Published in final edited form as:

Plasmid. 2007 July ; 58(1): 1–12. doi:10.1016/j.plasmid.2007.01.003.

The Plasmid Replicon of Epstein-Barr Virus: Mechanistic Insights Into Efficient, Licensed, Extrachromosomal Replication in Human Cells

Scott E. Lindner and Bill Sugden *

McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI 53706

Abstract

The genome of Epstein-Barr Virus (EBV) and plasmid derivatives of it are among the most efficient extrachromosomal replicons in mammalian cells. The latent origin of plasmid replication (*oriP*), when supplied with the viral Epstein-Barr Nuclear Antigen 1 (EBNA1) *in trans*, provides efficient duplication, partitioning and maintenance of plasmids bearing it. In this review, we detail what is known about the viral *cis* and *trans* elements required for plasmid replication. In addition, we describe how the cellular factors that EBV usurps are used to complement the functions of the viral constituents. Finally, we propose a model for the sequential assembly of an EBNA1-dependent origin of DNA synthesis into a pre-Replicative Complex (pre-RC), which functions by making use only of cellular enzymatic activities to carry out the replication of the viral plasmid.

Keywords

oriP; EBNA1; EBV; origin; DS; Rep*

1. General Background

Epstein-Barr Virus (EBV), a member of the herpesvirus family, is a surprisingly successful parasite, as are other human members of this family of viruses. It establishes a persistent, lifelong infection in greater than 90% of the world's population (Fields, Knipe et al. 2006). Primary infection by EBV causes infectious mononucleosis in some, usually adolescent people (Evans, Niederman et al. 1968). The latent cycle of the virus that follows infection is usually asymptomatic in the host, however in a small fraction of infected people, EBV contributes to several cancers including Burkitt's lymphoma, some T-cell lymphomas, Hodgkin's disease, post-transplant lymphoproliferative disease, nasopharyngeal carcinoma, and gastric carcinoma. EBV was the first human virus to be classified as a tumor virus (reviewed in Chapter 75 of (Fields, Knipe et al. 2006)) and has been studied because of its association with these diseases. EBV, in addition, has also served as a model to study DNA replication in human cells because of its ability both to maintain its genome as an extrachromosomal replicon in infected B-cells, and to appropriate the cellular DNA replication machinery needed for its licensed replication.

* To whom correspondence should be addressed: 1400 University Ave, Madison, WI 53706, Phone: 608.262.6697, Fax: 608.262.2824, Email: sugden@oncology.wisc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2. *Cis* Elements of EBV that Contribute to DNA Replication

The genome of EBV is approximately 165kbp (Bloss and Sugden 1994) and resides as a nucleosome-coated nuclear plasmid in infected cells (Wensing and Farrell 2000), (Shaw, Levinger et al. 1979), (Dyson and Farrell 1985). Initial genetic dissections of EBV identified one viral protein, Epstein-Barr Nuclear Antigen 1 (EBNA1), and one region of the viral genome, termed *oriP*, as being necessary and sufficient for replication of the viral plasmid (Yates, Warren et al. 1984)(Lupton and Levine 1985; Reisman, Yates et al. 1985), (Reisman and Sugden 1986). *oriP* is composed of two separable *cis* elements, the Family of Repeats (FR) and the Dyad Symmetry element (DS) (Baer, Bankier et al. 1984; Reisman, Yates et al. 1985), which both contain binding sites for EBNA1 *in vitro* (Rawlins, Milman et al. 1985) (Harrison, Fisenne et al. 1994), and *in vivo* (Hsieh, Camiolo et al. 1993), (Niller, Glaser et al. 1995) (Figure 1). FR is composed of 21 imperfect copies of a 30bp repeat and contains 20 high affinity EBNA1-binding sites (Figure 1). When FR is bound by EBNA1, it both serves as a transcriptional enhancer of promoters *in cis* up to 10kb away (Reisman and Sugden 1986), (Yates 1988), (Sugden and Warren 1989), (Wysokenski and Yates 1989), (Gahn and Sugden 1995), (Kennedy and Sugden 2003; Altmann, Pich et al. 2006), and contributes to the nuclear retention and faithful maintenance of FR-containing plasmids (Langle-Rouault, Patzel et al. 1998), (Kirchmaier and Sugden 1995), (Wang, Lindner et al. 2006) (Nanbo, Sugden et al. submitted). The efficient partitioning of *oriP* plasmids is also likely attributable to FR. Single cell experiments in which *oriP* plasmids are visualized in live cells have demonstrated that at least 88% of those plasmids that are duplicated are co-localized with one another in S-phase and are partitioned faithfully to each daughter cell (Nanbo, Sugden et al. submitted). While the virus has evolved to maintain 20 EBNA1-binding sites in FR, efficient plasmid maintenance requires only 7 of these sites, and can be reconstituted by a polymer of 3 copies of DS, having a total of 12 EBNA1-binding sites (Wysokenski and Yates 1989). The Dyad Symmetry element (DS) is sufficient for initiation of DNA synthesis in the presence of EBNA1 (Aiyar, Tyree et al. 1998), (Yates, Camiolo et al. 2000), and initiation occurs either at or near DS (Gahn and Schildkraut 1989); (Niller, Glaser et al. 1995). Termination of viral DNA synthesis is thought to occur at FR, because when FR is bound by EBNA1 it functions as a replication fork barrier as observed by 2D gel electrophoresis (Gahn and Schildkraut 1989), (Ermakova, Frappier et al. 1996), (Wang, Lindner et al. 2006). Initiation of DNA synthesis from DS is licensed to once-per-cell-cycle (Adams 1987), (Yates and Guan 1991), and is regulated by the components of the cellular replication system (Chaudhuri, Xu et al. 2001), (Ritzi, Tillack et al. 2003), (Dhar, Yoshida et al. 2001), (Schepers, Ritzi et al. 2001), (Zhou, Chau et al. 2005), (Julien, Polonskaya et al. 2004). Single cell analyses have shown that DS is a highly-efficient origin; at least 84% of plasmids carrying *oriP in cis* in HeLa cells expressing EBNA1 are duplicated in each S-phase (Nanbo, Sugden et al. submitted). DS contains four EBNA1-binding sites, albeit with lower affinity than those found in FR (Reisman, Yates et al. 1985). The topology of DS is such that the four binding sites are arranged as two pairs of sites, with 21bp center-to-center spacing between each pair and 33bp center-to-center spacing between the two non-paired internal binding sites (Figure 1.C) (Baer, Bankier et al. 1984; Rawlins, Milman et al. 1985). As there are 10.5bp per turn of B-form DNA, this arrangement positions two EBNA1 dimers on the same helical face of the DNA when bound to a pair of sites. The outer binding sites of each pair (Sites 1 and 4) are of higher affinity than the two internal binding sites (Sites 2 and 3), and several groups have demonstrated by an electrophoretic mobility shift assay (EMSA) that the apparent affinities of EBNA1 for Sites 2 and 3 are increased when their paired high-affinity sites are also occupied. (Harrison, Fisenne et al. 1994), (Summers, Barwell et al. 1996), (Bashaw and Yates 2001). Upon binding to sites in DS, EBNA1 bends the DNA and also causes a permanganate-accessible distortion in the outer binding sites (Harrison 1994, (Frappier and O'Donnell 1992), (Hsieh, Camiolo et al. 1993), (Bashaw and Yates 2001), (Bochkarev, Barwell et al. 1996). Additionally, there is a region of helically unstable DNA near DS that has been proposed to facilitate DNA unwinding and the initiation of DNA

