayward 1995). CBF1 is a sequence-specific DNA binding protein, which in the absence of EBNA2 recruits a corepressor complex to the promoter or enhancer of target genes. Constituents of this corepressor complex are SMRT/N-CoR, CIR, SKIP, Sin3A, SAP30, and HDAC1, which either directly or indirectly interfere with histone acetylation of target gene chromatin, thereby repressing transcription (reviewed in Lai 2002). Binding of EBNA2 relieves this repression by competition with corepressor binding as well as the recruitment of coactivators by virtue of its intrinsic transactivation domains (Hsieh and Hayward 1995).

Since CBF1 is also an important downstream element of the cellular Notch signal transduction pathway, the discovery of CBF1 in the context of the viral protein EBNA2 has provoked an intense search for potential parallels of Notch and EBNA2 signaling (Hayward et al. 2006). The crystal structure of the Notch/MAM/CBF1/DNA complex has been solved (Kovall 2007; Nam et al. 2006; Wilson and Kovall 2006). Although the structure of the EB viral proteins associated with CBF1 has not been published yet, there is a compelling biochemical and genetic evidence that EBNA2 and Notch contact the same hydrophobic pocket within the CSL protein, but in addition appear to bind to distinct amino acids in the vicinity of this hydrophobic pocket (Fuchs et al. 2001; Kovall and

Hendrickson 2004). Thus, EBNA2 or Notch binding to CBF-1 is mutually exclusive (Hsieh and Hayward 1995).

The second but less well characterized EBNA2 DNA adaptor is the PU.1 protein. Several laboratories have shown that PU.1 promoter binding is critical for activation of the viral LMP1 promoter (Johannsen et al. 1995; Laux et al. 1994a, b). However, complex formation of EBNA2 with endogenous PU.1 has only been reported once (Yue et al. 2004). The potential contact points of the interaction partners have not been mapped, and the interaction has been demonstrated in vitro using purified proteins.

## 5 The Genetic and Biochemical Analysis of Viral EBNA2-Responsive Promoter Elements Has Provided Major Insights into the Molecular Mechanisms of EBNA2 Action

Most of our knowledge on EBNA2 functions is based on the detailed genetic analysis of EBNA2-responsive cis-active elements within viral promoters, and the subsequent use of these insights was used to characterize the proteins that are involved biochemically.

In EBV-infected B-cells, the viral C promoter (Cp) as well as the promoters of the viral LMP1 (LMP1p), LMP2A (LMP2Ap), and LMP2B (LMP2Bp) genes is strongly activated by EBNA2 (Abbot et al. 1990; Fahraeus et al. 1990; Ghosh and Kieff 1990; Jin and Speck 1992; Sung et al. 1991; Wang et al. 1990). By deletion analysis of promoter reporter constructs or gel retardation assays, EBNA2-responsive elements (EBNA2-RE) have been identified. All these promoters carry at least one CBF1 binding site (Allday et al. 1993; Henkel et al. 1994; Laux et al. 1994b; Ling et al. 1994; Meitinger et al. 1994; Waltzer et al. 1994; Zimber-Strobl et al. 1991, 1993, 1994). The high-affinity CBF1 binding sites within the EBNA2-REs of Cp and LMP2Ap were used to identify CBF1 by four independent groups in 1994 (Grossman et al. 1994; Henkel et al. 1994; Waltzer et al. 1994; Zimber-Strobl et al. 1994).

All these CBF1 binding motifs are flanked by additional distinct transcription factor binding sites, which contribute to promoter activation but might not bind to EBNA2 directly.

Within the EBNA2-RE of Cp, a single CBF1 binding site is flanked by a binding site for the cyclic AMP-responsive AUF1/hnRNP D protein, also called CBF2. The binding sites for both factors are evolutionary conserved as shown by sequence comparison of EBV such as lymphocryptoviruses found in baboon and rhesus macaques (Fuentes-Panana and Ling 1998; Fuentes-Panana et al. 1999, 2000). Regions distal to the EBNA2-RE, both upstream and downstream, which bind additional cellular factors such as SP1, Egr-1, NF-Y, or the viral EBNA1/oriP complex modulate the basal activity of the C promoter in an EBNA2-independent fashion (Borestrom et al. 2003; Puglielli et al. 1996).

