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Epstein-Barr Virus DNA Replication

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Epstein-Barr virus (EBV) and its close relatives are the only known viruses that have two fully independent systems for replicating their genomes, one that supports virus production during active or "lytic" infection, and a second one that operates during latent stages of infection to allow the circular viral genomes to be duplicated during each cell division cycle. Although EBV's system of lytic DNA replication is for the most part a heritage of the herpesvirus family, its system for replication during latency is entirely its own. During latent infection, while the genes required for lytic replication and virus production are silent, the circular EBV chromosome is replicated by the cell's DNA replication machinery, apparently only once during each S phase. This arrangement of having the host cell replicate its genome during latency is probably related to the fact that EBV establishes latency in cells that are prone to divide. EBV appears to maintain its life-long infection of people by residing within B cells (Klein 1994; Miyashita et al. 1995), and, during the initial phase of latent infection of B cells, EBV expresses a small set of genes that together cause the cells to proliferate (Kieff and Leibowitz 1990; Miller 1990). EBV DNA replication during latency might also be important for the eventual productive infection of epithelial cells, because EBV appears to be most able to infect undifferentiated epithelial cells but to replicate lytically in differentiated cells (Sixbey 1989).

EBV replication during latency relies almost entirely on the cell. A single EBV-encoded protein, EBNA1, apparently guides replication to initiate at *oriP* on the EBV genome—more specifically, at one of two regions of *oriP* to which EBNA1 binds (Fig. 1). All enzymatic steps of replication, including the initial unwinding of duplex DNA, must be performed by cellular factors under cellular control. Initiation of replication on the EBV chromosome has been found to occur not only at *oriP*, but also at sites well away from *oriP*, over a broad region not dissimilar from the zones of initiation that have been observed at replication origins of mammalian chromosomes (Little and Schildkraut 1995). This and other

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evidence suggests that the specification of an initiation site by *oriP* may not be essential for replication of EBV genomes in latently infected cells. Instead, a second function of *oriP,* which provides for prolonged survival of DNAs in cells independently of replication, could be the central mechanism that stably maintains EBV chromosomes in proliferating cells.

Lytic replication for EBV initiates at specific lytic-phase origins and yields thousands of copies of viral genomes per cell for assembly into virus particles. EBV encodes an ensemble of lytic replication enzymes and accessory factors common to all herpes family viruses. For this reason, it used to be assumed that, at this final stage of infection, EBV could amplify its genome without depending on cellular factors. Yet recent studies have led to the conclusion that EBV lacks the means to initiate its lytic replication and, instead, relies heavily on the cell to conduct this critical step to end latency.

EBNA1-DEPENDENT PLASMID REPLICATION AND MAINTENANCE

Multiple Functions of *oriP* and EBNA1

oriP was identified by systematically testing cloned segments of the EBV genome for the ability to support stable autonomous maintenance

Figure 1 EBV replication during latent infection. (*A*) The positions of *oriP* and the two copies of *oriLyt* on the EBV circularized chromosome are shown relative to the joined terminal repeats (TR). During latent infection, replication initiates at *oriP*, but initiation has also been observed at sites dispersed over a large region, as indicated by the hyphenated arcs at the lower left. In non-B cells and in a restricted state of latency in B cells, the EBNA1 gene is expressed from an autoregulated promoter (*bent arrow*) located over 40 kbp upstream of its coding region. In EBV-immortalized B cells, promoters much farther upstream (*pair of bent arrows*) give rise to regulated levels of EBNA1 synthesis. (*B*) The bipartite structure of *oriP* and a comparison between EBV and a close relative, HVP. (DS) dyad symmetry region; (FR) family of repeats. Each black circle, or oval in the enlargements, represents a binding site for an EBNA1 dimer. At the OBR of both viruses, the spacing of 21 bp exists between neighboring pairs of binding sites. The two viruses have different arrangements of EBNA1-binding sites at the left end of *oriP*. The EBNA1-binding sites were predicted for the HVP sequence using the binding requirements that were determined experimentally for EBNA1 of EBV (Ambinder et al. 1990). The first site, at position 2089 (Loeb et al. 1990), might have low affinity and thus is indicated with an open circle. A partial tenth binding site was originally indicated by Loeb et al. at the left end of this cluster (position 2332), but it is not shown here because it is not expected to have significant affinity for EBNA1.