synthesis originating at DS (Polonskaya, Benham et al. 2004). Flanking the pairs of EBNA1-binding sites of DS are three 9bp elements that resemble telomeric repeats, and have been termed nonamers (Niller, Glaser et al. 1995), (Yates, Camiolo et al. 2000). Lieberman and colleagues have shown in several studies that these elements are bound by cellular proteins associated with the telomeres, including TTAGGG-Repeat binding Factors (TRF1, TRF2), the human repressor activator protein 1 (hRap1), and tankyrase (Deng, Lezina et al. 2002), (Deng, Atanasiu et al. 2003), (Deng, Atanasiu et al. 2005). Deletion or mutation of the nonamer elements decreases by twofold the average number of plasmids maintained per cell (Deng, Lezina et al. 2002), (Deng, Atanasiu et al. 2003). One protein found to bind these elements, TRF2, exhibits cooperative binding with a truncated form of EBNA1 at DS as measured in gel shift assays, and may contribute to the enhanced replication of DNA by increasing the apparent affinity of EBNA1 for its binding sites (Deng, Lezina et al. 2002). Despite this two-fold effect, the nonamer elements are not necessary for replicative function (Julien, Polonskaya et al. 2004), (Yates, Camiolo et al. 2000), (Koons, Van Scoy et al. 2001), (Deng, Lezina et al. 2002), (Deng, Atanasiu et al. 2003; Wang, Lindner et al. 2006). Genetic dissections of DS indicate that the minimal requirement for its replicative function is one pair of EBNA1-binding sites (Harrison, Fisenne et al. 1994), (Shirakata and Hirai 1998), (Yates, Camiolo et al. 2000), (Koons, Van Scoy et al. 2001), (Bashaw and Yates 2001). The spacing between a pair of EBNA1-binding sites is critical as well; changing the center-to-center spacing of the sites by inserting or deleting only 1bp eliminates DNA synthesis (Bashaw and Yates 2001). Insertions of 3, 5, or 10bp between the paired sites do not support DNA replication either (Harrison, Fisenne et al. 1994). Additionally, pairs of EBNA1-binding sites within the EBV genome that have center-to-center spacings other than 21bp, such as in FR (with 30bp spacing) and near the Q promoter (with 24bp spacing) (Figure 1.A), do not support DNA replication (Reisman, Yates et al. 1985), (Aiyar, Tyree et al. 1998), (Yates, Camiolo et al. 2000). The functional roles of the elements within DS have been confirmed by studies of another region of EBV's genome, termed Rep*, which was identified as an element that can substitute for DS inefficiently (Kirchmaier and Sugden 1998). Polymerizing Rep* eight times yielded an element as efficient as DS in its support of replication (Wang, Lindner et al. 2006). Biochemical dissection of Rep* identified a pair of EBNA1-binding sites with a 21bp center-to-center spacing critical for its replicative function (ibid). These sites are also bent when bound by EBNA1 (ibid). The minimal replicator of Rep* was found to be the pair of EBNA1-binding sites, as replicative function was retained even after all flanking sequences in the polymer were replaced with sequences derived from lambda phage which were known not to support DNA synthesis themselves (ibid). These findings indicate that neither the flanking nonamer elements nor the helically unstable regions are essential for initiation of DNA synthesis. Comparisons of DS and Rep* have revealed a common mechanism: these replicators support the initiation of DNA synthesis by recruiting the cellular replicative machinery via a pair of appropriately spaced sites, bent and bound by EBNA1.

Not all origins of DNA synthesis in the EBV genome are discrete, though. In the Raji strain of EBV, a 14kbp region is used predominantly to initiate licensed DNA synthesis (Adams 1987), (Little and Schildkraut 1995), (Norio, Schildkraut et al. 2000), (Norio and Schildkraut 2004). The initiation of DNA synthesis is spread across this large region, and is reminiscent of the broad zones of initiation found with many chromosomal origins (reviewed in (Bogan, Natale et al. 2000)). However, the mechanism of the initiation of DNA synthesis from Raji ori is unlike that of DS and Rep*; EBNA1 does not detectably bind to it and no single origin has been localized within it that would facilitate its analysis (C. Wang and Sugden unpublished results).