Analysis of LMP1p has revealed a complex pattern of transcription factor binding sites. PU.1/Spi-1 and CBF1 binding sites are both critical for EBNA2 transactivation (Johannsen et al. 1995; Laux et al. 1994a). In addition, ATF-2/c-Jun heterodimers enhance EBNA2 effects (Sjoblom et al. 1998). Further transcription factor binding sites such as an interferon-stimulated response element, a Sp1 binding site, and a yet undefined POU-Box protein contribute to LMP1p activity (Sjoblom et al. 1995a, b).

## 6 EBNA2 Binds to Cellular Promoter and Enhancer Regions and Can Promote the Formation of Chromatin Loops Within the Cellular Genome

In order to study the impact of EBNA2 on cellular target gene expression, genome-wide array-based screens or candidate approaches using either EBV-infected B-cells or EBNA2-expressing B-cell lines (Burgstahler et al. 1995; Calender et al. 1990; Johansen et al. 2003; Knutson 1990; Lucchesi et al. 2008; Maier et al. 2005; 2006; Mohan et al. 2006; Pegman et al. 2006; Sakai et al. 1998; Wang et al. 1987a; Zhao et al. 2006). These studies identified CD23, CD21, CCR7 (BLR2/EBI1), Hes-1, BATF, bfl-1, FcRH5, ABHD6, CCL3, CCL4, CDK5R1, DNASE1L3, MFN1, RAPGEF2, RHOH, SAMSN1, SLAMF1, and CXCR7 as EBNA2 target genes in EBV-negative B-cells. In EBV-infected B-cells, the proto-oncogene *MYC*, the p55α subunit of PIK3R1, CD21, CD23, AML-2, and FcRH5 were defined as a direct target gene, since their RNA can be induced by EBNA2 in the absence of de novo protein synthesis. In contrast, induction of cyclin D, cdk4 or tumor necrosis factor alpha (TNF-α), granulocyte colony-stimulating factor (G-CSF), and lymphotoxin (LT) requires additional cellular or viral functions (Kaiser et al. 1999; Mohan et al. 2006; Spender et al. 2001, 2002). Based on shRNA experiments, a small panel of selected EBNA2 target genes (CXCR7, Runx3 and p55α) has been identified that promote viability and proliferation of EBV-transformed B-cells (Lucchesi et al. 2008; Spender et al. 2005, 2006). For the majority of EBNA2 target genes, functional assays have not been performed. High-level expression of the EBNA2 target gene *MYC* in EBV-infected B-cells depleted for functional EBNA2 can promote cellular proliferation but leads to a switch of the viral and cellular transcription program from latency III to latency I (Pajic et al. 2000; Polack et al. 1996). Thus, it remains to be determined which of the EBNA2 target genes reflect the activated blast-like phenotype (latency III) but may not contribute to the success of immortalization process in vitro or the establishment of latency in vivo.

Since transcription factor binding sites in genomic regions can be mapped by chromatin immunoprecipitation combined with next-generation sequencing techniques, CBF1, EBNA2, and EBNA-LP binding to the cellular chromatin have been studied in B-cells (McClellan et al. 2012, 2013; Portal et al. 2013; Zhao et al. 2011). These studies mapped approximately 10 000 CBF1 binding site (Zhao et al.

2011) and 5000–20,000 EBNA2 binding sites (McClellan et al. 2013; Portal et al. 2013; Zhao et al. 2011). Obviously, the cellular background and alternative bioinformatics peak calling strategies can influence the results to a certain extent. 72 % of EBNA2 and CBF1 binding sites overlapped with each other confirming that CBF1 is the major DNA adaptor for EBNA2 (Zhao et al. 2011). All these studies benefitted from the comprehensive data sets provided by the ENCyclopedia Of DNA Elements (ENCODE) project on functional DNA elements obtained by the analysis of EBV-immortalized B-cells or primary B-cells. The comparative analysis of CBF1 and EBNA2 binding sites with regions annotated by the ENCODE project revealed that the majority of EBNA2/CBF1 binding sites were also enriched for B-cell transcription factors including ETS, RUNX, EBF, PU.1, and NκFB. Frequently, these cooccupied regions carried a characteristic enhancer chromatin signature that was also established in primary B-cells prior to infection indicating that EBNA2 is recruited to B-cell-specific open chromatin regions (Zhao et al. 2011).