of plasmids under selection in cells that were latently infected by EBV (Yates et al. 1984). A single EBV-encoded protein, EBNA1, was found to be both necessary and sufficient for *oriP* function (Yates et al. 1985; Lupton and Levine 1985). Stable maintenance of *oriP*-containing plasmids in proliferating cells seems to involve two EBNA1-dependent functions: initiation of DNA replication and a distinct, plasmid-maintenance function that can be demonstrated to operate in the absence of replication. Correspondingly, *oriP* has two essential regions, each containing multiple EBNA1-binding sites and separated by almost 1000 bp of nonessential DNA (Reisman et al. 1985; Lupton and Levine 1985). *oriP* of herpesvirus papio (HVP), an EBV-like virus that infects baboons, has two analogous clusters of EBNA1-binding sites, implying a conserved, bipartite functional arrangement (Fig. 1B) (Loeb et al. 1990).

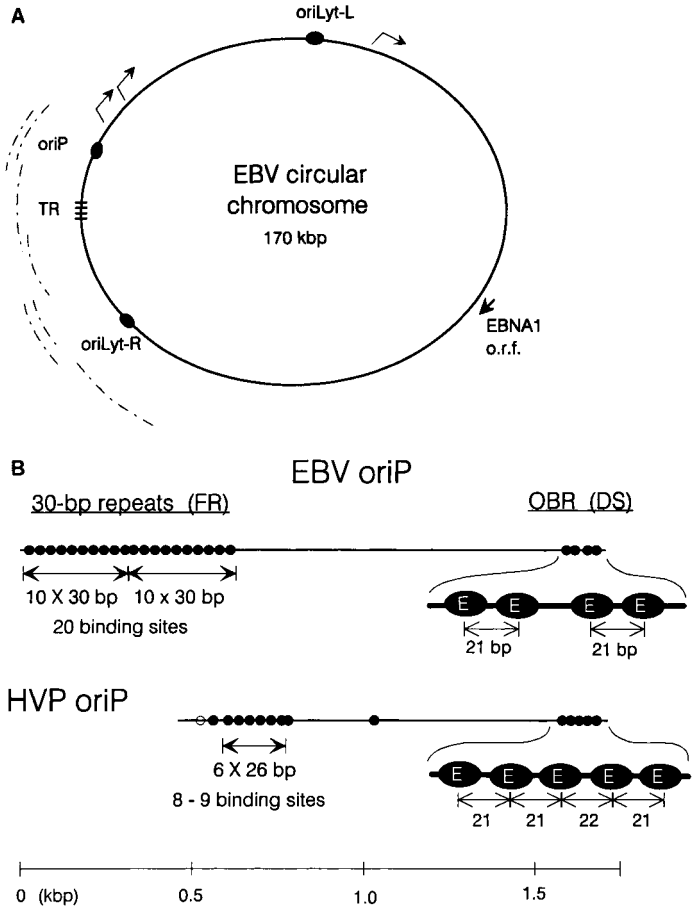

Figure 1 (See facing page for legend.)

One of *oriP*'s essential regions contains four EBNA1-binding sites, suitably arranged to serve as an origin of bidirectional replication, or OBR (Gahn and Schildkraut 1989; Wysokenski and Yates 1989; Harrison et al. 1994). The other essential region is an array of high-affinity EBNA1-binding sites within a family of 30-bp repeats, which form a large, stable complex with EBNA1 (Jones et al. 1989; Ambinder et al. 1990). The 30-bp repeats activate replication initiation at the OBR (Reisman et al. 1985; Wysokenski and Yates 1989), and they are also responsible for the nonreplicational activities associated with *oriP*. The 30-bp repeats, in the presence of EBNA1, prevent plasmids from being lost rapidly from dividing cells, and indications are that this replication-independent maintenance function is as important as replication for episomal maintenance in mammalian cells (Reisman et al. 1985; Krysan et al. 1989). The 30-bp repeats also act as an EBNA1-dependent transcriptional enhancer, a property of unknown significance to replication or maintenance of the viral genome (Reisman and Sugden 1986; Sugden and Warren 1989; Gahn and Sugden 1995).