3. EBNA1: The Sole *Trans*-Acting Element of EBV Required for Its Plasmid Replicon

Epstein-Barr Nuclear Antigen 1 (EBNA1) is the viral initiator protein of EBV (Lupton and Levine 1985; Yates, Warren et al. 1985), because binding of EBNA1 to a pair of sites in DS or Rep* converts that DNA into an origin of DNA synthesis (Gahn and Schildkraut 1989), (Wang, Lindner et al. 2006). The 641 amino acids (AA) of EBNA1 have been categorized into domains associated with its varied functions by mutational and deletional analyses (Figure 2). Two regions, between AA40-89 and AA329-378 are capable of linking two DNA elements *in cis* or *in trans* when bound by EBNA1, and have thus been termed Linking Region 1 and 2 (LR1, LR2) (Middleton and Sugden 1992), (Frappier and O'Donnell 1991), (Su, Middleton et al. 1991), (Mackey, Middleton et al. 1995). Fusing these domains of EBNA1 to GFP homes the GFP to mitotic chromosomes (Marechal, Dehee et al. 1999), (Kanda, Otter et al. 2001). LR1 and LR2 are functionally redundant for replication; a deletion of either one yields a derivative of EBNA1 capable of supporting DNA replication (Mackey and Sugden 1999), (Sears, Ujihara et al. 2004). LR1 and LR2 are rich in arginine and glycine residues, and resemble the AT-hook motifs that bind A/T rich DNA (Aravind and Landsman 1998), (Sears, Ujihara et al. 2004). An *in vitro* analysis of LR1 and LR2 of EBNA1 has demonstrated their ability to bind to A/T rich DNA (Sears, Ujihara et al. 2004). When LR1, containing one such AT-hook, was fused to the DNA-binding and dimerization domain of EBNA1, it was found to be sufficient for DNA replication of *oriP* plasmids, albeit less efficiently than the wild-type EBNA1 (ibid). LR1 and LR2 do differ, though. The C-terminal half of LR1 is composed of amino acids other than the repeated Arg-Gly of the N-terminal half, and is termed Unique Region 1 (UR1). UR1 is necessary for EBNA1 to activate transcription efficiently from transfected and integrated reporter DNAs containing FR (Wu, Kapoor et al. 2002; Kennedy and Sugden 2003; Altmann, Pich et al. 2006). UR1 is also essential for the efficient transformation of B-cells infected by EBV. When a derivative of EBNA1 lacking this domain replaces the wild-type protein in the context of the whole virus, these derivative viruses have 0.1% of the transforming ability of the wild-type virus (Altmann, Pich et al. 2006). The presence of the UR1 domain in EBNA1 allows a shift from the Wp promoter, which is used immediately after infection, to the Cp promoter 5 days post-infection (Figure 1.A) (ibid). This shift in promoter usage correlates with the transcription of several viral genes and a prevention of the infected cell from dying by apoptosis, events likely underlying the role of transcriptional activation by UR1 in transforming infected B-cells. Additionally, UR1 is necessary for the continuing survival of EBV-positive B-cells; derivatives of EBNA1 lacking UR1 function as dominant-negative mutants and inhibit cell survival (Kennedy, Komano et al. 2003).

A yeast two-hybrid assay found that LR2 of EBNA1 associates with the cellular protein EBP2, an essential nucleolar protein implicated in ribosome biogenesis (Shire, Ceccarelli et al. 1999). The maintenance of *oriP* plasmids in *S. cerevisiae* is made possible when human EBP2 is co-expressed with EBNA1 (Kapoor, Shire et al. 2001), (Kapoor and Frappier 2003). These findings in yeast have not been extended to human cells, though. EBP2 was found to bind LR2 of EBNA1, and LR2 is not required for EBNA1's support of *oriP* replication (Shire, Ceccarelli et al. 1999), (Mackey and Sugden 1999), (Sears, Ujihara et al. 2004). Additionally, the N-terminal half of EBNA1 can be replaced with cellular proteins containing AT-hook motifs, such as HMGA1a, and still retain replicative function (Hung, Kang et al. 2001), (Sears, Kolman et al. 2003) (Altmann, Pich et al. 2006). These findings indicate that it likely is the AT-hook activities of LR1 and LR2 and not LR2's binding of EBP2 that are required for the maintenance of *oriP* in human cells.

A third of EBNA1's residues (AA91-328) consist of glycine-glycine-alanine (GGA) repeats, implicated in EBNA1's ability to evade the host immune response by inhibiting proteosomal degradation and presentation (Levitskaya, Coram et al. 1995), (Levitskaya, Sharipo et al.

1997). These repeats have also been found to inhibit translation of EBNA1 *in vitro* and *in vivo* (Yin, Manoury et al. 2003). However, the deletion of much of this domain has no apparent effect on functions of EBNA1 in cell culture, making the role that this domain plays difficult to elucidate.

A nuclear localization signal (NLS) is encoded by AA379–386, which also associates with the cellular nuclear importation machinery (Kim, Maher et al. 1997), (Fischer, Kremmer et al. 1997). Sequences within the Arg-Gly rich regions of LR1 and LR2 may also function as NLSs due to their highly basic content. The ubiquitin specific protease 7 has been found to bind to a region of EBNA1 downstream of its identified NLS (Saridakis, Sheng et al. 2005).

Lastly, the C-terminus (AA458–607) encodes the overlapping DNA-binding and dimerization domains of EBNA1. The structure of these domains bound to DNA has been solved by x-ray crystallography, and was found to be similar to the DNA-binding domain of the E2 protein of papillomaviruses (Hegde, Grossman et al. 1992), (Kim, Tam et al. 2000), (Bochkarev, Barwell et al. 1996). Each monomer of this domain of EBNA1 is a mirror-image of its partner, with the two-fold axis of the dimer aligning with the two-fold axis of its 16bp binding site (*ibid*). The dimerization domain is formed by an eight-stranded, anti-parallel, beta barrel, with four strands being contributed by each monomer. The residues that contribute to sequence-specific DNA binding lie predominantly between AA458–477 as an extended chain that threads through the minor grooves of each half of the binding site, as well as the N-terminal cap of an alpha helix (*ibid*). An additional alpha helix (AA514–527) with structural similarity to that used by papillomaviral E2 proteins to contribute to their DNA-binding has also been implicated in site-specific binding (Bochkarev, Barwell et al. 1996), (Cruickshank, Shire et al. 2000). A symmetrical underwinding and compensatory overwinding of the DNA helix is present when bound by EBNA1, as well as a subtle bending of the DNA toward the protein (Bochkarev, Barwell et al. 1996). However, the extent of bending is not as pronounced as was observed in biochemical assays in which the DNA and protein were in solution (Bashaw and Yates 2001), (Wang, Lindner et al. 2006). *In silico* modeling of a pair of EBNA1-binding sites with 21bp center-to-center spacing with the degree of bending observed in the co-crystal structure (~10°) predicted steric hindrance between the two EBNA1 dimers resulting from significant overlap of the calculated electron densities (Bochkarev, Barwell et al. 1996). The measured bending by EBNA1 of two sites is on the order of 45° which indicates that the exact structure of EBNA1 bound to DNA is uncertain (Bashaw and Yates 2001), (Wang, Lindner et al. 2006).