A physical and functional link between an enhancer bound by EBNA2 to the promoter of the *MYC* target gene was recently established by chromatin conformation capture technologies. A chromatin loop links an EBNA2-bound enhancer more than 400 kb upstream of the *MYC* transcription start site in the presence of EBNA2 (Zhao et al. 2011). Novel technologies that integrate the analysis of the nuclear architecture with biochemical binding studies will be required for the correct assignment of cellular transcription initiation sites of target genes to remote EBNA2 binding enhancers.

Most recently binding sites of the coactivator of EBNA2, the EBNA-LP protein, in the chromatin of EBV-infected B-cells have been identified and mapped in a genome-wide ChIP-seq approach (Portal et al. 2013). These studies identified genomic binding sites shared by both viral factors, EBNA2 and EBNA-LP, but also identified sites bound by either factor, EBNA2 or EBNA-LP. According to ENCODE data sets, these binding sites colocalize to clusters of B-cell-specific transcription factor binding sites and exhibit chromatin signatures which characterize promoter and enhancer regions in the cellular genome of EBV-infected B-cells. In contrast to EBNA2, EBNA-LP binding sites preferentially occupied promoter rather than enhancer regions. Since EBNA-LP is not known to bind to DNA, the molecular mechanism by which EBNA-LP is targeted to DNA still needs to be explored.

## 7 Target Genes Which Are Down-Regulated in the Presence of EBNA2

EBNA2 not only induces but also actively down-regulates expression of target genes. Notably, EBNA2 interferes with the B-cell and germinal center phenotype by down-regulating IgM- or BCR-associated signal transduction moieties such as CD79A and CD79B, BCL6, TCL1A, and AID (Boccellato et al. 2007; Maier

et al. 2005, 2006; Tobollik et al. 2006). Down regulation of IgM by EBNA2 appears to be at least partially independent of CBF1 signaling (Maier et al. 2005). In the context of Burkitt's lymphoma cell lines, which carry a chromosomal translocation that juxtaposes the IgM and the *MYC* gene locus on chromosome 8, repression of IgM coincides with *MYC* repression and a potent growth-inhibitory activity of EBNA2 (Jochner et al. 1996; Kempkes et al. 1996). This function of EBNA2 is mimicked by activated Notch (Strobl et al. 2000), which is somewhat surprising given that this EBNA2 function appeared to be partially CBF1-dependent.

Apparently, EBNA2- and *MYC*-driven proliferation programs are incompatible with each other (Pajic et al. 2001). In fact, EBNA2 downregulation is positively selected in Burkitt's lymphoma cells in vivo for at least two reasons: Firstly, it down-regulates a translocated *MYC* gene (Jochner et al. 1996), and secondly, it drives expression of LMP1 which promotes antigen presentation and T-helper-specific chemokines thus rendering the cells strongly immunogenic (Kelly et al. 2002).

## 8 EBNA-LP, the Coactivator of EBNA2

The most widely confirmed EBNA-LP function has been its ability to cooperate with EBNA2 and will be the focus in this review. However, EBNA-LP has been reported to mediate apoptosis and other cellular pathways through associations with a variety of cellular proteins. We refer the reader to another previous comprehensive review for details about these potential functions (Ling, P.D. EBNA-LP function. Epstein-Barr virus Latency 2010).

One of the first clues that EBNA-LP might have a role in gene regulation came from a study showing that expression of EBNA2 together with EBNA-LP in primary B-cells induced the expression of the cellular cyclin D2 gene (Sinclair et al. 1994). Subsequent studies by other investigators confirmed that EBNA-LP was a strong coactivator of EBNA2 (Harada and Kieff 1997; Nitsche et al. 1997; Peng et al. 2000a, b; Yokoyama et al. 2001a). These studies utilized two types of assays: (1) transient reporter gene assays with EBNA2-responsive reporter plasmids and (2) induction of endogenous EBNA2-responsive genes in Burkitt's lymphoma cell lines. Further validation of EBNA-LP coactivator function came from experiments showing that EBNA-LP from the rhesus LCV also coactivated EBNA2, demonstrating that this function was evolutionarily conserved (Peng et al. 2000a).

