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Contributions of the EBNA1 Protein of Epstein-Barr Virus Toward B-Cell Immortalization and Lymphomagenesis

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1. Introduction

Epstein-Barr Virus (EBV) is a human herpesvirus, infecting 95% of humans, that is causally associated with the benign B-cell proliferative disorder, infectious mononucleosis. EBV has also been etiologically associated with several cancers, such as Burkitt's lymphoma, AIDS-related immunoblastic lymphomas, post-transplant lymphomas, nasopharyngeal carcinoma, and gastric carcinoma. Very few viral genes are expressed in these malignancies and infectious virus is rarely released, defining a state of infection termed latency. Many EBV-associated malignancies respond poorly to treatment with large variability in success rates dependent on disease stage, viral gene expression patterns, and concurrent immunosuppressive therapy. Developing broadly effective strategies to suppress cell proliferation induced by EBV requires careful consideration of viral factors common to many malignancy types. One interesting viral therapeutic target is the viral protein Epstein-Barr nuclear antigen 1 (EBNA1), which is expressed in all EBV-associated malignancies and has been extensively characterized. Upon association with a region on EBV's genome, termed *oriP*, EBNA1 facilitates the licensed replication and mitotic segregation of EBV genomes in proliferating tumor cells. EBNA1 bound to *oriP* also activates transcription from two major EBV promoters. Finally, EBNA1 is known to suppress apoptosis of EBV-positive tumor cells. In this review, we have described the molecular mechanisms by which EBNA1's functions are operant.

2. Malignancies associated with Epstein-Barr virus (EBV)

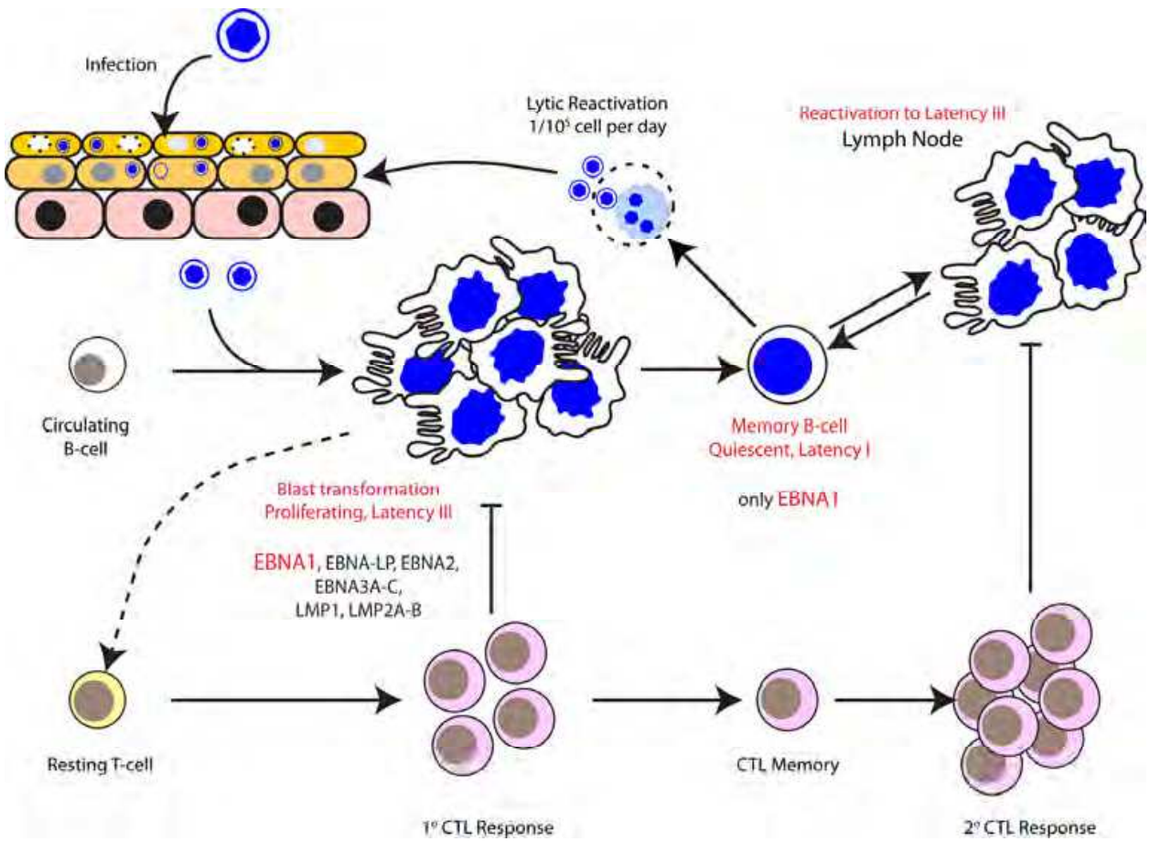
EBV is a double-stranded lymphotropic gammaherpesvirus with a 172-kb linear DNA genome that is widely distributed in the human population (Kieff and Rickinson 2007; Rickinson and Kieff 2007). EBV was discovered through its association with B-cell lymphomas in children and young adults in sub-Saharan Africa. These tumors, now termed Burkitt's lymphoma, were recognized by Denis Burkitt, a British surgeon, as occurring at an unusually high rate in regions of Africa where malaria was endemic. Postulating that an infectious agent was the cause, Burkitt provided tumor biopsies to Anthony Epstein and Yvonne Barr, who screened them for the presence of viral-like particles using electron

microscopy (Epstein, Achong et al. 1964). EBV subsequently became the first virus to be isolated from human cancer (Henle, Henle et al. 1968). Soon after this discovery, epidemiological studies revealed that a primary acute infection with EBV was causally associated with the development of infectious mononucleosis in adolescence and adulthood (Niederman, McCollum et al. 1968). Similar studies revealed a causal association between EBV and Burkitt's lymphoma (de-The, Geser et al. 1978). Molecular studies conducted since 1975 have revealed EBV genomes and expressed genes to be present in several other malignancies.

EBV is currently associated with Hodgkin's lymphoma, non-Hodgkin's lymphoma, AIDS-related immunoblastic lymphomas, primary effusion lymphomas, post-transplant lymphomas, CNS lymphomas, gastric carcinoma, and nasopharyngeal carcinoma (Crawford, Thomas et al. 1980; Purtilo and Klein 1981; Ernberg and Altiok 1989; Glaser, Lin et al. 1997; Dockrell, Strickler et al. 1998; Taylor, Marcus et al. 2005; Navarro and Kaplan 2006). Indeed, together with Kaposi's sarcoma, lymphomas caused by EBV were the first malignancies identified as AIDS-defining clinical conditions (1987). In Figure 1, we have depicted a brief overview of the EBV life-cycle. During primary infection, EBV is transmitted by saliva to oral epithelial cells in which it replicates lytically (Kieff and Rickinson 2007; Rickinson and Kieff 2007). Released virus infects circulating B-lymphocytes using the B-cell surface proteins MHC class II and complement receptor 2 (CR2/CD21) as viral receptors (Fingeroth, Weis et al. 1984; Li, Spriggs et al. 1997). A small subset of viral genes, including EBNA1, are expressed in these infected B-cells that concurrently home to the closest lymphoid tissue, such as the Waldeyer's tonsillar ring, where they proliferate (Laichalk, Hochberg et al. 2002). These latently infected B-cells proliferate rapidly, a process driven by the expression of viral proteins that are described in the next section.

It is pertinent to note that these infected B-cells are latently infected; while they express viral genes that drive cell proliferation, they do not express the large number of viral genes required for production of infectious virus. Cytokines released by rapidly proliferating infected B-cells promote a strong primary CTL response that suppresses their proliferation (Rickinson, Lee et al. 1996; Steven, Leese et al. 1996). Infected B-cells that are not deleted by the CTL response exist in peripheral circulation as quiescent memory B-cells in which EBNA1 is typically the only viral protein expressed (Babcock, Decker et al. 1999). With a very low frequency, EBV's lytic replication is activated in these latently infected cells, releasing virus that is ultimately transmitted through oral mucosa (Laichalk, Hochberg et al. 2002). If EBV-positive memory B-cells transit to a regional lymph node or back to the tonsils, they can return to a highly proliferative state (*ibid*). When this occurs, a strong secondary CTL response limits their proliferation.

Under immunosuppressive conditions, such as a prior malarial infection, HIV-disease, or post-transplantation immunosuppressive therapy, an impaired CTL response permits the unimpeded proliferation of EBV-infected cells (Rickinson, Lee et al. 1996; Kieff and Rickinson 2007). Mutations in cellular genes caused by errors in DNA replication initially result in oligoclonal proliferative disorders, such as post-transplant lymphoproliferative disease; the progressive acquisition of additional mutations ultimately results in clonal malignancies, such as Burkitt's lymphoma (Hammerschmidt 2011; Vereide and Sugden 2011)



After lytic replication in epithelial cells, the virus is transmitted to circulating B-cells that undergo blast transformation in the nearest lymphoid tissue. A subset of viral genes, including EBNA1, is expressed in these proliferating blasts. A primary CTL response limits blast proliferation, and the surviving cells reside as quiescent, memory B-cells in the peripheral circulation. EBNA1 is the only viral protein expressed in EBV-positive memory B-cells. Additional details are in the text.