Assays measuring EBNA1's binding to various DNA sequences have demonstrated that it recognizes many related 16-mers, making *in silico* prediction of its binding sites difficult (Ambinder, Shah et al. 1990), (Wang, Lindner et al. 2006). Each monomer of EBNA1 contacts portions of DNA extending over eight base pairs and although many 16-mers known to bind EBNA1 dimers are palindromes, they need not be (Baer, Bankier et al. 1984), (Rawlins, Milman et al. 1985), (Wang, Lindner et al. 2006). This complexity has also made it difficult to predict those sites bound by EBNA1 under physiologic conditions. All known transcriptional and replicative functions of EBNA1 depend on its binding of DNA site-specifically. For example, a derivative of EBNA1 that is capable of localizing to the nucleus and binding DNA, but lacks all other functions, behaves dominant-negatively for all of EBNA1's activities (Kirchmaier and Sugden 1997).

4. EBV usurps cellular factors for its own ends

EBNA1 lacks any enzymatic activities despite all functions attributed to it and must rely entirely on cellular factors to carry out these functions (Yates 1988). Many proteins associated with EBNA1's replicative functions have been identified recently, while the cellular factors

associated with its ability to modulate transcription are unknown. EBNA1-dependent DNA synthesis from both DS and Rep* is licensed to occur once-per-cell-cycle, and several groups have shown by chromatin immunoprecipitation (ChIP) that this process is mediated and regulated by the cellular replicative machinery (Schepers, Ritzi et al. 2001), (Dhar, Yoshida et al. 2001), (Ritzi, Tillack et al. 2003), (Chaudhuri, Xu et al. 2001), (Wang, Lindner et al. 2006). When EBNA1 binds to a pair of binding sites of DS or Rep*, the protein-DNA structure formed by this interaction recruits members of the pre-Replicative Complex (pre-RC) to it (ibid). The Origin Recognition Complex (ORC1–6) and the putative replicative helicase (MCM2–7) have both been found to associate with these EBV origins in a similar cell-cycle dependent manner as is thought to occur generally in human cellular origins and been found for the human lamin B2 origin (Chaudhuri, Xu et al. 2001), (Dhar, Yoshida et al. 2001), (Ritzi, Tillack et al. 2003), (Schepers, Ritzi et al. 2001), (Zhou, Chau et al. 2005), (Julien, Polonskaya et al. 2004), (Wang, Lindner et al. 2006), (Abdurashidova, Danailov et al. 2003). The replication of *oriP* plasmids is significantly inhibited in cells hypomorphic for ORC2 or overexpressing the cellular protein geminin that inhibits full assembly of the pre-RC, confirming EBV's reliance on these cellular pathways for the replication of its own genome (Dhar, Yoshida et al. 2001). While a pair of EBNA1-binding sites is sufficient for DNA synthetic function, the presence of the nonamer elements flanking the origin and the binding of TRF2 to them stimulates the initiation of DNA synthesis from that origin (Deng, Lezina et al. 2002; Deng, Atanasiu et al. 2003; Atanasiu, Deng et al. 2006). Some of this stimulation likely results from TRF2's increasing EBNA1's apparent affinity for DS; in addition, some may also result from the direct interaction of TRF2 with the BAH domain of ORC1, allowing increased recruitment of ORC1 to DS at the G1/S border of the cell cycle (Ritzi, Tillack et al. 2003; Atanasiu, Deng et al. 2006). The BAH domain of ORC1 is important for the activation of EBNA1-dependent origins of DNA synthesis. Deletion of the entire domain (AA1–169), as well as a single point mutation (E111K), reduced the association of ORC1 with DS to background as measured by Chromatin ImmunoPrecipitation (ChIP) (Noguchi, Vassilev et al. 2006). These same modifications also reduced the short-term replication of *oriP* plasmids to background levels, and the overexpression of the BAH domain alone (AA 1–315) functioned dominant-negatively to inhibit *oriP* replication (ibid). Cellular components in addition to ORC used for DNA synthesis, such as polymerases, single-stranded DNA-binding proteins and other accessory proteins must also be recruited to EBNA1-dependent origins of DNA synthesis. EBV does not express viral proteins with these functions during the latent phase of its life cycle.

Although herpesviruses do not use histones to coat their genomes when compacted in viral particles, EBV does use cellular histones to condense its 55 microns of duplex DNA into nucleosomes while resident in the host (Wensing and Farrell 2000), (Shaw, Levinger et al. 1979), (Dyson and Farrell 1985). The nucleosomal structure of *oriP* is defined by EBNA1 binding to it; EBNA1 can displace histones covering its binding sites *in vitro* and can maintain DS and FR free of nucleosomes when binding to them (Avolio-Hunter, Lewis et al. 2001; Avolio-Hunter and Frappier 2003; Zhou, Chau et al. 2005). The positioning of nucleosomes by EBNA1 on *oriP* plasmids occurs as quickly as 6 days post-transfection, and the histones flanking DS undergo cell-cycle-dependent remodeling via acetylation and methylation, apparently by using the cellular SNF2 and HDAC2 proteins (Zhou, Chau et al. 2005). The role of this chromatin remodeling at DS on its replicative function is uncertain, though; it occurs both in Raji cells in which DS apparently is not used as the predominant origin of DNA synthesis (Norio, Schildkraut et al. 2000) and in an engineered variant of EBV DNA in which DS is the only origin shown to function (ibid). However, some epigenetic events such as nucleosomal remodeling may be important for the replication of *oriP* plasmids and by inference for the EBV genome too (Leight and Sugden 2001). The introduction of *oriP* plasmids into cells is followed by their precipitous loss at a rate of $\geq 25\%$ of these plasmids per generation from the cell population, despite their being replicated (Leight and Sugden 2001). This phenomenon is observed with plasmids derived from either mammalian or bacterial sources

where the associated proteins are removed from the plasmid DNA during its purification (ibid). By 2–3 weeks after their introduction the remaining plasmids (1–10% of those initially introduced) that are duplicated are stably maintained in >99.7% of mitoses and are thus described as being “established” (Leight and Sugden 2001; Nanbo, Sugden et al. submitted). Single-cell analysis of established plasmids in live cells has demonstrated that any subsequent loss of plasmids results from defects in DNA synthesis, which lead to 16% of plasmids failing to duplicate each S-phase (Nanbo, Sugden et al. submitted). These observations indicate a requirement for an epigenetic change to occur for these plasmids to be established, potentially by creating the appropriate nucleosomal architecture near FR and DS to allow efficient DNA synthesis and plasmid maintenance.