A major question is whether or not EBNA-LP is a global transcriptional coactivator. While independent studies from several groups have confirmed that EBNA-LP coactivates EBNA2-responsive genes LMP-1, LMP2B, and the Cp, other known EBNA2 target genes such as LMP2A, CD21, CD23, and Hes-1 appear not to be affected by EBNA-LP (Peng et al. 2005). There have been some reports that EBNA-LP can coactivate GAL4-EBNA2 fusion proteins or GAL4 acidic activation domain fusions in transient mammalian 2-hybrid systems

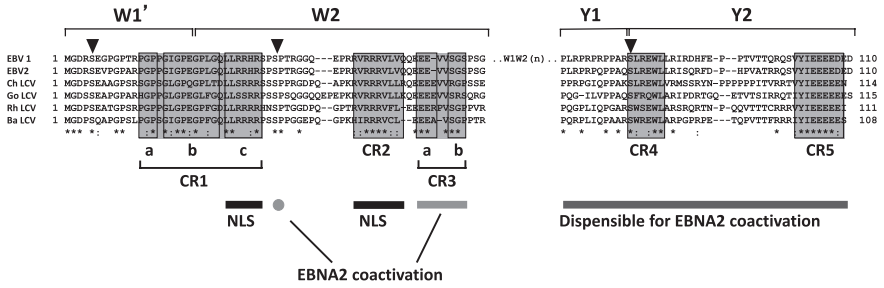


**Fig. 3** Sequence comparison of LCV EBNA-LP proteins. Conserved or similar amino acid residues are indicated by *asterisks* or *dotted lines*, respectively. The corresponding exons encoding EBNA-LP sequences and regions of conservation between the EBV and nonhuman primate LCVs are shown. Below, the *black* and *gray bars* indicate residues conferring nuclear localization (NLS), EBNA2 coactivation, and coactivation dispensable domains. For simplicity, amino acid numbering is for an EBNA-LP protein with only a single W1/W2 repeat. However, as indicated, the residues encoded by the W1/W2 repeats are present in multiple copies in wild-type EBNA-LP proteins

(Han et al. 2002; Harada and Kieff 1997), but subsequent studies have found that EBNA-LP acts as a repressor in some of these assays (Peng et al. 2004a). EBNA-LP was also unable to coactivate GAL4 fusions with other transcriptional activating proteins (Inman and Farrell 1995). A single report suggested that EBNA2 stimulation of endogenous Hes-1 in EBV-negative Burkitt lymphoma was enhanced twofold by EBNA-LP (Portal et al. 2011). Unlike a previous study, however, where the effects of EBNA2 and EBNA-LP were determined from multiple experiments in the same cells (Peng et al. 2005), the latter study was done by comparing cell lines constitutively expressing EBNA2 and EBNA-LP to cell lines expressing only one of these proteins and did not take into account clonal variation in these lines (Portal et al. 2011). Collectively, the available evidence suggests that EBNA-LP only coactivates EBNA2 on a subset of EBNA2-responsive genes.

The mechanism(s) by which EBNA-LP coactivates EBNA2 have not been fully elucidated. However, a methodical approach to understanding this process was to identify important functional domains within EBNA-LP that mediated this function. One helpful tool was the availability of known EBNA-LP protein sequences from human and nonhuman primate LCVs (McCann et al. 2001; Peng et al. 2000a, b). Comparison of these proteins revealed the presence of 5 conserved regions (CR1–5) (Fig. 3; McCann et al. 2001; Peng et al. 2000b). Deletion or alanine-scanning mutations introduced into these conserved regions showed that CR3 and a single serine residue within the W2 repeat were important for EBNA2 coactivation (McCann et al. 2001; Peng et al. 2000b). The mutational analyses also identified a nuclear localization signal (CR1 and CR2) (Peng et al. 2000b). The Y1- and Y2-encoded segments of EBNA-LP are not required for coactivation function, although it has been hypothesized that they might impose both negative and positive regulatory effects under certain conditions (Peng et al. 2007).