Fig. 1. Schematic overview of the EBV life-cycle.

2.1 EBV genes necessary for B-cell immortalization and patterns of viral gene expression

The capacity of EBV to drive unfettered B-cell proliferation is recapitulated during infections of naive B-cells in cell culture. EBV efficiently immortalizes naive B-cells to yield cell-lines termed lymphoblastoid cell-lines (LCLs) (Sugden and Mark 1977). Genetic studies have been used to identify the viral proteins required to immortalize naive B-cells in cell culture. Although the EBV genome encodes for approximately 90 proteins, only eight of these proteins are expressed when EBV infects naive B-cells (Mark and Sugden 1982). Of these, only six are required for naive B-cells' immortalization (Kieff and Rickinson 2007). Five of these genes are nuclear proteins, collectively referred to as the Epstein-Barr nuclear antigens (EBNAs). The five proteins are expressed from the same EBNA1-responsive viral promoter (Woisetschlaeger, Strominger et al. 1989). The sixth protein is membrane-associated, and termed latent membrane protein 1 (LMP1) that is also expressed from an EBNA1-responsive promoter (Abbot, Rowe et al. 1990).

The EBV proteins expressed during immortalization have numerous functions that are described briefly here. LMP1 is a homolog of the B-cell membrane protein CD40. While CD40 requires a ligand to be activated, LMP1 is constitutively active (Kaye, Izumi et al. 1993; Zimber-Strobl, Kempkes et al. 1996; Gires, Zimber-Strobl et al. 1997). Akin to activated CD40, it activates NFκB, AP-1, and JNK pathways, and their cellular targets, to sustain B-cell proliferation (Laherty, Hu et al. 1992; Mosialos, Birkenbach et al. 1995; Kieser, Kaiser et al. 1999). The five EBNA proteins have distinct, as well as some overlapping, functions. EBNA2 subverts the cellular Notch pathway (Grossman, Johannsen et al. 1994; Henkel, Ling et al. 1994). Like the intracellular domain of Notch, Notch-IC, EBNA2 associates with the transcription repressor human suppressor of hairless (hSH) converting it into an activator. This complex activates transcription from the same two viral promoters activated by EBNA1, and the cellular Enhancer of Split complex genes. While EBNA-LP greatly augments the efficiency with which EBV immortalizes naive B-cells, it is not required for immortalization (Hammerschmidt and Sugden 1989). In either event, it acts in concert with EBNA2 at specific viral promoters (Harada and Kieff 1997; Ling, Peng et al. 2005; Peng, Moses et al. 2005). EBNA3A and EBNA3C are similar in sequence and have some overlapping functions. They can both act to modulate the activation of hSH-responsive genes by EBNA2 by interacting with hSH (Robertson, Lin et al. 1996; Zhao, Marshall et al. 1996; Dalbies-Tran, Stigger-Rosser et al. 2001). However, only EBNA3C alters the expression of the metastatic suppressor Nm23-H1 (Murakami, Kaul et al. 2009). Finally, recent evidence indicates that EBNA3A and EBNA3C can individually, and cooperatively, down-modulate the expression of the pro-apoptotic cellular protein Bim (Paschos, Smith et al. 2009).

Latency Type	Active Promoter(s)	Genes Expressed	Condition(s)
0	Qp	EBNA1 (upon cell division)	Memory B-cells
I	Qp	EBNA1, EBERs, BARTs	Burkitt's lymphoma
II	Qp, LMP1p, LMP2p	EBNA1, LMP1, LMP2A, LMP2B, EBERs, BARTs	nasopharyngeal carcinoma, T-cell lymphoma
III	Wp, Cp, LMP1p, LMP2p	EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2A, LMP2B, EBERs, BARTs	PTL, AIDS-related immunoblastic lymphoma, CNS lymphoma

Subsets of viral genes are expressed in cells infected latently with EBV. Different viral promoters are used to express EBNA1 in Type III and Type 0/I/II latency. The EBERs and BARTs are expressed from their own promoters that are not listed above.

Table 1. EBV gene expression programs in latency.

In addition to the five EBNA proteins and LMP1, there are three other EBV proteins expressed during latent infection. These proteins are EBNA3B, LMP2A and LMP2B (Kieff and Rickinson 2007; Rickinson and Kieff 2007). Two non-coding RNAs, termed EBER1 and EBER2, as well as a variable number of viral microRNAs, are also expressed during latency (*ibid*). The six proteins required for immortalization (EBNA1, EBNA2, EBNA3A, EBNA3C, EBNA-LP, LMP1) are expressed in four distinct programs termed Latency types 0, I, II, and III (*ibid*). These programs, indicated in Table I, are associated with specific cellular phenotypes. All six proteins are expressed in type III latency, which is a gene expression pattern observed in lymphoblastoid cell lines immortalized by EBV. Type III latency is also observed in post-transplant lymphomas and AIDS-related immunoblastic lymphomas. A more restricted pattern of gene expression is observed during the other latency types. Only EBNA1 is expressed in most Burkitt's lymphoma cells, which is a pattern termed type I latency. Latency type 0 is observed in infected memory B-cells where only EBNA1 expression is detected, and that too only when cells divide. No other viral proteins are expressed during latency type 0. Finally, latency type II is observed in rare T-cell lymphomas and EBV-associated carcinomas. In this pattern, the expression of EBNA1, LMP1, LMP2A, and LMP2B is detected.

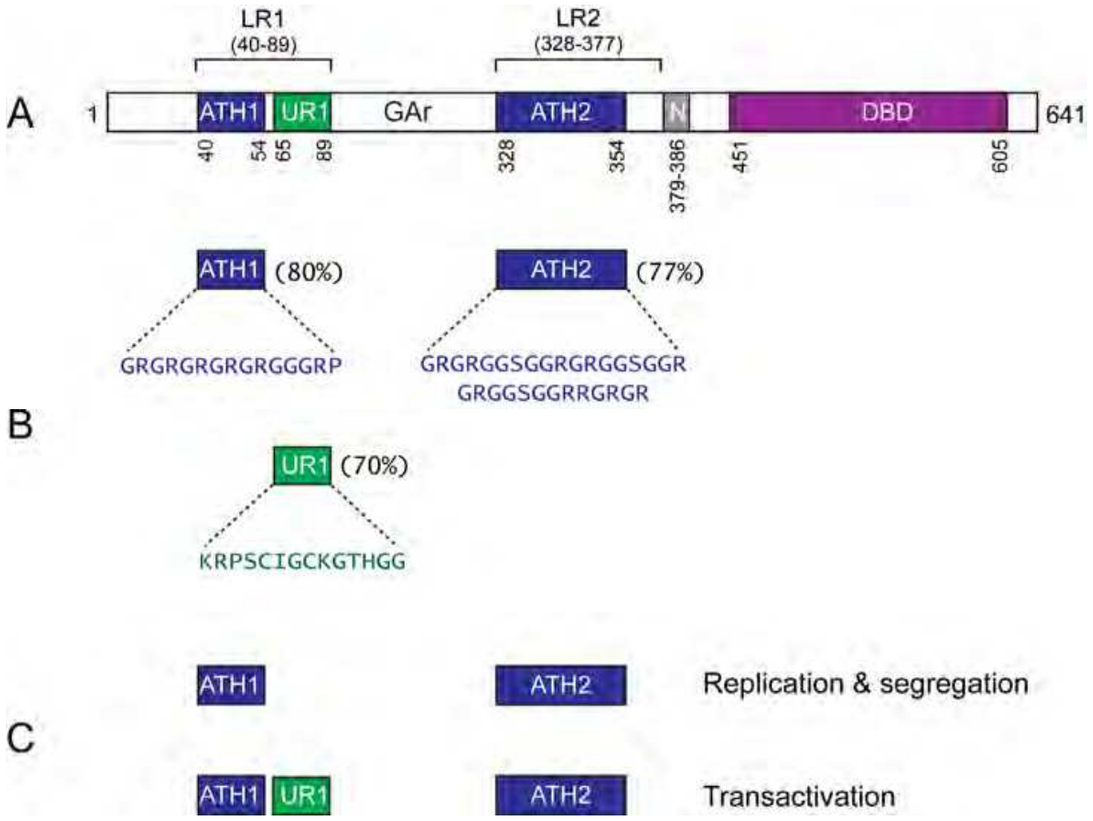
The differential expression of the EBNA proteins in these latency types results from the use of two different promoters for the expression of EBNA1. During latency type III, the chromatin conformation and epigenetic markup of the viral genome favors the activation of the viral BamHI-C promoter (BamHI-Cp) by EBNA1 and EBNA2 (Day, Chau et al. 2007; Tempera, Klichinsky et al. 2011). All the six EBNA proteins are expressed from spliced transcripts that originate at BamHI-Cp. During latency type I, an alternative conformation and markup represses BamHI-Cp (*ibid*). When this occurs, the viral BamHI-Q promoter (BamHI-Qp) is activated; splicing of BamHI-Qp transcripts permits the expression of EBNA1 but not the other EBNAs (*ibid*). Irrespective of the promoter used, EBNA1 is the only viral protein expressed in all four latency types, rendering it an excellent target for therapies directed against EBV-infected cells.