Replication from *oriP*

The position within *oriP* from which replication forks originate and diverge bidirectionally, the OBR, was found by Gahn and Schildkraut, using two-dimensional gel electrophoresis, to coincide with the functional element of *oriP* containing four EBNA1-binding sites, within a resolution of several hundred bp (Gahn and Schildkraut 1989). This functional element was originally named the "dyad symmetry region" for a large inverted repeat that spans two of the EBNA1-binding sites (Reisman et al. 1985). In the homologous region of *oriP* of HVP, no significant amount of dyad symmetry is evident except that which exists among the EBNA1-binding sites due to the inherent symmetry of each site, as shown in Figure 1. I prefer OBR as a more descriptive term for this functional element of *oriP*, keeping in mind the possibility that the physical origin and this functional cluster of EBNA1-binding sites might not coincide precisely.

Why does replication initiate at the OBR and not at the 30-bp repeats where EBNA1-binding sites are more numerous? Evidence favors a requirement for a proper arrangement of EBNA1-binding sites, as well as a preference for an optimal number of sites (Wysokenski and Yates 1989; Platt et al. 1993; Harrison et al. 1994). To date, no evidence has been found that any protein other than EBNA1 must interact with the OBR in

a sequence-specific manner to support replication.

oriP of EBV and of HVP are likely to function through similar mechanisms, since *oriP* of either virus is active when supported by EBNA1 from either virus (Loeb et al. 1990; J.L. Yates et al., unpubl.). Interestingly, the only obvious features that are common to the OBRs of the two viruses are (1) the presence of EBNA1-binding sites and (2) a center-to-center spacing of 21 nucleotides for adjacent pairs of sites. For EBV, the four EBNA1-binding sites have spacings of 21 bp, 30 bp, and 21 bp, and thus can be viewed as two pairs of sites with each pair having a 21-bp spacing. For HVP, the five EBNA1-binding sites have spacings of 21 bp, 21 bp, 22 bp, and 21 bp for neighboring sites (Fig. 1). The B-form DNA double helix completes two full turns in 21 bp, so EBNA1 dimers bind to these adjacent sites on the same side of the double helix. Mutational studies have shown for EBV that either the left or the right pair of sites spaced 21 bp apart can support a significant level of OBR activity in the absence of the other pair of sites, whereas two improperly spaced sites (16 bp, 26 bp, or 30 bp apart) are inactive (Harrison et al. 1994). However, unpublished studies in my own laboratory have shown that the proximity of a third site to either active pair (regardless of the precise distance) improves the efficiency of replication and that all four sites are required for full activity. Binding of EBNA1 to the OBR causes a distortion of the DNA double helix, presumably a bend or a twist, at each pair of 21-bp-spaced EBNA1 sites, as revealed by increased reactivity of specific thymine bases to oxidation by permanganate, both in vitro and in vivo (Frappier and O'Donnell 1992; Hearing et al. 1992; Hsieh et al. 1993).

The above observations suggest that a specific geometric arrangement of EBNA1 molecules and bound DNA forms a substrate which allows cellular proteins to initiate DNA unwinding and replication. DNA of the OBR is relatively easily unwound, a feature common to replication origins of several organisms (Williams and Kowalski 1993).

Most studies have found that *oriP*-specific replication requires the 30-bp repeats, which activate the OBR with little regard to distance or orientation between the two functional elements (Reisman et al. 1985; Wysokenski and Yates 1989). One study found that when certain cell lines were used for transient transfection assays, the OBR was fully active without being linked to the 30-bp repeats (Harrison et al. 1994). Several attempts to reproduce this result in my own laboratory have failed, but the result suggests that there might be conditions under which EBNA1 can activate the OBR without the aid of the 30-bp repeats. EBNA1 binds to the OBR with lower affinity than to the 30-bp repeats

(Jones et al. 1989; Ambinder et al. 1990; Frappier and O'Donnell 1991a). EBNA1 dimers bound to the 30-bp repeats appear to coalesce into a stable spherical mass as viewed by electron microscopy, and this complex interacts with EBNA1 bound at the OBR to form a DNA "loop." This interaction between the two EBNA1/DNA complexes stabilizes the association of EBNA1 with the OBR (Frappier and O'Donnell 1991b; Su et al. 1991; Middleton and Sugden 1992). The 30-bp repeats act in a highly cooperative manner to activate replication and to form a transcriptional enhancer, with at least seven repeats (EBNA1-binding sites) needed to function effectively. In contrast to the spatial requirements of the OBR, a sufficient number of EBNA1-binding sites can assume the activities of the 30-bp repeats with substantial indifference to their spatial arrangement (Chittenden et al. 1989; Wysokenski and Yates 1989). The homologous cluster of EBNA1-binding sites at the left end of *oriP* of HVP is arranged quite differently than within the family repeats of *oriP* of EBV (Fig. 1).