5. A Proposed Model for the Sequential Assembly of Cellular Proteins at an EBNA1-Dependent Origin of DNA Synthesis

The study of the replicative mechanisms by which DS functions has been tractable and fruitful because of the several key attributes of DS described above. These experiments elucidating both the *cis* (short and long-term replication assays) and *trans* elements (mass spectrometry, chromatin immunoprecipitation) that contribute to the licensed initiation of DNA synthesis at DS have led us to propose the following model (Figure 3). A pair of EBNA1-binding sites, spaced precisely 21bp center-to-center, are bound and bent in the same direction by two dimers of EBNA1. EBNA1 binds to a pair of sites in DS interacting cooperatively with the TTAGGG-repeat Binding Factor 2 (TRF2), which binds to an adjacent 9bp element termed a ‘nonamer.’ EBNA1 and TRF2 associate with DS throughout the cell cycle, as do subunits 2 through 6 of the Origin Recognition Complex (ORC2–6) (Figure 3.A). ORC1 associates with DS in G1/S, as it does with cellular origins. The recruitment of ORC1 to DS does not require TRF2-binding sites. However, the association of the N-terminal basic domain of TRF2 with the N-terminal BAH domain of ORC1 enhances the association of ORC1 with DS. The regulatory protein Cdc6 is also likely recruited to DS in G1/S (Figure 3.B). ORC1–6 and Cdc6 function as a clamp loader for the reiterative recruitment and loading of the putative replicative helicase MCM2–7 to yield a head-to-head double hexamer at DS. The association of MCM2–7 with DS is dependent upon the regulatory protein Cdt1, which possibly associates with DS in complex with MCM2–7 (Figure 3.C). Taken together, this protein/DNA complex progresses from a pre-Replicative Complex (pre-RC) to a preInitiation Complex (pre-IC), allowing the efficient initiation of DNA synthesis to occur at or near DS in at least 84% of S-phases.

6. Conclusions

EBV infects small, dense B-lymphocytes which are quiescent, induces them to enter S-phase, and maintains them in a proliferating state in cell culture. EBV introduces its DNA into these resting cells covered only with polyamines and must insure that its replicon is handled by its host cell as it transits from G0 to G1 to insure its being recognized and used by the cellular replicative machinery in the first S-phase. These requirements appear to be met by EBNA1 binding to and bending DS, along with the positioning of nucleosomes adjacent to it. This bent protein/DNA structure, which is aided by cellular proteins such as TRF2 binding adjacent to it, efficiently recruits the cellular replicative machinery for the duplication of the viral genome. Moreover, this EBNA1-dependent origin functions under the same regulatory constraints imposed upon cellular origins of DNA synthesis. As there are no known consensus sequences that define an origin of DNA synthesis in human cells, an overall protein/DNA structure may be the predominant identifying mark of an origin, which EBV has mimicked by using its own *cis* and *trans* elements.

Acknowledgements

We thank Chen-Yu Wang for providing her unpublished findings. We thank too the members of the Sugden lab for critically reviewing this manuscript. Our work has been supported by grants from the NIH: CA22443 and T32CA09135. B.S. is an American Cancer Society Research Professor.

References

- Abdurashidova G, Danailov MB, et al. Localization of proteins bound to a replication origin of human DNA along the cell cycle. *EMBO J* 2003;22(16):4294–303. [PubMed: 12912926]
- Adams A. Replication of latent Epstein-Barr virus genomes in Raji cells. *J Virol* 1987;61(5):1743–6. [PubMed: 3033303]
- Aiyar A, Tyree C, et al. The plasmid replicon of EBV consists of multiple cis-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element. *EMBO J* 1998;17(21):6394–403. [PubMed: 9799247]
- Altmann M, Pich D, et al. Transcriptional activation by EBV nuclear antigen 1 is essential for the expression of EBV's transforming genes. *Proc Natl Acad Sci U S A* 2006;103(38):14188–93. [PubMed: 16966603]
- Ambinder RF, Shah WA, et al. Definition of the sequence requirements for binding of the EBNA-1 protein to its palindromic target sites in Epstein-Barr virus DNA. *J Virol* 1990;64(5):2369–79. [PubMed: 2157891]
- Aravind L, Landsman D. AT-hook motifs identified in a wide variety of DNA-binding proteins. *Nucleic Acids Res* 1998;26(19):4413–21. [PubMed: 9742243]
- Atanasiu C, Deng Z, et al. ORC binding to TRF2 stimulates OriP replication. *EMBO Rep* 2006;7(7):716–21. [PubMed: 16799465]
- Avolio-Hunter TM, Frappier L. EBNA1 efficiently assembles on chromatin containing the Epstein-Barr virus latent origin of replication. *Virology* 2003;315(2):398–408. [PubMed: 14585343]
- Avolio-Hunter TM, Lewis PN, et al. Epstein-Barr nuclear antigen 1 binds and destabilizes nucleosomes at the viral origin of latent DNA replication. *Nucleic Acids Res* 2001;29(17):3520–8. [PubMed: 11522821]
- Baer R, Bankier AT, et al. DNA sequence and expression of the B95–8 Epstein-Barr virus genome. *Nature* 1984;310(5974):207–11. [PubMed: 6087149]
- Bashaw JM, Yates JL. Replication from oriP of Epstein-Barr virus requires exact spacing of two bound dimers of EBNA1 which bend DNA. *J Virol* 2001;75(22):10603–11. [PubMed: 11602702]
- Bloss TA, Sugden B. Optimal lengths for DNAs encapsidated by Epstein-Barr virus. *J Virol* 1994;68(12):8217–22. [PubMed: 7966614]
- Bochkarev A, Barwell JA, et al. Crystal structure of the DNA-binding domain of the Epstein-Barr virus origin-binding protein, EBNA1, bound to DNA. *Cell* 1996;84(5):791–800. [PubMed: 8625416]
- Bogan JA, Natale DA, et al. Initiation of eukaryotic DNA replication: conservative or liberal? *J Cell Physiol* 2000;184(2):139–50. [PubMed: 10867638]
- Chaudhuri B, Xu H, et al. Human DNA replication initiation factors, ORC and MCM, associate with oriP of Epstein-Barr virus. *Proc Natl Acad Sci U S A* 2001;98(18):10085–9. [PubMed: 11517328]
- Cruikshank J, Shire K, et al. Two domains of the Epstein-Barr virus origin DNA-binding protein, EBNA1, orchestrate sequence-specific DNA binding. *J Biol Chem* 2000;275(29):22273–7. [PubMed: 10801810]
- Deng Z, Atanasiu C, et al. Telomere repeat binding factors TRF1, TRF2, and hRAP1 modulate replication of Epstein-Barr virus OriP. *J Virol* 2003;77(22):11992–2001. [PubMed: 14581536]
- Deng Z, Atanasiu C, et al. Inhibition of Epstein-Barr virus OriP function by tankyrase, a telomere-associated poly-ADP ribose polymerase that binds and modifies EBNA1. *J Virol* 2005;79(8):4640–50. [PubMed: 15795250]
- Deng Z, Lezina L, et al. Telomeric proteins regulate episomal maintenance of Epstein-Barr virus origin of plasmid replication. *Mol Cell* 2002;9(3):493–503. [PubMed: 11931758]
- Dhar SK, Yoshida K, et al. Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. *Cell* 2001;106(3):287–96. [PubMed: 11509178]