3. EBNA1, its domains, and their functions

A schematic representation of EBNA1 is shown in Figure 2. EBNA1 from the prototypic B95-8 strain of EBV is 641 amino-acids (a.a.) long, and can be divided into several functional domains. EBNA1 associates with several cognate binding sites on the EBV genome through its DNA binding and dimerization domain (DBD), which lies from a.a. 451-641 (Ambinder, Shah et al. 1990; Ambinder, Mullen et al. 1991). The capacity of this domain, when expressed by itself, to bind EBNA1 binding sites was first demonstrated by the studies of Ambinder and Hayward (Ambinder, Shah et al. 1990) . While sufficient to bind EBNA1 binding sites, DBD alone does not support the replication & mitotic segregation of EBV genomes, nor can it activate EBNA1-responsive EBV promoters (Kirchmaier and Sugden 1997; Aiyar and Sugden 1998; Sears, Ujihara et al. 2004) . Indeed, when co-expressed with wild-type EBNA1, DBD functions as a dominant-negative by displacing EBNA1 from its binding sites (Kirchmaier and Sugden 1997).

The DBD is also the only domain of EBNA1 whose structure has been determined (Bochkarev, Barwell et al. 1995); it has a beta-barrel core region that is used for dimerization, flanked by a long alpha-helix that extends into the major groove of the binding site (Bochkarev, Barwell et al. 1996). Surprisingly, this structure is remarkably close to the

structure of the DNA binding domain of the E2 protein (E2DBD) from bovine papilomavirus (BPV) (Hegde, Grossman et al. 1992), despite a lack of sequence conservation. In this context, it should be noted that BPV, a small DNA virus, is very distantly related to EBV, and the related structure of DBD and E2DBD is proposed to result from convergent evolution (Grossman and Laimins 1996). The modular nature of the DBD is reflected by several observations. Sugden and co-workers demonstrated that the DBD could be substituted by DNA-binding domain from the yeast GAL4 protein (Mackey, Middleton et al. 1995; Mackey and Sugden 1997). The chimeric protein displayed several biochemical properties of EBNA1 when bound to GAL4 binding sites. In a similar observation, we have shown that a chimeric protein in which a.a. 1-450 of EBNA1 was fused to E2DBD activated transcription from a cluster of E2 binding sites in a manner similar to WT EBNA1 (Aras, Singh et al. 2009). We have also demonstrated that a chimeric protein in which a strong heterologous acidic activation domain was fused to DBD activated transcription with the characteristics of the heterologous activation domain (*ibid*).



(A) EBNA1 is 641 a.a. in length. ATH1 and ATH2 are two AT-hooks, and UR1 is a domain necessary for transactivation. The GAr is a repeat of glycine and alanine, which can vary in length between EBV isolates. The DBD is used to bind defined sites on EBV's genome. N represents EBNA1's NLS. (B) ATH1 and ATH2 are highly conserved, 80% and 77% respectively, in EBNA1 orthologs from other EBV-like gammaherpesviruses. These regions have a repeated sequence of glycine and arginine. A portion of UR1 is also highly conserved in EBNA1 orthologs. UR1 contains a conserved cys-x-x-cys motif. (C) In addition to DBD, genome replication and segregation requires ATH1 and ATH2. For transactivation, EBNA1 needs both AT-hooks and UR1.

Fig. 2. Schematic diagram of EBNA1 and its domains.

EBNA1 contains at least one nuclear localization sequence (NLS) between a.a. 379-386. When fused to a cytoplasmic protein, the NLS is sufficient to render it nuclear (Ambinder, Mullen et al. 1991). Consistent with its function, the NLS interacts with the nuclear transporters karyopherin alpha1 and alpha2 (Fischer, Kremmer et al. 1997; Kim, Maher et al. 1997). However, an EBNA1 NLS mutant that is substantially reduced in its association with the karyopherins remains nuclear and is functional (Kim, Maher et al. 1997). Therefore, it is likely that EBNA1 has additional NLS signals that are currently unknown.

While B95-8 EBNA1 is 641 a.a. long, EBNA1 proteins from other EBV strains and isolates vary in size. This difference in size arises from differences in the length of a central glycine-alanine repeat region (GAr) (Falk, Gratama et al. 1995). Reductions in the length of the GAr to just 15 a.a. have no effect on the efficiency of B-cell immortalization by EBV (Lee, Diamond et al. 1999), EBNA1's capacity to activate transcription (Aiyar and Sugden 1998), or its ability to support replication and mitotic segregation of EBV genomes (Yates, Warren et al. 1985). It is now appreciated that the GAr reduces the efficiency with which EBNA1 epitopes are presented on the surface of EBV-infected cells. This reduction in efficiency is either a consequence of the GAr affecting processing by the proteosome (Levitskaya, Sharipo et al. 1997), or by reducing the efficiency with which EBNA1 is translated (Yin, Manoury et al. 2003; Apcher, Komarova et al. 2009; Apcher, Daskalogianni et al. 2010). Peculiarly, while cell-culture studies indicate that long GAr sequences substantially reduce proteosome processing and EBNA1 translation, the presence of a long GAr reduces the recognition of EBV-infected cells by EBNA1-specific CTLs by 50% or less (Lee, Brooks et al. 2004). Therefore, the conservation of long GArs in various EBV isolates may reflect other GAr functions, in addition to reduced epitope presentation, that are not recapitulated in cell culture.

The GAr is flanked by positively charged domains. These domains were originally termed linking regions 1 and 2 (LR1/LR2) (Figure 2) because they had the capacity to link DNAs bound by EBNA1 into large multimeric complexes (Mackey, Middleton et al. 1995; Mackey and Sugden 1997; Mackey and Sugden 1999) . Linking is dependent on the capacity of LR1 and LR2 to directly bind nucleic acids. LR1 and LR2 contain within them repeats of glycine and arginine (GR repeats) that can associate specifically with AT-rich DNA and G-quadruplex RNA (Sears, Ujihara et al. 2004; Norseen, Thomae et al. 2008) . These nucleic acid binding properties are observed for cellular AT-hook proteins that also contain GR repeats (Huth, Bewley et al. 1997; Reeves 2001; Norseen, Thomae et al. 2008). For this reason, the GR repeats of EBNA1 are referred to as AT-hook 1 and 2 (ATH1, ATH2) in this review. Deletion of ATH1 or ATH2 reduces the capacity of EBNA1 to transactivate EBV promoters and to support genome replication/segregation (Sears, Ujihara et al. 2004; Singh, Aras et al. 2009). Deletion of both ATH1 and ATH2 eliminates both functions (Sears, Kolman et al. 2003). A chimeric protein in which ATH1 and ATH2 are replaced by the cellular AT-hook protein HMGA1 supports transactivation and genome replication/segregation when bound to EBNA1 binding sites (Hung, Kang et al. 2001; Sears, Kolman et al. 2003). In contrast, a chimeric protein in which EBNA1's AT-hooks were replaced by a non-sequence specific cellular DNA binding protein, HMG1, does not support either function (Sears, Kolman et al. 2003). Therefore, an association between EBNA1's AT-hooks and specific nucleic acids, such as AT-rich DNA or G-quadruplex RNA, is necessary for EBNA1's functions.

While initially considered to be a single positively-charged domain, it is now appreciated that LR1 contains two distinct domains: 1) ATH1; and 2) A short unique region (UR1) that

lies between ATH1 and GAr (Figure 2) (Kennedy and Sugden 2003; Singh, Aras et al. 2009). Deletion mutagenesis has revealed that UR1 is essential for EBNA1 to transactivate, but is not required for EBNA1 to support genome replication/segregation (Kennedy and Sugden 2003). Recent studies have revealed that UR1 contains a short sequence with a cys-x-x-cys motif that is conserved in the EBNA1 orthologs from other EBV-like primate gammaherpesviruses (Aras, Singh et al. 2009). Mutation of the conserved cysteines is sufficient to abrogate EBNA1's capacity to activate transcription, emphasizing the importance of the conserved cys-x-x-cys motif (*ibid*).