Pausing and Termination

The large, stable complex formed by EBNA1 at the 30-bp repeats causes replication forks to slow down when they reach it, and the accumulation of forked molecules is easily recognized by two-dimensional gel analysis (Gahn and Schildkraut 1989; Dhar and Schildkraut 1991). Fork progression through the 30-bp repeats is slow enough that, for recombinant plasmids, the fork that has reached the 30-bp repeats by traversing the short distance from the OBR is often met by the opposite fork that has traveled several kilobase pairs around the plasmid, leading replication to terminate there. Although the 30-bp repeats delay fork progression, they do not seem to act as a specific terminator. Placement of an additional set of 30-bp repeats on an *oriP*-replicated plasmid caused only a slight decrease in plasmid stability (Kirchmaier and Sugden 1995). Pausing and termination structures containing bi-forked molecules have also been detected at the 30-bp repeats of EBV genomes in latently infected cells (Little and Schildkraut 1995). Interestingly, in this study, pausing and termination were also detected near or at the OBR of *oriP* on chromosomes for which *oriP* was being replicated passively because initiation had occurred elsewhere (see below). Pausing was also detected at or near each of the genes for the EBV-encoded small RNAs, EBER1 and EBER2, which are located just to the left of *oriP* on the EBV chromosome (Little and Schildkraut 1995).

Regulation of Initiation of Replication at *oriP*

EBV genomes in latently infected cells and plasmids that are replicated from *oriP* are duplicated in apparent synchrony with cell DNA and are only replicated once per S phase (Hampar et al. 1974; Adams 1987; Yates and Guan 1991). Plasmid copy levels are insensitive to increased levels of EBNA1 in cells and are insensitive to placement of an additional copy of *oriP* on the plasmid, indicating that it is neither the availability of EBNA1 nor the inherent activity of *oriP* that limits copy number (Sugden and Warren 1988; Yates and Gaun 1991). In addition, all EBNA1-binding sites at *oriP* appear to be fully occupied by EBNA1 in asynchronously dividing cells, as revealed by in vivo footprinting (Hsieh et al. 1993). Thus, EBNA1 appears to remain bound to both elements of *oriP* throughout most of the cell cycle, as does the origin recognition complex, ORC, at replication origins of budding yeast (Diffley and Cocker 1992). Initiation of replication from *oriP* presumably awaits the regulatory events that initiate replication from cellular origins and seems to be fully subservient to the cellular controls that limit chromosome replication to once during S phase.

Selectable plasmids carrying *oriP* can attain high copy levels in cells during the transfection process. However, it is not clear how EBV chromosomes increase in number from a single infecting genome to several copies or to hundreds of copies in cells that are latently infected. Unequal segregation of replicated genomes during mitosis clearly occurs and could provide a slow increase. Transient and reversible activation of lytic replication might account for high copy levels. A more explicit consideration of these issues has been presented elsewhere (Yates 1993).

Plasmid Maintenance Function of the 30-bp Repeats

When the chromosomes of latently infected cells are viewed at metaphase using fluorescence in situ hybridization to detect EBV DNA, EBV chromosomes are seen decorating the condensed human chromosomes at seemingly random positions (Hurley et al. 1991; see also Harris et al. 1985). By associating with human chromosomes during mitosis, EBV chromosomes are ensured of being included within daughter nuclei when nuclear membranes re-form around the segregated human chromosomes. The association of EBV chromosomes with human chromosomes during mitosis must have an EBV determinant, since extrachromosomal DNA elements of cellular origin (e.g., double minutes) do not share this property. EBNA1 protein appears to localize exclusively to the condensed human chromosomes during mitosis (Grogan et al.

1983). It often has been assumed that it is EBNA1, bound to the 30-bp repeats of *oriP*, that causes EBV chromosomes to associate with human chromosomes during mitosis. This notion is due to an interesting activity of the 30-bp repeats and EBNA1, a plasmid maintenance activity which is often called a "nuclear retention" or "segregation" function.