- Dyson PJ, Farrell PJ. Chromatin structure of Epstein-Barr virus. *J Gen Virol* 1985;66(Pt 9):1931–40. [PubMed: 2993484]
- Ermakova OV, Frappier L, et al. Role of the EBNA-1 protein in pausing of replication forks in the Epstein-Barr virus genome. *J Biol Chem* 1996;271(51):33009–17. [PubMed: 8955146]
- Evans AS, Niederman JC, et al. Seroepidemiologic studies of infectious mononucleosis with EB virus. *N Engl J Med* 1968;279(21):1121–7. [PubMed: 4879667]
- Fields, BN.; Knipe, DM., et al. *Fields' fundamental virology*. Philadelphia, Pa. ; London: Lippincott Williams & Wilkins; 2006.
- Fischer N, Kremmer E, et al. Epstein-Barr virus nuclear antigen 1 forms a complex with the nuclear transporter karyopherin alpha2. *J Biol Chem* 1997;272(7):3999–4005. [PubMed: 9020106]
- Frappier L, O'Donnell M. Epstein-Barr nuclear antigen 1 mediates a DNA loop within the latent replication origin of Epstein-Barr virus. *Proc Natl Acad Sci U S A* 1991;88(23):10875–9. [PubMed: 1660154]
- Frappier L, O'Donnell M. EBNA1 distorts oriP, the Epstein-Barr virus latent replication origin. *J Virol* 1992;66(3):1786–90. [PubMed: 1310782]
- Gahn TA, Schildkraut CL. The Epstein-Barr virus origin of plasmid replication, oriP, contains both the initiation and termination sites of DNA replication. *Cell* 1989;58(3):527–35. [PubMed: 2547525]
- Gahn TA, Sugden B. An EBNA-1-dependent enhancer acts from a distance of 10 kilobase pairs to increase expression of the Epstein-Barr virus LMP gene. *J Virol* 1995;69(4):2633–6. [PubMed: 7884916]
- Harrison S, Fisenne K, et al. Sequence requirements of the Epstein-Barr virus latent origin of DNA replication. *J Virol* 1994;68(3):1913–25. [PubMed: 8107251]
- Hegde RS, Grossman SR, et al. Crystal structure at 1.7 Å of the bovine papillomavirus-1 E2 DNA-binding domain bound to its DNA target. *Nature* 1992;359(6395):505–12. [PubMed: 1328886]
- Hsieh DJ, Camiolo SM, et al. Constitutive binding of EBNA1 protein to the Epstein-Barr virus replication origin, oriP, with distortion of DNA structure during latent infection. *EMBO J* 1993;12(13):4933–44. [PubMed: 8262037]
- Hung SC, Kang MS, et al. Maintenance of Epstein-Barr virus (EBV) oriP-based episomes requires EBV-encoded nuclear antigen-1 chromosome-binding domains, which can be replaced by high-mobility group-I or histone H1. *Proc Natl Acad Sci U S A* 2001;98(4):1865–70. [PubMed: 11172042]
- Julien MD, Polonskaya Z, et al. Protein and sequence requirements for the recruitment of the human origin recognition complex to the latent cycle origin of DNA replication of Epstein-Barr virus oriP. *Virology* 2004;326(2):317–28. [PubMed: 15302216]
- Kanda T, Otter M, et al. Coupling of mitotic chromosome tethering and replication competence in Epstein-Barr virus-based plasmids. *Mol Cell Biol* 2001;21(10):3576–88. [PubMed: 11313483]
- Kapoor P, Frappier L. EBNA1 partitions Epstein-Barr virus plasmids in yeast cells by attaching to human EBNA1-binding protein 2 on mitotic chromosomes. *J Virol* 2003;77(12):6946–56. [PubMed: 12768013]
- Kapoor P, Shire K, et al. Reconstitution of Epstein-Barr virus-based plasmid partitioning in budding yeast. *Embo J* 2001;20(1–2):222–30. [PubMed: 11226172]
- Kennedy G, Komano J, et al. Epstein-Barr virus provides a survival factor to Burkitt's lymphomas. *Proc Natl Acad Sci U S A* 2003;100(24):14269–74. [PubMed: 14603034]
- Kennedy G, Sugden B. EBNA-1, a bifunctional transcriptional activator. *Mol Cell Biol* 2003;23(19):6901–8. [PubMed: 12972608]
- Kim AL, Maher M, et al. An imperfect correlation between DNA replication activity of Epstein-Barr virus nuclear antigen 1 (EBNA1) and binding to the nuclear import receptor, Rch1/importin alpha. *Virology* 1997;239(2):340–51. [PubMed: 9434725]
- Kim SS, Tam JK, et al. The structural basis of DNA target discrimination by papillomavirus E2 proteins. *J Biol Chem* 2000;275(40):31245–54. [PubMed: 10906136]
- Kirchmaier AL, Sugden B. Plasmid maintenance of derivatives of oriP of Epstein-Barr virus. *J Virol* 1995;69(2):1280–3. [PubMed: 7815506]
- Kirchmaier AL, Sugden B. Dominant-negative inhibitors of EBNA-1 of Epstein-Barr virus. *J Virol* 1997;71(3):1766–75. [PubMed: 9032305]