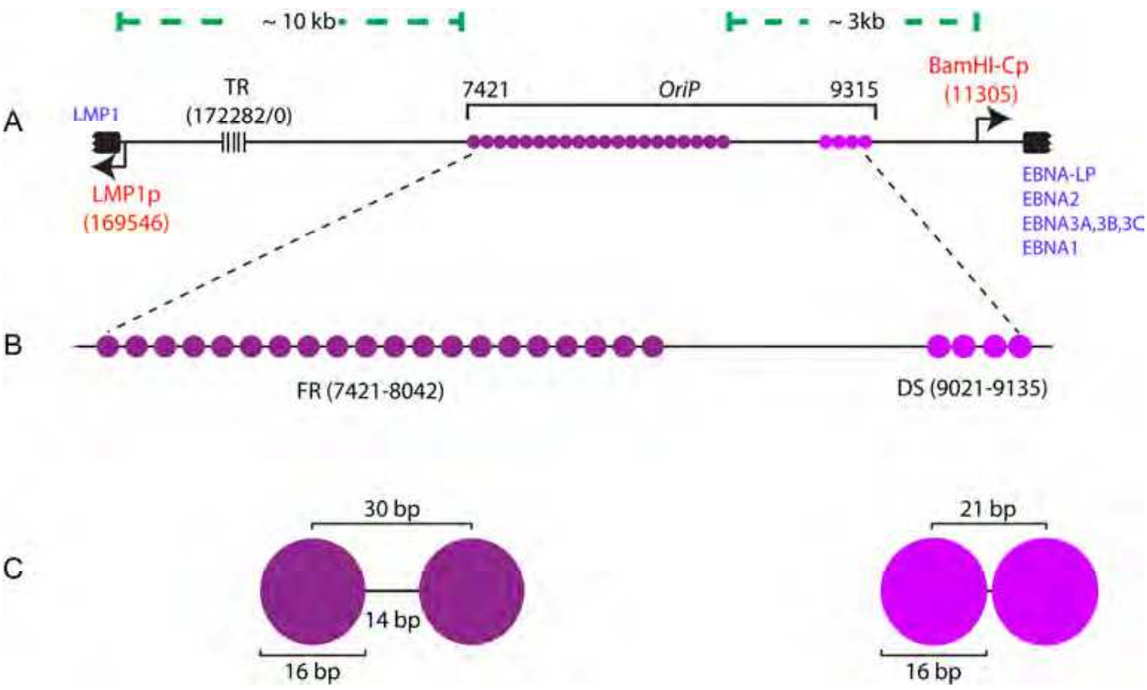
EBNA1's ability to transactivate EBV promoters and to support EBV genome replication/segregation is dependent upon its association with two clusters of cognate binding sites on the EBV genome (Figure 3). The organization of these two clusters is critical to EBNA1's EBV-specific functions and therefore is detailed below.

4. *OriP*, the family of repeats (FR), and the dyad symmetry element (DS)

Adams observed that akin to eukaryotic chromosomes, EBV genomes are replicated once per cell-cycle during latency, by a process termed licensed DNA replication (Lindner and Sugden 2007). This pattern of genome replication in which genomes are precisely duplicated during S phase had not been observed previously for other DNA viruses such as polyomaviruses, papilomaviruses, and alphaherpesviruses. EBV's unique mode of replication, coupled with an efficient segregation mechanism, permits viral genomes to be distributed equally to daughter cells when latently infected cells proliferate (Figure 4) (Sears, Kolman et al. 2003; Sears, Ujihara et al. 2004; Nanbo, Sugden et al. 2007; Norseen, Thomae et al. 2008). To identify EBV sequences necessary for licensed replication and mitotic segregation, Yates and Sugden screened for EBV fragments that conferred these properties to small plasmids introduced into EBV-positive cells (Yates, Warren et al. 1984). Their screen identified a fragment with 24 similar sequences arranged in two clusters. This fragment was termed *oriP* and is depicted in Figure 3. Upon determining *oriP* to be sufficient for the licensed replication and mitotic segregation of plasmids in EBV-positive cells, these investigators identified EBNA1 as the sole EBV protein necessary for licensed replication and mitotic segregation of *oriP*-plasmids (Yates, Warren et al. 1985). Later, it was determined that EBNA1 bound each of the repeated sequences within *oriP* as a dimer (Rawlins, Milman et al. 1985; Ambinder, Shah et al. 1990; Ambinder, Mullen et al. 1991). The repeats in *oriP* are arranged in two clusters (Figure 3): 1) A cluster with 20 EBNA1-binding sites, termed the Family of Repeats (FR); and 2) A cluster with four EBNA1-binding sites arranged as a dyad (DS) (Lupton and Levine 1985; Reisman, Yates et al. 1985).

Deletion and complementation experiments revealed that EBNA1 bound to FR and DS has distinct functions. Plasmids containing only DS undergo DNA replication in cells expressing EBNA1, but are mitotically unstable and lost within 1-2 cell-cycles. Plasmids containing FR alone are distributed as EBNA1-expressing cells divide, but do not replicate, and therefore ultimately diluted out of a proliferating culture (Yates, Camiolo et al. 2000). These studies indicate that DS functions primarily as a licensed replication origin, and FR functions as an element similar to a chromosomal centromere in that it permits newly-replicated *oriP*-plasmids to be mitotically stable and segregated (Wysokenski and Yates 1989; Aiyar, Tyree et al. 1998). The studies of Calos and co-workers reiterate these functional assignations (Krysan, Haase et al. 1989; Krysan and Calos 1993). Similar to DS-only plasmids, plasmids

containing putative chromosomal replication origins undergo licensed replication but are mitotically unstable and lost within 1-2 cell cycles. Introducing FR into these plasmids permitted them to undergo licensed replication and become mitotically stable in EBNA1-expressing cells (*ibid*).



(A) *OriP* contains two clusters of EBNA1 binding sites, represented by the purple and pink filled circles. *OriP* is approximately 3 kb removed from the BamHI-C promoter, which is used to transcribe all the EBNA proteins, and 10 kb removed from the LMP1 promoter that is used to transcribe LMP1. (B) An expanded view of the two clusters of EBNA1 binding sites with *oriP*. The family of repeats (FR) contains 20 high-affinity binding sites, and is approximately 1 kb removed from the dyad symmetry element (DS) that contains four low-affinity binding sites. (C) Each EBNA1 binding site is 16 bp is length, and represented by a purple or pink filled circle. The distance between adjacent binding sites in FR is 14 bp, and therefore EBNA1 dimers bound to adjacent sites are on the same phase of the DNA double helix. The four sites in DS are configured as two pairs of two sites. The EBNA1 binding sites within each pair are 5 bp apart, and this distance is essential for DS to function as a replication origin.

Fig. 3. Schematic representation of *OriP*, and the two EBV promoters transactivated by EBNA1 bound to *OriP*.

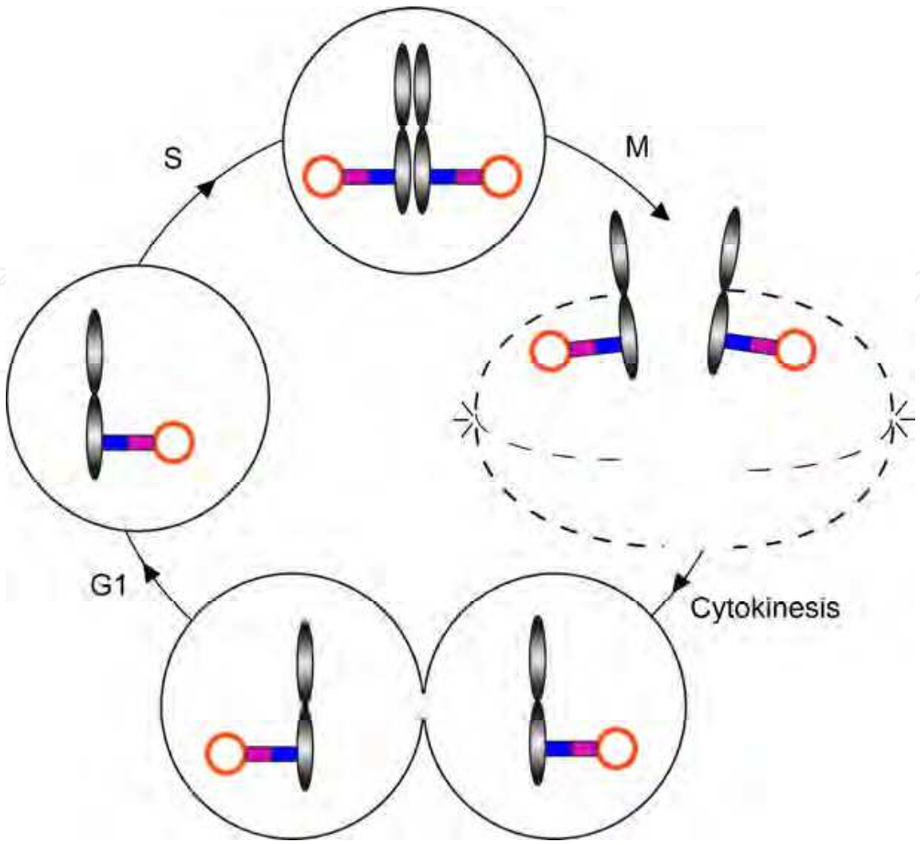
Although FR contains 20 EBNA1-binding sites, numerous studies by others and us have revealed that it must contain a minimum of 7 EBNA1-binding sites to provide mitotic stability in EBNA1-expressing cells. These studies also revealed that plasmids with a modified FR, which only contained 10 EBNA1-binding sites, were more stable than plasmids containing a wild-type FR (Wysokenski and Yates 1989; Hebner, Lasanen et al. 2003). This is because EBNA1 dimers bound to 20 contiguous EBNA1-binding sites in FR impede, but do not completely abrogate, the migration of replication forks through FR (Aiyar, Aras et al. 2009). 10 contiguous EBNA1-binding sites are not an effective replication fork-block; therefore, replication initiating from DS is completed efficiently (*ibid*). Because EBV isolates contain a minimum of 20 EBNA1-binding sites, it is likely that