Plasmids carrying the 30-bp repeats of *oriP* but lacking the OBR replicate very poorly but show prolonged survival when introduced into EBNA1-containing cells (Reisman et al. 1985; Krysan et al. 1989). This form of specific plasmid maintenance has been inferred to occur without any requirement for DNA replication (Reisman et al. 1985; Krysan et al. 1989; Middleton and Sugden 1994), and unpublished experiments in my own laboratory have demonstrated this directly. If EBNA1 in a complex with the 30-bp repeats were to keep plasmids from being lost to the cytoplasm during each mitosis, as described above, this would explain the maintenance effect if, and only if, it were also assumed that plasmids are less likely to find the nucleus by other means and that plasmids left in the cytoplasm are more prone to degradation. However, transfected plasmids appear to reach the nuclei of cells and to be retained in the nuclear fraction for a few cell divisions equally well whether supported by the 30-bp repeats and EBNA1 or not (our unpublished data), so EBNA1 bound to the 30-bp repeats might protect plasmids from destruction in some other way. Perhaps related to either possibility is the observation by Jankelevich et al. (1992) that the region of the EBV genome including the 30-bp repeats, and only this region, attaches to the nuclear matrix (isolated from interphase cells). Regardless of the mechanisms involved, observations described below imply that the nonreplicative maintenance function of the 30-bp repeats and EBNA1 could be the central feature of EBV's system for maintaining its chromosomes in latently infected cells.

EBNA1

EBNA1 is presumed to carry out all of its functions at *oriP* through interactions with cellular factors. Purified EBNA1 lacks ATPase or DNA helicase activities (Frappier and O'Donnell 1991a; Middleton and Sugden 1992). To initiate replication at *oriP*, EBNA1 requires a species-specific factor that is not required for enhancer activation or for nonreplicative plasmid maintenance, indicating that EBNA1 makes at least two distinct functional interactions with cellular factors (Wysokenski and Yates 1989; Krysan and Calos 1993). It has not been possible to separate EBNA1's three activities by introducing deletions into the protein (Yates and Camiolo 1988 and unpubl.), but consideration of the structural properties of EBNA1 might explain this failure.

EBNA1 dimerizes in solution and binds as a dimer to its symmetric binding sites on DNA, perhaps changing its conformation in the process (Ambinder et al. 1991; Frappier and O'Donnell 1991a; Shah et al. 1992). Approximately 160 amino acids of the carboxy-terminal domain of EBNA1 comprise a structurally complex DNA-binding/dimerization domain (Chen et al. 1993). Crystallographic studies of this part of EBNA1 have recently revealed a structure strikingly similar to that of the E2 DNA-binding protein of bovine papillomavirus, with four antiparallel β strands of each molecule combining to form an eight-stranded β barrel with α helices available for contacting the DNA (Bochkarev et al. 1995). EBNA1 dimers that are bound to DNA, particularly at clusters of sites, are prone to adhere to other EBNA1/DNA complexes. Thus, EBNA1 brings the 30-bp repeats and the OBR of *oriP* together, forming a DNA loop (Frappier and O'Donnell 1991b; Su et al. 1991; Middleton and Sugden 1992). This "looping" or "linking" activity is mediated by two separate regions of the amino-terminal two-thirds of EBNA1 (Fig. 2) (Goldsmith et al. 1993; Mackey et al. 1995) and appears to be required for all three activities associated with *oriP:* DNA replication, plasmid maintenance, and activation of transcription (J.L. Yates and S.M. Camiolo, unpubl.).

One-third of the EBNA1 polypeptide is a peculiar, repetitive arrangement of glycines and alanines over 200 amino acids long that is not important for any known function of EBNA1 (Yates et al. 1985; Yates and Camiolo 1988). A recent study found that attaching the glycine, alanine repeats to a heterologous protein would prevent cells expressing the protein from being recognized and lysed by specific cytotoxic T lymphocytes, accounting for the apparent lack of a cellular immune response against EBNA1 (Klein 1994; Levitskaya et al. 1995). In EBV's most restricted state of latent infection, EBNA1 may be the only EBV-encoded protein that is synthesized (Tierney et al. 1994; Miyashita et al. 1995), so in such cells the infection is truly *latent* (from Latin, meaning hidden).

INITIATION OF LATENT REPLICATION AWAY FROM *oriP*

Is the Initiation Site Function of *oriP* Essential?