Further insight into the pathways utilized by EBNA-LP to mediate EBNA2 coactivation has come from the identification of associated cellular cofactors. At the current time, only a single cofactor has been identified that interacts with an EBNA-LP domain required for transcriptional coactivation. Ling et al. (2005) first identified a strong association between EBNA-LP CR3 and the cellular protein Sp100A, which is predominantly localized in PML NBs. EBNA-LP can displace Sp100A from PML NBs, and this correlates with its ability to coactivate EBNA2, while the noncoactivating EBNA-LP with a mutation in CR3 neither interacts with Sp100A nor displaces it from PML NBs. Furthermore, expression of an amino-terminal deletion mutant of Sp100A, which prevents it from entering PML NBs (i.e., it localizes as if EBNA-LPs were coexpressed), was sufficient to coactivate EBNA2 in the absence of EBNA-LP. Additionally, Sp100A is known to associate with the transcriptional repressor heterochromatin protein 1 (HP1). Deletion of the HP1 interaction domain ablates Sp100A coactivation function. The data are consistent with a model in which EBNA-LP-Sp100A interactions facilitate coactivation by a mechanism involving chromatin modification.

A second potential mechanism by which EBNA-LP might work to cooperate with EBNA2 is through direct interactions with EBNA2, which have been detected in vitro using small fragments of EBNA2 and EBNA-LP (Peng et al. 2004b). However, several investigators have been unable to detect EBNA2-EBNA-LP interactions in mammalian or yeast two-hybrid systems or using traditional coimmunoprecipitation assays (Kashuba et al. 2003; Peng et al. 2004a, 2005). In addition, it is unclear what EBNA-LP-associated cofactors or intrinsic property of EBNA-LP might be providing coactivation function in this context.

A third mechanism for EBNA-LP coactivation might involve displacement of NCoR-repressive complexes from enhancers (Portal et al. 2011). In addition, Chip-seq identified several thousand sites in which EBNA-LP was associated although interestingly, less than a third of these were also associated with EBNA2, consistent with the idea that EBNA-LP might not be a global EBNA2 coactivator (Portal et al. 2013). A limitation to these studies, as discussed previously, is that the DNA and transcription factor associations measured were done in an cellular environment where it is unclear whether EBNA-LP is functioning as an active coactivator or if this function is being masked because of pleotropic effects on transcription from other viral (e.g., LMP1) or cellular factors.

## 9 EBNA3A, B and C Proteins Can Counteract or Enhance EBNA2 Activity

The EBNA3 proteins, EBNA3A, 3B, and 3C, are all coexpressed with EBNA2 and EBNA-LP in latency III. The EBNA3 proteins score as transcriptional repressors when tethered to DNA by heterologous DNA adaptors like the GAL4 DNA binding domain (Bain et al. 1996; Cludts and Farrell 1998). All EBNA3 proteins

bind to CBF1 and can interfere with EBNA2-mediated transactivation of the CBF1-dependent Cp, LMP2A, and LMP1 promoters in transient reporter assays (Le Roux et al. 1994). Further, it was reported that EBNA3C can cooperate with EBNA2 to activate PU.1-dependent transcription from LMP1p (Marshall and Sample 1995; Zhao and Sample 2000). However, expression of EBNA3C in EBV-positive Raji cells does not impair Cp-driven EBNA2 expression but significantly induces endogenous LMP1 levels indicating that the viral genome embedded in the context of the cellular chromatin reacts differently (Allday and Farrell 1994; Jimenez-Ramirez et al. 2006). The retrospect analysis of EBNA2 and EBNA3 target genes published by different laboratories showed a significant overlap indicating that EBNA2 and EBNA3 might indeed regulate similar target gene populations (Hertle et al. 2009; McClellan et al. 2013). In addition, EBNA2, EBNA3, and CBF1 binding sites in the cellular genome show a significant overlap (McClellan et al. 2013), and EBNA2 and EBNA3A can directly compete for CBF1 binding as shown for the CXCL9 and CXCL10 gene locus (Harth-Hertle et al. 2013).