the capacity of 20 or more contiguous binding sites to limit genome replication is of importance to EBV.

The four EBNA1-binding sites in DS are of lower affinity than the binding sites in FR (Ambinder, Shah et al. 1990). The four sites are arranged in two pairs in which the distance between the centers of the two sites in each pair is 21 base-pairs (bp) (Figure 3). Either pair is sufficient for DS to function as an origin, but alterations of the spacing between them blocks origin function (Harrison, Fisenne et al. 1994; Bashaw and Yates 2001). When EBNA1 binds the sites in DS, it induces a large symmetrical bend in the DNA and forms a structure necessary for the cellular licensed DNA replication machinery to function at DS (Bashaw and Yates 2001). There is an additional sequence juxtaposed 3' to DS, termed Rep*, which can associate with EBNA1 (Kirchmaier and Sugden 1998). The non-canonical EBNA1 binding sites in Rep* are also 21 bp apart, and multiple copies of Rep* can substitute for DS for the licensed replication of *oriP*-plasmids (Wang, Lindner et al. 2006).

5. Contributions of EBNA1 to the licensed replication and segregation of oriP-plasmids

Although it bends DS, DBD alone is not sufficient to support replication from DS (Kirchmaier and Sugden 1998). The latter requires contributions from other EBNA1 domains, particularly ATH1 and ATH2. Investigations into the mechanism by which DS functions as a cell-cycle controlled licensed replication origin have revealed it is similar to the mechanism that restricts chromosomal replication origins to "fire" only once per cell-cycle (Lindner and Sugden 2007). During licensed replication of chromosomal DNA, the cellular origin recognition complex (ORC) marks replication origins throughout the cell-cycle. Late in mitosis or early in G1, the cellular minichromosome maintenance complex (MCM) associates with ORC. Phosphorylation events at the G1/S boundary convert the MCM complex into an active helicase that opens the replication origin and permits DNA polymerase α/primase to be recruited to the origin. MCM functions as the leading strand helicase and can reassociate with ORC only at the end of mitosis. The inability for MCM to be re-used during S phase prevents any single origin from being used more than once in a single cell-cycle. Studies by the groups of Dutta, Lieberman, Schepers, and Yates have provided insights into the mechanism which DS functions as an origin through (Chaudhuri, Xu et al. 2001; Dhar, Yoshida et al. 2001; Schepers, Ritzi et al. 2001; Ritzi, Tillack et al. 2003; Zhou, Chau et al. 2005; Norseen, Thomae et al. 2008). It is now clear that EBNA1 recruits ORC to DS, with the subsequent cell-cycle dependent recruitment of MCM, thus ensuring that *oriP* is subject to the same cell-cycle controlled replication as cellular chromosomes. Studies from several groups, including ours, indicated that EBNA1's AT-hooks were essential to recruit ORC to DS (Sears, Ujihara et al. 2004; Norseen, Thomae et al. 2008). This conclusion has been reiterated by our observations that chimeric proteins in which ATH1 and ATH2 were substituted by cellular AT-hook proteins support licensed replication from DS (Sears, Kolman et al. 2003; Kelly, Singh et al. 2011). Lieberman and co-workers have demonstrated that EBNA1 uses its AT-hooks to recruit the ORC complex to DS via a G-quadruplex RNA intermediate, underscoring a critical role for these domains in *oriP*-replication (Norseen, Thomae et al. 2008).



EBV genomes, represented by an orange circle, are tethered by EBNA1 to cellular chromosomes. The AT-hooks of EBNA1 are shown in blue, and the DBD in purple. EBNA1 recruits ORC/MCM to permit duplication of EBV genomes in S-phase. Replicated genomes are tethered to sister chromatids by EBNA1 and piggy-back on the mitotic spindle to partition into daughter cells.

Fig. 4. Model for piggy-back partitioning of EBV genomes.

ATH1 and ATH2 are also essential for the mitotic stability and partitioning of newly-replicated *oriP*-plasmids (Sears, Kolman et al. 2003; Sears, Ujihara et al. 2004). The studies of Miller and co-workers were the first to indicate that EBNA1 associates with mitotic chromosomes in a punctate manner (Grogan, Summers et al. 1983). This observation, which has been recapitulated by others and us, is used as the basis of the piggy-back partitioning model depicted in Figure 4 (Sears, Kolman et al. 2003; Sears, Ujihara et al. 2004; Nanbo, Sugden et al. 2007). In this model, *oriP*-plasmids are proposed to be tethered to cellular chromosomes throughout the cell-cycle. Tethered plasmids are duplicated during chromosomal replication, and distributed to sister chromatids after replication. Plasmids then piggy-back on chromatids being partitioned by the mitotic apparatus. The association of EBNA1 with chromosomes is central to this model. Two alternative mechanisms have been proposed for this association. In the first, it was observed using a yeast two-hybrid screen that the ATH2 domain of EBNA1 associates with the cellular nucleolar protein EBP2/p40 (Shire, Ceccarelli et al. 1999). Expression of human EBP2 in budding yeast permitted EBNA1 to partition FR-containing plasmids in yeast, suggesting a role for EBP2 in partitioning of *oriP*-plasmids in human cells (Kapoor, Shire et al. 2001). The second model relies on the nucleic acid binding properties of ATH1 and ATH2. EBNA1 mutants in which

either ATH1 or ATH2 is deleted retain the capacity to bind mitotic chromosomes and support the segregation of *oriP*-plasmids (Sears, Ujihara et al. 2004). Further, both ATH1 and ATH2 can be substituted by cellular AT-hook proteins, and the resulting chimeras associate with mitotic chromosomes and support segregation of *oriP*-plasmids (Hung, Kang et al. 2001; Sears, Kolman et al. 2003; Kelly, Singh et al. 2011). Because both ATH1 and ATH2 function equivalently to support *oriP*-plasmid segregation, but only ATH2 associates with EBP2/p40, it is likely that EBP2 does not mediate the partitioning of *oriP*-plasmids or EBV genomes. This interpretation is supported strongly by recent observations examining the segregation of *oriP*-plasmids in live cells. Derivatives of EBNA1 lacking ATH2 were found to segregate *oriP*-plasmids in a mechanism that could not be distinguished from the function of wild-type EBNA1 (Nanbo, Sugden et al. 2007). It remains to be determined whether an association with AT-rich DNA or G-quadruplex RNA underlies EBNA1's binding of cellular chromosomes.

5.1 Transcription activation by EBNA1

Shortly after identification of *oriP*, it was recognized that FR functioned as an enhancer when placed 5' to a promoter-luciferase reporter in cells that expressed EBNA1, but not control cells (Reisman and Sugden 1986). Transactivation by FR-bound EBNA1 is not restricted to specific promoters from EBV, but has also been observed for multiple heterologous promoters when they are juxtaposed to FR (*ibid*). In the context of the EBV genome and promoter-reporter constructs, EBNA1 bound to FR transactivates EBV's BamHI-C promoter (BamHI-Cp) that is approximately 3 kb distal to FR, and the LMP1 promoter (LMP1p), which is more than 10 kb removed from FR (Figure 3) (Gahn and Sugden 1995; Puglielli, Woisetschlaeger et al. 1996). All the proteins necessary for EBV to immortalize naive B-cells are expressed from these two promoters, and therefore EBNA1's capacity to transactivate is essential for B-cell immortalization (Altmann, Pich et al. 2006).