Because *oriP* was shown to be the only element in the viral genome (strain B95-8) capable of supporting efficient, stable replication of recombinant plasmids introduced into cells under selection (Yates et al. 1984), for a long time it was assumed that *oriP* is essential for replication of EBV chromosomes during latent infection. *oriP* may yet prove to be

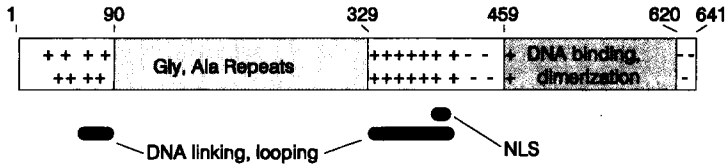

Figure 2 Structure and function of the EBNA1 polypeptide. The regions involved in DNA linking and looping are indicated beneath a rectangular depiction of the polypeptide chain. Most of the polypeptide chain outside of the glycine, alanine repetitious region is involved in binding to DNA and linking it into higher-order structures. Regions of significant net positive or negative charge are indicated with multiple plus and minus signs. (NLS) Nuclear localization sequence (Ambinder et al. 1991).

essential for maintaining circular EBV genomes in proliferating, latently infected cells, but it now appears that its function as an initiation site for replication is not always needed.

Transcriptional studies of an EBV-transformed B-cell line known as X50-7 a few years ago revealed that the EBV strain (of unknown origin) carried by these cells had a genomic rearrangement that deleted all of *oriP* except for the 30-bp repeats (Yandava and Speck 1992). The variant EBV genomes of X50-7 cells are maintained as circular molecules (Cho and Tran 1993) and are capable of transforming human B lymphocytes and, thus, of establishing new latent infections (Gradoville et al. 1990). We cannot be sure that a short sequence derived from *oriP* is not present elsewhere in the viral genome and functioning as an EBNA1-dependent OBR, but otherwise, these results suggest that the replication initiation function of *oriP* is not required for latent replication of EBV genomes in B cells.

One possible explanation for the replication of X50-7 EBV genomes is that replication of large DNA molecules in mammalian cells may not require highly specific initiation sites. Support for this idea came from an attempt to isolate sequences from the human genome that could function as replication origins when inserted into a plasmid that carried only the 30-bp repeats of *oriP*. In the presence of EBNA1, the 30-bp repeats would allow the plasmid to be retained by cells, but without the OBR of *oriP*, replication would be much too inefficient for sustained maintenance unless a segment of human DNA, from a library of randomly inserted fragments, could provide that function. The surprising outcome of such studies was that most randomly tested fragments of human DNA of about 10 kb or longer would allow the plasmids to replicate rather stably in cells (Heinzel et al. 1991). Replication was found to ini-

tiate at multiple sites over most regions of the plasmids (Krysan and Calos 1991). If EBNA1 and the 30-bp repeats contribute to the replication of these plasmids, the contribution is probably indirect. Although EBNA1 and *oriP* cannot support replication in cell lines derived from rodents, the maintenance function of the 30-bp repeats and EBNA1 does function in rodent cells (Wysokenski and Yates 1989; J.L. Yates and N. Guan, unpubl.). Plasmids whose OBR has been substituted by large fragments of human DNA replicate stably in rodent cells (Krysan and Calos 1993), implying that the mechanism for initiating replication on such plasmids is different from that of *oriP*-replicated plasmids and does not require a specific replication function of EBNA1. It should be mentioned that the OBR-substituted plasmids are not as stable as *oriP*-replicated plasmids even under selection, and they generally work well only in certain laboratory cell lines such as 293 (transformed by adenovirus) and HeLa (transformed by human papillomavirus) (J.L. Yates and N. Guan, unpubl.). Nevertheless, the results clearly show that, in the proper context, replication can initiate rather nonspecifically with adequate efficiency in mammalian cells.