- Kirchmaier AL, Sugden B. Rep*: a viral element that can partially replace the origin of plasmid DNA synthesis of Epstein-Barr virus. *J Virol* 1998;72(6):4657–66. [PubMed: 9573229]
- Koons MD, Van Scoy S, et al. The replicator of the Epstein-Barr virus latent cycle origin of DNA replication, oriP, is composed of multiple functional elements. *J Virol* 2001;75(22):10582–92. [PubMed: 11602700]
- Langle-Rouault F, Patzel V, et al. Up to 100-fold increase of apparent gene expression in the presence of Epstein-Barr virus oriP sequences and EBNA1: implications of the nuclear import of plasmids. *J Virol* 1998;72(7):6181–5. [PubMed: 9621086]
- Leight ER, Sugden B. Establishment of an oriP replicon is dependent upon an infrequent, epigenetic event. *Mol Cell Biol* 2001;21(13):4149–61. [PubMed: 11390644]
- Levitskaya J, Coram M, et al. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* 1995;375(6533):685–8. [PubMed: 7540727]
- Levitskaya J, Sharipo A, et al. Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1. *Proc Natl Acad Sci U S A* 1997;94(23):12616–21. [PubMed: 9356498]
- Little RD, Schildkraut CL. Initiation of latent DNA replication in the Epstein-Barr virus genome can occur at sites other than the genetically defined origin. *Mol Cell Biol* 1995;15(5):2893–903. [PubMed: 7739569]
- Lupton S, Levine AJ. Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. *Mol Cell Biol* 1985;5(10):2533–42. [PubMed: 3016506]
- Mackey D, Middleton T, et al. Multiple regions within EBNA1 can link DNAs. *J Virol* 1995;69(10):6199–208. [PubMed: 7666521]
- Mackey D, Sugden B. The linking regions of EBNA1 are essential for its support of replication and transcription. *Mol Cell Biol* 1999;19(5):3349–59. [PubMed: 10207059]
- Marechal V, Dehee A, et al. Mapping EBNA-1 domains involved in binding to metaphase chromosomes. *J Virol* 1999;73(5):4385–92. [PubMed: 10196336]
- Middleton T, Sugden B. EBNA1 can link the enhancer element to the initiator element of the Epstein-Barr virus plasmid origin of DNA replication. *J Virol* 1992;66(1):489–95. [PubMed: 1309258]
- Nanbo, A.; Sugden, A., et al. EMBO J. The coupling of synthesis and partitioning of EBV's plasmid replicon is revealed in live cells. in press
- Niller HH, Glaser G, et al. Nucleoprotein complexes and DNA 5'-ends at oriP of Epstein-Barr virus. *J Biol Chem* 1995;270(21):12864–8. [PubMed: 7759544]
- Noguchi K, Vassilev A, et al. The BAH domain facilitates the ability of human Orc1 protein to activate replication origins in vivo. *EMBO J*. 2006
- Norio P, Schildkraut CL. Plasticity of DNA replication initiation in Epstein-Barr virus episomes. *PLoS Biol* 2004;2(6):e152. [PubMed: 15208711]
- Norio P, Schildkraut CL, et al. Initiation of DNA replication within oriP is dispensable for stable replication of the latent Epstein-Barr virus chromosome after infection of established cell lines. *J Virol* 2000;74(18):8563–74. [PubMed: 10954558]
- Polonskaya Z, Benham CJ, et al. Role for a region of helically unstable DNA within the Epstein-Barr virus latent cycle origin of DNA replication oriP in origin function. *Virology* 2004;328(2):282–91. [PubMed: 15464848]
- Rawlins DR, Milman G, et al. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. *Cell* 1985;42(3):859–68. [PubMed: 2996781]
- Reisman D, Sugden B. trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. *Mol Cell Biol* 1986;6(11):3838–46. [PubMed: 3025615]
- Reisman D, Yates J, et al. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. *Mol Cell Biol* 1985;5(8):1822–32. [PubMed: 3018528]
- Ritzi M, Tillack K, et al. Complex protein-DNA dynamics at the latent origin of DNA replication of Epstein-Barr virus. *J Cell Sci* 2003;116(Pt 19):3971–84. [PubMed: 12953058]