The failure to find cellular co-activators that bound EBNA1 led to the proposal that EBNA1's capacity to "transactivate" reflected its capacity to retain FR-containing plasmids or the EBV genome, in the nuclei of proliferating cells. In this model, retention of FR-containing plasmids in nuclei provides additional transcription templates and therefore increased reporter activity. The plasmid retention model was tested by querying whether EBNA1 could transactivate a chromosomally integrated FR-dependent reporter (Kennedy and Sugden 2003; Sears, Kolman et al. 2003). The outcome from these studies indicated that EBNA1 transactivated the integrated reporter (*ibid*). Further, EBNA1 mutants that are replication/segregation competent, but transactivation defective, do not transactivate the integrated reporter or episomal reporters (*ibid*). While it is now accepted that EBNA1 is a genuine transactivator, the precise mechanism of transactivation remains to be clearly defined. Nevertheless, recent studies identifying domains of EBNA1 necessary for transactivation, and the regulation of transactivation, have provided glimpses into this mechanism. These studies, detailed below, support a structural role for EBNA1 in the activation of transcription, similar to the formation of enhanceosomes by the cellular AT-hook protein, HMGA1.

In addition to DBD, two other EBNA1 domains are necessary to activate FR-dependent transcription: 1) The AT-hooks (ATH1/ATH2); and 2) UR1. Deletion of either ATH1 or ATH2 reduces transactivation by about 50% (Aras, Singh et al. 2009; Singh, Aras et al. 2009); deletion of both ATH1 and ATH2 reduced transactivation to 5% of wild-type EBNA1 (*ibid*).

Similarly, deletion of UR1 reduced transactivation to 5% of WT EBNA1 (Kennedy and Sugden 2003; Aras, Singh et al. 2009; Singh, Aras et al. 2009). These studies also indicate that UR1 and the AT-hooks are individually insufficient to activate transcription; deletion of either domain eliminates the capacity of the remaining domain to promote transactivation (Singh, Aras et al. 2009). Studies using chimeric proteins indicate the nucleic-acid binding properties of ATH1/ATH2 are critical for EBNA1 to transactivate. The chimeric protein HMGA1-UR1-DBD, in which ATH1/ATH2 are replaced by the cellular AT-hook protein, HMGA1, can transactivate when bound to FR (Altmann, Pich et al. 2006).

The UR1-deleted EBNA1 mutant was used to establish definitively that EBNA1's capacity to transactivate was necessary for EBV to immortalize naive B-cells. A recombinant EBV in which WT EBNA1 was replaced by this protein established latent infection in transformed B-cell lymphomas, but failed to immortalize naive B-cells (Altmann, Pich et al. 2006). Consistent with immortalization requiring both AT-hooks and UR1, a recombinant EBV in which WT EBNA1 was replaced by HMGA1-UR1-DBD was competent to immortalize naive B-cells (*ibid*).

5.2 AT-hooks and transactivation

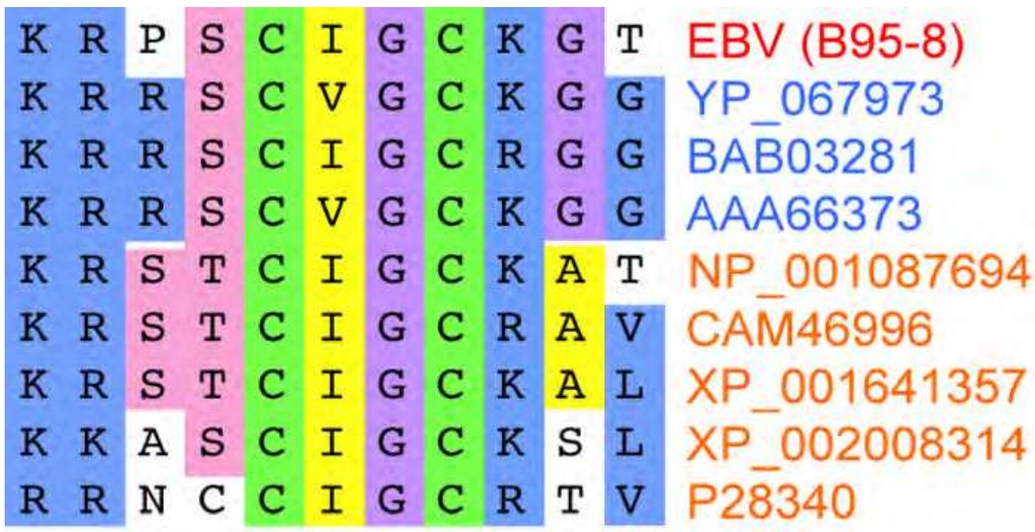
Cellular AT-hook proteins function as architectural proteins in transactivation. This structural function was first revealed for the cellular AT-hook protein, HMGA1, at the ®-interferon enhancer by the studies of Thanos and Maniatis (Kim and Maniatis 1997; Yie, Liang et al. 1997; Yie, Merika et al. 1999). The β-interferon enhancer contains sites bound by the transactivators ATF2, NFκB, and IRF1, and contains four short AT-rich sites bound by HMGA1, previously referred to as HMG-I(Y) (Kim and Maniatis 1997; Yie, Merika et al. 1999). A basal level of transcription was observed during i*n vitro* transcription reactions in which ATF2, NFκB, and IRF1 were provided individually, or as a combination of all three. Similarly, addition of HMGA1 alone also resulted in basal transcription. However, a dramatic increase in transcription was observed when all four factors were provided at the same time. DNA phasing experiments were used to establish a structural role for HMGA1 in transactivation. A six bp deletion that changed the phasing between two HMGA1-binding sites by a half-turn of DNA double helix decreased transactivation by 50%. In contrast, a 10 bp deletion, which restored the original DNA phasing, also restored transactivation (*ibid*). On this basis, it was proposed that structural changes imposed in the enhancer by HMGA1-induced DNA bending, and HMGA1 self-association, formed a transactivation complex termed an "enhanceosome" (Maniatis, Falvo et al. 1998). Other proteins that bind the β-interferon enhancer, namely ATF2, IRF1, and NFκB, recruited transcription co-activators only in the context of this enhanceosome (Merika, Williams et al. 1998). The role of HMGA1 at the β-interferon enhanceosome has been recapitulated at other promoters, such as the early promoter of human papilomavirus type 18 (Bouallaga, Massicard et al. 2000; Bouallaga, Teissier et al. 2003). Six bp deletions or insertions between HMGA1 binding sites at this promoter reduce transcription by about 50%. In contrast, no change in transcription is observed consequent to 10 bp deletions or insertions (Bouallaga, Massicard et al. 2000). At this enhanceosome as well, HMGA1 is necessary to recruit transcription co-activators (Bouallaga, Teissier et al. 2003).

The unusual property of phasing dependent transactivation that was previously observed only for HMGA1 has been observed for EBNA1 (Hebner, Lasanen et al. 2003). The center-to-center distance between adjacent EBNA1-binding sites in FR is 30 bp, or three turns of the

DNA double-helix (Figure 3). Reduction of this distance to 24 bp alters transactivation by 50% without affecting EBNA1's capacity to bind adjacent sites (*ibid*). EBNA1's phasing-dependent transactivation provided the first indication that it functions as an architectural transactivator. Studies with inhibitors indicate the AT-rich DNA binding property of ATH1/ATH2 to be critical for EBNA1 to transactivate. The peptidomimetic netropsin, which displaces AT-hook proteins from AT-rich DNA (Wartell, Larson et al. 1974; Freyer, Buscaglia et al. 2007), also reduces the capacity of EBNA1 to transactivate at BamHI-Cp (Sears, Ujihara et al. 2004). Deletion of ATH1 reduces transactivation to 50% of WT EBNA1, and the reduction is reversed when ATH1 is substituted by AT-hooks from HMGA1 (Singh, Aras et al. 2009). While it is apparent that EBNA1's AT-hooks are necessary for transactivation, how they function bears clarification. AT-hook proteins typically bind AT-rich sequences that are close to the transcription start-site. Although AT-rich sequences are found juxtaposed to BamHI-Cp, and increase BamHI-Cp activity in reporter assays (Walls and Perricaudet 1991), it is yet to be determined if this increase is EBNA1-dependent and if EBNA1 associates with these sequences.

5.3 The role of UR1 in transactivation

Because the UR1-deleted EBNA1 mutant is transactivation defective, efforts have focused on cellular transcription co-activators that associate with UR1. However, several yeast two-hybrid screens (Fischer, Kremmer et al. 1997; Kim, Maher et al. 1997; Aiyar, Tyree et al. 1998), and proteomic analyses, have failed to identify co-activators that bind EBNA1. It has been reported recently that the chromatin remodeling protein Brd4 interacts with EBNA1 in yeast (Lin, Wang et al. 2008). While both over-expression and depletion of Brd4 reduced EBNA1's capacity to transactivate (*ibid*), these conditions also cause a striking reduction in cell viability (Schweiger, Ottinger et al. 2007). Therefore, it is currently not possible to assign a role for Brd4 in EBNA1's capacity to transactivate.