An EBV–EBV interactome based on binary interactions identified by yeast two-hybrid high-throughput screening has been published (Calderwood et al. 2007). The study confirmed binding of EBNA2 to EBNA-LP and described EBNA2 binding of EBNA3A, BZLF1, the inducer of the lytic viral life cycle, and BDLF2, a tegument protein. To which extent the biological activity of EBNA2 is modulated by the viral context remains to be analyzed further using specific viral mutants during all stages of the growth transformation process and the lytic viral life cycle.

## 10 EBNA2-Associated Cellular Proteins, Which Highlight Additional Functions of EBNA2

Chromatin immunoprecipitation assays using EBNA2 and histones H3- and H4-specific antibodies proved that the LMP1p- and Cp-associated chromatin is differentially acetylated in the presence of EBNA2 (Alazard et al. 2003). A further histone acetylation-independent mechanism of Cp activation by EBNA2 is dependent on cdk9 activity, which phosphorylates Ser-5 of the C-terminal tail of polymerase II (Bark-Jones et al. 2006). In addition, EBNA2 forms a complex with a novel cellular coactivator, p100, which can bind to the general transcription factor TFIIE and thereby bridges STAT6/RNA polymerase II interactions (Tong et al. 1995b; Yang et al. 2002) (Table 1).

Apart from recruiting HAT activity and general transcription factors, phosphorylated EBNA2 also interacts with hSNF5/Ini, a component of the hSWI/SNF chromatin remodeling complex and potential tumor suppressor gene. EBNA2 recruits this protein to target promoters. This interaction is conferred by less conserved regions of EBNA2 and depends on the integrity of IPP285 and DQQ111 as well as phosphorylation of SS469 adjacent to the transactivation domain of EBNA2 (Kwiatkowski et al. 2004; Wu et al. 1996, 2000). The EBNA2 interaction

**Table 1** EBNA2-associated cellular proteins associated cellular proteins

| Protein | References |
|---|---|
| CBF1/RBP-J/ RBP-Jκ | Grossman et al. (1994), Henkel et al. (1994), Waltzer et al. (1994), Zimber-Strobl et al. (1994) |
| PU.1/Spi-1 | Yue et al. (2004) |
| TFIIB | Tong et al. (1995c) |
| TAF40 | Tong et al. (1995c) |
| CBP/p300 | Wang et al. (2000) |
| PCAF/GCN5 | Wang et al. (2000) |
| P100 | Tong et al. (1995b) |
| Nur77 | Lee et al. (2002) |
| SKIP | Zhou et al. (2000) |
| DP103 | Grundhoff et al. (1999) |
| SMN | Barth et al. (2003) |
| hSNF5/Ini1 | Wu et al. (1996) |
| BS69 | Ansieau and Leutz (2002) |
| p34cdc2 | Yue et al. (2004) |
| ATF-2/c-Jun | Sjoblom et al. (1998) |
| FOE | Kwiatkowski et al. (2004) |
| Nucleophosmin | Liu et al. (2012) |

with hSNF5/Ini1 could potentially serve a second function. It might interfere with the growth-suppressing activities of hSNF5/Ini1 in heterotrimeric complexes with GADD34 and PP-1 (Wu et al. 2002). A potential chromatin association of EBNA2 has been further suggested by the interaction of FOE (friend of EBNA), the human homologue of the *Drosophila* homologue of wap1, with EBNA2 (Kwiatkowski et al. 2004).

The carboxyl terminus, CR7 and CR8, can be further targeted by the Mynd domain protein and corepressor BS69, a cellular protein which was first described as one that binds to the adenovirus E1A protein (Hateboer et al. 1995). A potential function of EBNA2 in RNA processing has been suggested by the identification of the DEAD box protein DP103 (Gemin3/DDX20) which binds to (AA:121–213) (Grundhoff et al. 1999). The RG repeat region of EBNA2 is methylated at arginine residues and recruits the survival motor neuron (SMN) (Barth et al. 2003). SMN, a protein involved in RNA splicing, directly interacts with DP103 and can enhance LMP1 promoter activation by EBNA2 (Voss et al. 2001). The Ski-interacting protein (SKIP) is a multifunctional protein, which is a component of the spliceosome, a coactivator or corepressor of transcription and a pRB and E7 binding protein (reviewed in (Folk et al. 2004). SKIP binds to CBF1 and either facilitates binding of the SMRT, CIR, Sin3A, and HDAC2 corepressor complex or potentiates binding of EBNA2 to CBF1 by interacting with CR5 (Zhou et al. 2000). The chaperone nucleophosmin directly binds to EBNA2 and supports complex formation with CBF1 and promoter recruitment (Liu et al. 2012). Recently, a novel anti-apoptotic function of EBNA2, based on the finding that EBNA2 binds to Nur77,