- Saridakis V, Sheng Y, et al. Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein-Barr nuclear antigen 1 implications for EBV-mediated immortalization. *Mol Cell* 2005 Apr 1;18(1):25–26. [PubMed: 15808506]
- Schepers A, Ritzi M, et al. Human origin recognition complex binds to the region of the latent origin of DNA replication of Epstein-Barr virus. *EMBO J* 2001;20(16):4588–602. [PubMed: 11500385]
- Sears J, Kolman J, et al. Metaphase chromosome tethering is necessary for the DNA synthesis and maintenance of oriP plasmids but is insufficient for transcription activation by Epstein-Barr nuclear antigen 1. *J Virol* 2003;77(21):11767–80. [PubMed: 14557661]
- Sears J, Ujihara M, et al. The amino terminus of Epstein-Barr Virus (EBV) nuclear antigen 1 contains AT hooks that facilitate the replication and partitioning of latent EBV genomes by tethering them to cellular chromosomes. *J Virol* 2004;78(21):11487–505. [PubMed: 15479791]
- Shaw JE, Levinger LF, et al. Nucleosomal structure of Epstein-Barr virus DNA in transformed cell lines. *J Virol* 1979;29(2):657–65. [PubMed: 219253]
- Shirakata M, Hirai K. Identification of minimal oriP of Epstein-Barr virus required for DNA replication. *J Biochem (Tokyo)* 1998;123(1):175–81. [PubMed: 9504426]
- Shire K, Ceccarelli DF, et al. EBP2, a human protein that interacts with sequences of the Epstein-Barr virus nuclear antigen 1 important for plasmid maintenance. *J Virol* 1999;73(4):2587–95. [PubMed: 10074103]
- Su W, Middleton T, et al. DNA looping between the origin of replication of Epstein-Barr virus and its enhancer site: stabilization of an origin complex with Epstein-Barr nuclear antigen 1. *Proc Natl Acad Sci U S A* 1991;88(23):10870–4. [PubMed: 1660153]
- Sugden B, Warren N. A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. *J Virol* 1989;63(6):2644–9. [PubMed: 2542577]
- Summers H, Barwell JA, et al. Cooperative assembly of EBNA1 on the Epstein-Barr virus latent origin of replication. *J Virol* 1996;70(2):1228–31. [PubMed: 8551585]
- Wang J, Lindner SE, et al. Essential elements of a licensed, mammalian plasmid origin of DNA synthesis. *Mol Cell Biol* 2006;26(3):1124–34. [PubMed: 16428463]
- Wensing B, Farrell PJ. Regulation of cell growth and death by Epstein-Barr virus. *Microbes Infect* 2000;2(1):77–84. [PubMed: 10717544]
- Wu H, Kapoor P, et al. Separation of the DNA replication, segregation, and transcriptional activation functions of Epstein-Barr nuclear antigen 1. *J Virol* 2002;76(5):2480–90. [PubMed: 11836426]
- Wysokinski DA, Yates JL. Multiple EBNA1-binding sites are required to form an EBNA1-dependent enhancer and to activate a minimal replicative origin within oriP of Epstein-Barr virus. *J Virol* 1989;63(6):2657–66. [PubMed: 2542579]
- Yates J, Warren N, et al. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. *Proc Natl Acad Sci U S A* 1984;81(12):3806–10. [PubMed: 6328526]
- Yates JL, Camiolo SM, et al. The minimal replicator of Epstein-Barr virus oriP. *J Virol* 2000;74(10):4512–22. [PubMed: 10775587]
- Yates JL, Guan N. Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells. *J Virol* 1991;65(1):483–8. [PubMed: 1845903]
- Yates JL, Warren N, et al. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* 1985;313(6005):812–5. [PubMed: 2983224]
- Yates JLC, S M. Dissection of DNA replication and enhancer activation functions of Epstein-Barr virus nuclear antigen 1. *Cancer Cells* 1988;(6):197–205.
- Yin Y, Manoury B, et al. Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1. *Science* 2003;301(5638):1371–4. [PubMed: 12958359]
- Zhou J, Chau CM, et al. Cell cycle regulation of chromatin at an origin of DNA replication. *EMBO J* 2005;24(7):1406–17. [PubMed: 15775975]

Figure 1.

The EBV genome and the latent origin of plasmid replication (*oriP*). (A) The genome of EBV is depicted here in its covalently closed circular form of ~165kbp. The original genomic fragments produced by *Bam*HI digestion are labeled with letters on the interior. Promoters, transcription units, and gene products of the latent cycle are labeled on the exterior as arrows, dashed lines, and open boxes, respectively. In bold boxes are the lytic origin of DNA synthesis (*oriLyt*), the latent origin of DNA synthesis (*oriP*), the terminal repeats (TR), and the ~14kbp region shown to support initiation of DNA synthesis in the Raji strain of EBV (Raji ori). (B) The origin of plasmid replication (*oriP*) is composed of the Family of Repeats (FR) and the Dyad Symmetry (DS) element. The Rep* element is located 240bp downstream of DS, and

the original mapping of this element is shown as a lightly shaded box. EBNA1-binding sites are depicted as open boxes in all three *cis* elements; the dyad present in DS is denoted by the head-to-head arrows above it. The base positions of each of these elements are listed below them with respect to the sequence of the B95–8 strain of EBV. (C) A more detailed depiction of the DS element. The two pairs of EBNA1-binding sites of DS have an intra-pair spacing of 21bp from the center of one site to the center of the paired site, and an inter-pair spacing of 33bp between the centers of the adjacent binding sites. Nine base pair binding sites for the TTAGGG-repeat Binding Factor 2 (TRF2), also referred to as 'nonamers,' flank the pairs of EBNA1-binding sites and are denoted by the shaded boxes. The arrows above the TRF2-binding sites denote the N-to-C terminal orientation that TRF2 would use to bind to these sites.

Figure 2.

A Domain-Based Model and Partial Structural Representation of Epstein-Barr Nuclear Antigen 1 (EBNA1). EBNA1 is depicted as modules corresponding to functional domains identified by mutational or deletional analyses. Amino acid (AA) positions are denoted below the junction of adjacent domains. G-R denotes a glycine-arginine rich region within the linking regions 1 (AA40–89) and 2 (AA325–379). Two regions composed of more unique amino acids within LR1 and LR2 are denoted as Unique Region 1 (UR-1) and Unique Region 2 (UR-2), respectively. G-G-A denotes a glycine-glycine-alanine region that composes over a third of the molecule. NLS denotes a nuclear localization signal. The DNA-binding and dimerization domain is labeled according to its functions, and has been crystallized and its structure determined by x-ray diffraction. The acidic tail is rich in aspartic and glutamic acids. Functions attributed to EBNA1 are denoted in rectangles below the modular depiction of EBNA1; additional proteins shown to interact with specific regions of EBNA1 are denoted in rounded rectangles above it.

Figure 3.

A sequential model of the licensing process for DNA synthesis at an EBNA1-dependent origin. (A) A pair of EBNA1 dimers binds to a pair of 16bp binding sites with a precise 21bp center-to-center spacing, placing the dimers on the same helical face of the DNA. The binding event bends the DNA toward the DNA-binding and dimerization domain in an additive manner and in the same direction. The TTAGGG-repeat Binding Factor 2 (TRF2) is found as a dimer in the cell, and one monomer can bind to a 9bp element that flanks the pairs of EBNA1-binding sites in wtDS. The binding of TRF2 to these elements (also termed nonamers) permits the cooperative binding of EBNA1 to its binding sites, thereby increasing EBNA1's apparent affinity. In addition, ORC2–6 were found to be bound to such an EBNA1-dependent origin

throughout the cell cycle by Chromatin Immunoprecipitation (ChIP). (B) ORC1 has been shown to interact with EBNA1-dependent origins in the presence and absence of the TRF2 binding sites, although ORC1's association with these origins is likely enhanced by the interaction of its BAH domain with the N-terminal basic domain of TRF2. Cdc6 is also likely recruited to the origin in G1/S. (C) Taken together, these assembled proteins at the EBNA1-dependent origin allow the subsequent recruitment of, and likely the reiterative loading of, the putative replicative helicase MCM2–7 as a head-to-head double hexamer, possibly in a complex with the regulatory protein Cdt1.