UR1 is highly conserved in the EBNA1 orthologs from other gammaherpesviruses, whose Genbank accession numbers are in blue. The conserved domain closely matches one-half a zinc finger from the DNA polymerase δ active subunit from many eukaryotic species, whose accession numbers are shown in orange

Fig. 5. The UR1 domain of EBNA1.

During our studies to identify co-activators that associated with UR1, we observed that a sub-sequence within UR1 was highly conserved in the UR1 domains of EBNA1 orthologs from other EBV-like gammaherpesviruses (Aras, Singh et al. 2009). The conserved sequence, KRPSCIGCK, also resembles one-half of a C4 zinc-finger present in the catalytic subunit of DNA polymerase δ from multiple eukaryotes (Figure 5). In zinc fingers, the group 12 metal zinc is coordinated between two cys-x-x-cys containing sequences of a protein, bringing these two segments together. Because EBNA1 and its orthologs do not contain another pair of cysteines in a cys-x-x-cys configuration, it is unlikely that zinc is coordinated by a single molecule of EBNA1. Sometimes, zinc coordination between dicysteine motifs in two separate proteins is used to mediate an association between the two proteins. This is exemplified by the interaction between the C-terminal cytoplasmic tail of CD4 or CD8α with the Lck kinase (Huse, Eck et al. 1998; Lin, Rodriguez et al. 1998; Kim, Sun et al. 2003). Therefore, we postulated, and experimentally confirmed, that: 1) UR1 bound zinc; and 2) Zinc coordination resulted in UR1 self-association (Aras, Singh et al. 2009). The role of the cys-x-x-cys motif within UR1 for both properties was confirmed by replacing both cysteines with serines. This mutant UR1 did not bind zinc or self-associate (*ibid*).

Zinc is essential for EBNA1 to transactivate. Chelation of zinc using the cell-permeable zinc chelator **N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine** (TPEN) severely reduced EBNA1's capacity to transactivate without affecting levels of EBNA1 or cell viability (*ibid*). The specific effect of TPEN on EBNA1-dependent transactivation was confirmed by determining it had no effect on transcription from EBNA1-independent promoters, on the function of zinc-independent transactivators. Consistent with the chelation studies, an EBNA1 mutant in which both UR1 cysteines were altered to serine transactivates as poorly as UR1-deleted EBNA1 (*ibid*). Biochemical studies indicate that zinc is coordinated intermolecularly between adjacent EBNA1 dimers bound to adjacent sites in FR, rather than intramolecularly within an EBNA1 dimer bound to a single site in FR (*ibid*). Intermolecular coordination results in a large structured array of EBNA1 at FR that is essential for EBNA1 to transactivate cooperatively. Confirming this conclusion, the EBNA1 mutant in which UR1 cysteines were changed to serines does not transactivate cooperatively. On this basis, it is proposed that a zinc-coordinated array of EBNA1 at FR creates a structured complex within which EBNA1's AT-hooks can form an enhanceosome at promoter proximal sequences (Aras, Singh et al. 2009; Singh, Aras et al. 2009). The model mechanistically explains the co-dependent roles of UR1 and the AT-hooks in transactivation. In the absence of UR1, EBNA1's AT-hooks cannot form a structured complex at promoter proximal AT-rich sequences. In the absence of the AT-hooks, EBNA1 bound to FR lacks the domains necessary to bind such sequences. This model also explains why alteration of phasing between adjacent EBNA1-binding sites in FR affects transactivation. Adjacent EBNA1 dimers bound to sites that lie on opposite sides of DNA will coordinate zinc with different kinetics than dimers bound to adjacent sites on the same side of DNA. This in turn will alter the structure necessary for effective enhanceosome formation, as observed previously for the function of HMGA1 in enhanceosomes (Kim and Maniatis 1997; Bouallaga, Massicard et al. 2000).

5.4 Regulation of transactivation by phosphorylation

EBNA1 contains multiple serine and threonine residues in sequence contexts that resemble recognition sites for protein kinases including cAMP-dependent protein kinase, protein kinase C, glycogen synthase kinase, and mitogen activated protein kinase (PKA)

(Duellman, Thompson et al. 2009). Indeed, a sequence recognized by PKA, KRxS, is conserved in UR1 (Figure 5), and the conserved serine is phosphorylated *in vivo* (*ibid*). Because this sequence is juxtaposed to the critical cys-x-x-cys motif within UR1, we created three mutants of EBNA1 in which this serine was altered to alanine, aspartic acid, or threonine (Singh, Aras et al. 2009). These substitutions were chosen because alanine resembles an unphosphorylated serine, aspartic acid resembles a phosphorylated serine, and threonine restores a PKA recognition site. All three substitutions did not affect the half-life of EBNA1, its nuclear localization, or the efficiency of transactivation (*ibid*). Therefore, although this site is phosphorylated *in vivo*, phosphorylation does not affect any property or function of EBNA1. Subsequent to our analysis, Duellman and Burgess created a mutant derivative of EBNA1 in which all 10 serines known to be phosphorylated were simultaneously substituted by alanine (Duellman, Thompson et al. 2009). This mutant EBNA1 displayed no defects in half-life, expression level, or nuclear localization. There were minor reductions in the efficiency of replication/segregation of *oriP*-plasmids and transactivation (*ibid*). Therefore, it appears unlikely that phosphorylation is a major mechanism that regulates the activities of EBNA1. This conclusion was also drawn from studies in which activators and inhibitors of various kinases were observed to not affect EBNA1's capacity to transactivate.

5.5 Transactivation is sensitive to oxidative stress and regulated by cellular redox effectors

Many viral and mammalian transactivators, including AP-1, NFκB, the Tat protein of human immunodeficiency virus (HIV), and the E2 protein of papillomavirus, are regulated by redox (Hutchison, Matic et al. 1991; Xanthoudakis and Curran 1992; Xanthoudakis, Miao et al. 1992; Huang and Adamson 1993; Mitomo, Nakayama et al. 1994; Xanthoudakis and Curran 1996; Jayaraman, Murthy et al. 1997; Okamoto, Tanaka et al. 1999; Kalantari, Narayan et al. 2008; Wan, Ottinger et al. 2008; Washington, Singh et al. 2010). These proteins contain one or several cysteine residues susceptible to oxidation, whose redox status regulates the capacity to transactivate, due to the effect of oxidative stress on protein structure. Upon exposure to superoxide and hydroxyl radicals, the thiol groups of cysteine can either be oxidized to form a disulfide bond, or progressively oxidized to form sulfenic acid, sulfinic acid, and finally sulfonic acid via the Fenton reaction (Held, Sylvester et al. 1996). Proteins containing zinc fingers essential for their function are especially sensitive to oxidative stress because the thiol group, but not oxidized thiol adducts, can coordinate zinc (Kiley and Storz 2004). In light of EBNA1's UR1 domain containing two conserved cysteines that are critical for transactivation, we tested whether transactivation was susceptible to oxidative stress or alterations in environmental oxygen tension (Aras, Singh et al. 2009; Washington, Singh et al. 2010). Exposure of EBNA1-expressing cells to low levels of agents that generate intracellular hydroxyl and superoxide radicals, such as menadione and paraquat, dramatically reduced transactivation without substantially affecting cell survival or proliferation. In contrast, lowering the oxygen tension by placing EBNA1 expressing cells in hypoxic conditions extended the half-life of transactivation (*ibid*). The latter result suggests that although EBNA1 is very stable, the critical cysteines within UR1 are subject to intracellular oxidative stress, reducing EBNA1's capacity to transactivate over time. Hypoxic conditions reduce the generation of intracellular oxidative radicals, and therefore prolong the activity of EBNA1 as a transactivator (*ibid*).

Cellular and viral transactivators with redox-sensitive cysteines are often regulated by cellular redox effectors such as thioredoxin, thioredoxin reductase and the bifunctional enzyme AP-endonuclease 1 (APE1) (Hutchison, Matic et al. 1991; Xanthoudakis and Curran 1992; Xanthoudakis, Miao et al. 1992; Huang and Adamson 1993; Mitomo, Nakayama et al. 1994; Xanthoudakis and Curran 1996; Jayaraman, Murthy et al. 1997; Okamoto, Tanaka et al. 1999; Kalantari, Narayan et al. 2008; Wan, Ottinger et al. 2008; Washington, Singh et al. 2010). Thioredoxin and thioredoxin reductase can reduce disulfide bridges that result from cysteine oxidation, and therefore function similarly to small molecules such as b-mercaptoethanol (b-ME) and dithiothreitol (DTT) (Lothrop, Ruggles et al. 2009). APE1 is a bifunctional enzyme with two activities: 1) It is an AP-endonuclease that participates in DNA damage repair (Bhakat, Mantha et al. 2009); and 2) It can reduce the oxidized cysteine adduct sulfenic acid back to a thiol (*ibid*). Therefore, oxidized cysteine adducts reduced by APE1 are not acted on by b-ME and DTT.