has been described (TR3, NGFI-B) (Lee et al. 2002). Nur77 is an orphan member of the nuclear hormone receptor superfamily and a bifunctional molecule. Nur77, a nuclear protein, either acts as a transcription factor or can be translocated from the nucleus into the cytoplasm and trigger cytochrome c release in response to apoptotic stimuli (Li et al. 2000; Philips et al. 1997). EBNA2 can protect cells from apoptotic cell death by retaining Nur77 in the nucleus upon apoptotic stimuli (Lee et al. 2002). Whether EBNA2 also modulates functions of Nur77 related to its role as a transcription factor has not been analyzed to date.

## 11 Final Conclusions and Open Questions

In summary, EBNA2 acts as a key determinant of the activated phenotype of EBV-infected B-cells. The systematic and in-depth analysis of EBNA2 viral target genes has provided important clues to the molecular mechanism by which EBNA2 exerts its function as a transcriptional activator and has shown that CBF1 is the central effector of EBNA2 function. Within the cellular genome, EBNA2 preferentially binds to enhancers of cellular target genes which also preferentially recruit multiple B-cell-specific transcription factors. Since promoter and enhancers can reside in distant parts of a chromosome, the assignment of functional pairs of promoters and enhancers that are activated by EBNA2 and EBNA-LP will require intensive further investigations that combine studies on transcription factor binding, chromatin state, and nuclear architecture. Activation of *MYC* by EBNA2 is the major rate-limiting step for initiation and maintenance of the proliferation of EBV-infected B-cell cultures. The potential contribution of further cellular EBNA2 target genes to the growth transformation process in vitro will need to be rigorously tested in large-scale RNAi-based screens. It is also to be expected that several EBNA2 target genes are critical for the establishment of viral latency in vivo. With regard to EBNA-LP, it appears that one of its principal functions is to coactivate a subset of EBNA2-regulated viral latency genes. Thus, EBNA-LP might be required to activate EBNA2 target genes, which exhibit a specific chromatin configuration in naïve B-cells. The exact mechanism remains to be elucidated, but the observed interactions with Sp100 and cellular repressors or repressor complexes suggest that it facilitates EBNA2 coactivation through the modulation of repressors or facultative heterochromatin. Through its interaction with Sp100, EBNA-LP shares features with other herpesvirus immediate early proteins that modulate PML NBs or PML NB-associated proteins. One hypothesis is that PML NBs exert a repressive effect on viral gene expression, referred to as an intrinsic antiviral defense mechanism, which is counteracted by viral immediate early or tegument proteins (Everett 2013; Everett and Chelbi-Alix 2007). An intriguing notion is that EBNA-LP provides similar function(s) to help jump start viral latency gene expression immediately following infection. Due to the complex nature of the IR1 repeats for both Wp and EBNA-LP functions, it has been technically challenging to generate EBNA-LP null EBV recombinants or recombinants that express

EBNA-LP coactivation mutants to interrogate its role in EBV-induced B-cell immortalization. Such reagents will be needed to confirm and extend the previous observations concerning cellular cofactor interactions under the physiological conditions of EBV infection in primary B-cells. Moreover, the emergence of CRISPR/Cas9 technology should enable investigators to generate targeted knockout of cellular genes to assess their importance for EBNA2 and EBNA-LP function in the near future. However, a serious limitation of all the results discussed in this review is the fact that there is no small animal model available, which allows assessing the specific contribution of a target gene to the pathogenesis of EBV-associated diseases.

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