We determined that treating cells with b-ME or DTT did not alter EBNA1's ability to transactivate, or ameliorate the effect of oxidative stress on transactivation (Washington, Singh et al. 2010). Therefore, it is unlikely that oxidative stress reduces EBNA1's ability to transactivate by creating intra- or intermolecular disulfide bridges. Consistent with this, over-expression of thioredoxin or thioredoxin reductase also did not increase transactivation, or rescue transactivation from intracellular oxidative stress generated by menadione or paraquat exposure (*ibid*). In striking contrast, over-expression of APE1 increases EBNA1's ability to transactivate, and curtails the effect of oxidative stress on EBNA1's function as a transactivator (Aras, Singh et al. 2009; Washington, Singh et al. 2010). It is relevant to note that cysteine residues regulated by APE1 are often adjacent to arginine or lysine residues, as observed for the cysteines in UR1 (Figure 5).

6. Functions of EBNA1 in EBV-positive lymphomas

Two patterns of gene expression predominate in EBV-positive lymphomas: 1) The type III latency pattern of gene expression; and 2) The type I latency pattern of gene expression, in which EBNA1 is the only viral protein expressed. In type III lymphomas, EBNA1 drives the expression of other EBV genes necessary for cell proliferation and facilitates virus genome replication/segregation into daughter cells. It is not clear what functions are provided by EBNA1 in type I lymphomas. It is possible that EBNA1 itself provides functions necessary for cell survival and proliferation. One function indicated by the studies of Kennedy and Sugden is that EBNA1 can inhibit or reduce p53-dependent apoptosis (Kennedy, Komano et al. 2003). In addition, it is possible that by facilitating genome replication/segregation, EBNA1 permits the expression of EBV microRNAs in type I lymphoma cells (Cai, Schafer et al. 2006; Choy, Siu et al. 2008; Pratt, Kuzembayeva et al. 2009). Finally, as described in the next section, several studies indicate that EBNA1 can transactivate some cellular genes, and it is possible that one or a combination of these genes is necessary for lymphoma survival/proliferation (Wood, O'Neil et al. 2007; Baumforth, Birgersdotter et al. 2008; Flavell, Baumforth et al. 2008; O'Neil, Owen et al. 2008; Gruhne, Sompallae et al. 2009).

In either event, it is of interest that introduction of a dominant-negative derivative of EBNA1 into EBV-positive Burkitt's lymphoma cells severely decreases their rate of proliferation, and dramatically increases their apoptosis, reiterating the utility of devising therapies against EBNA1 (Kennedy, Komano et al. 2003; Nasimuzzaman, Kuroda et al. 2005).

6.1 Modulation of cellular gene expression by EBNA1

Recent studies show that EBNA1 alters the transcriptional pattern of several cellular genes (Dawson, Rickinson et al. 1990; Falk, Gratama et al. 1995; Wood, O'Neil et al. 2007; Baumforth, Birgersdotter et al. 2008; Flavell, Baumforth et al. 2008; O'Neil, Owen et al. 2008). These include increasing the expression of STAT1 and AP-1 family transcription factors c-Jun and ATF2. EBNA1 has been demonstrated to bind the promoters of the latter two genes in carcinoma cells (Wood, O'Neil et al. 2007; O'Neil, Owen et al. 2008). If these genes are also activated in the context of an EBV infection, they may contribute to the proliferation of cells transformed by EBV and the immortalization of naive B-cells by EBV. One pro-oncogenic function of EBNA1 is predicted by the recent observation that EBNA1 induces the expression of *NOX2*, resulting in the generation of reactive oxygen species (ROS) that cause genome instability (Gruhne, Sompallae et al. 2009). The cellular enzyme complex, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, produces ROS by transferring electrons from cytosolic NADPH to O_2 (Geiszt and Leto 2004). *NOX2* is the β-subunit of flavocytochrome b_{558}, which forms the catalytic core of NADPH oxidase (D'Autreaux and Toledano 2007). While all the components of NADPH oxidase are expressed in B-cells, *NOX2* is expressed at very low levels (Suzuki and Ono 1999). Therefore, by inducing *NOX2* expression, EBNA1 causes a significant increase in ROS production, which in turn causes pro-oncogenic DNA damage (Gruhne, Sompallae et al. 2009; Gruhne, Sompallae et al. 2009).

Several sequences bound by EBNA1's DBD have been identified in the human genome (Canaan, Haviv et al. 2009; Dresang, Vereide et al. 2009; Lu, Wikramasinghe et al. 2010), permitting the creation of a position-weighted matrix that has been used to predict additional binding sites (Dresang, Vereide et al. 2009). The relevance of EBNA1 binding to these sites is yet to be established because it does not transactivate any genes within 10 kb of the sites bound with highest affinity (*ibid*). Curiously, there are no sequences predicted to be recognized by EBNA1's DBD within the promoter/enhancer regions of genes that are transactivated by EBNA1, suggesting that EBNA1 may associate with these promoters without using its DBD. It is possible that EBNA1 associates with the promoters for these genes through its AT-hooks, similar to the association of HMGA1 with AT-rich sequences at several promoters (Skalnik and Neufeld 1992; Siddiqa, Sims-Mourtada et al. 2001; Martinez Hoyos, Fedele et al. 2004; Tesfaye, Di Cello et al. 2007; Henriksen, Stabell et al. 2010). In this context, it may be of relevance that HMGA1 binds to the *NOX2* promoter (Skalnik and Neufeld 1992).

7. Conclusions

In this review, we have focused on the biological functions and biochemical properties of the EBNA1 protein of EBV. Genetic studies have indicated that EBNA1 is essential for EBV to immortalize naive B-cells, and EBNA1 is the only EBV protein that is expressed in all cells infected latently by EBV, including lymphomas. EBNA1 provides two major functions necessary for EBV to immortalize naive B-cells: 1) It permits replication/segregation of virus genomes; and 2) It activates transcription from two critical viral promoters. Current therapies against EBV have severe side-effects, and recurrent tumors are often resistant to therapy (O'Reilly, Connors et al. 1997; Khanna, Moss et al. 1999; Gottschalk, Rooney et al. 2005; Heslop 2005; Mounier, Spina et al. 2006; Mounier, Spina et al. 2007; Spina, Simonelli et al. 2007). It is therefore desirable to develop new therapies that are specific to EBV-positive

cells, and are highly effective at disrupting their proliferation and survival. Its unique expression pattern and critical functions render EBNA1 an excellent target for the development of such therapies.

Molecular and biochemical analyses have provided insights into the mechanisms by which EBNA1 functions to support replication/segregation and transactivate viral promoters. For both replication and transactivation, EBNA1 needs to bind the *oriP* region of EBV's genome. Therefore, small molecules that interfere with the capacity of DBD to bind its cognate binding sites are predicted to block EBNA1's functions, and indeed, several candidate inhibitors have already been identified (Li, Thompson et al. 2010). EBNA1 needs its AT-hook domains for replication, segregation and transactivation, and therefore these domains are good targets for therapies against EBV-positive lymphomas. Laemmli and co-workers have designed peptidomimetics that interfere with the function of specific AT-hook proteins in Drosophila, with three desirable properties: 1) high specificity; 2) low toxicity; and 3) oral delivery (Janssen, Cuvier et al. 2000; Janssen, Durussel et al. 2000; Blattes, Monod et al. 2006). They also provide a framework for the development of small molecules that disrupt the functions of EBNA1's AT-hooks.

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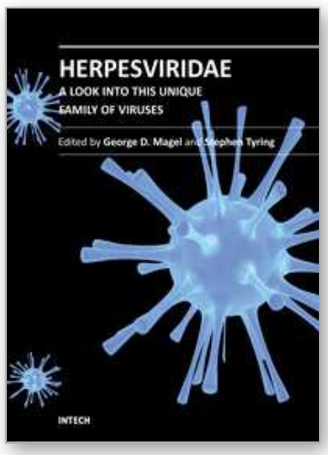
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In order to fully understand the nature of viruses, it is important to look at them from both, their basic science and clinical, standpoints. Our goal with this book was to dissect Herpesviridae into its biological properties and clinical significance in order to provide a logical, as well as practical, approach to understanding and treating the various conditions caused by this unique family of viruses. In addition to their up-to-date and extensive text, each chapter is laced with a variety of diagrams, tables, charts, and images, aimed at helping us achieve our goal. We hope that this book will serve as a reference tool for clinicians of various specialties worldwide.

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