Initiation of Replication at *oriP* and Elsewhere on the EBV Chromosome

Clearly, it is important to determine just where replication initiates on EBV chromosomes during latent infection, and Little and Schildkraut (1995) have begun to do so using two-dimensional gels to analyze replication intermediates. With four different latently infected B-cell lines, replication intermediates representing initiation events at *oriP* were seen in all cases. But intermediates due to passive replication by forks that had initiated outside of the region indicated that replication was initiating at sites away from *oriP* a significant fraction of the time in all four cell lines. In one of the cell lines, Raji, most initiations appeared to occur elsewhere. As a control, cells carrying a 19-kb, *oriP*-replicated plasmid were analyzed in parallel, and in this case the expected pattern consistent with predominant or exclusive initiation at *oriP* was observed. Using Raji cells, EBV sequences to the left of *oriP* were examined, and weak signals indicating infrequent initiation events were detected in every region examined over an expanse of at least 50 kb. One region, centered near position 153,000 (B95-8 coordinate), seemed to contain a preferred site of initiation, but initiation was not detected in this region when another cell line was examined. Not all regions of the EBV genome were examined, but at least one region, about 30 kb to the right of *oriP*, lacked

detectable initiation events, indicating that initiation does not occur over the entire EBV chromosome in Raji cells. Consistent with these findings, an earlier electron microscopic study of replicating EBV chromosomes of Raji cells revealed some molecules with more than two forks, implying that initiation had occurred at more than one place on those DNA molecules (Gussander and Adams 1984).

The delocalized initiation events detected with Raji cells are similar to the broad initiation zones that have been observed at replication origins on mammalian chromosomes (Vaughn et al. 1990; Dijkwel and Hamlin 1992; Little et al. 1993; Heintz, this volume). Might EBV have a replication origin of the mammalian chromosomal kind, with a *cis*-acting replicator (distinct from *oriP*), a preferred site of initiation, and a broad initiation zone? Genetic studies are needed to determine whether EBV has a distinct function of this kind, to what extent initiation of replication at *oriP* is important, whether specific initiation sites are required at all, and whether a nuclear anchoring function of the 30-bp repeats and EBNA1 is indeed the most essential feature of autonomous chromosome maintenance for EBV.

REPLICATION DURING LYTIC INFECTION

Components of EBV Lytic Replication: Different Strategies of Initiation for EBV and HSV

Whereas EBV DNA replication during latency serves to duplicate the number of circular viral genomes prior to cell division, during lytic infection viral genomes are amplified to thousands of copies per cell in long concatemers, perhaps through a rolling-circle mechanism, for assembly into virus particles. In contrast to its complete dependence on the cell for its replication during latency, EBV, like other herpesviruses, is much more self-reliant at replicating its genome during lytic infection. All herpesviruses specify six common, conserved replication proteins: DNA polymerase and an accessory factor, a single-stranded DNA-binding protein, and a helicase/primase complex with an associated factor (Table 1). These replication proteins are expected to function similarly for all herpesviruses, and they have been extensively investigated in the case of HSV (Challberg, this volume). However, cellular factors must perform a central role during initiation of EBV lytic replication, and this may be true for other herpesviruses as well, although the different herpesviruses have diverged greatly in their lytic initiation mechanisms.

At a glance, the viral components required for initiation of lytic replication for EBV and HSV bear little resemblance to each other. For

Table 1 Lytic replication proteins of EBV and HSV

EBV ORF	HSV gene	Protein, function
BALF5	UL30	DNA polymerase
BMRF1	UL42	polymerase processivity factor
BALF2	UL29	single-stranded DNA-binding protein
BBLF4	UL5	helicase
BSLF1	UL52	primase
BBLF2/3	UL8	primase-associated factor
—	UL9	HSV origin-binding protein; helicase
BZLF1	—	Z transcriptional activator (EBV); activates *oriLyt* promoter, replication

HSV, the protein product of the UL9 gene binds to each HSV lytic origin at two sites and is essential for replication; the UL9 protein has ATPase and DNA helicase activities, consistent with a role in unwinding DNA at the origin (Challberg, this volume). The EBV genes that are required for lytic replication were identified using the "Challberg approach," that is, by determining which EBV genomic segments must be transfected into noninfected cells in order to activate specific replication of a test plasmid carrying an EBV lytic replication origin, *oriLyt* (Fixman et al. 1992). Because EBV-encoded regulatory proteins are necessary for expression of all "early" genes, including lytic replication genes, the list was then refined by expressing the EBV replication genes using heterologous promoters, so that the EBV early gene *trans*-activators could be omitted. Under these conditions, one EBV transcriptional activator, Z (also called ZEBRA, EB1, BZLF1, and Zta), was still required for replication (Fixman et al. 1995). Z binds to and activates a strong promoter that serves as an integral component of *oriLyt* (Fig. 3). Z and the set of EBV homologs of the six conserved herpesvirus replication proteins complete the list of EBV gene products that are necessary and sufficient for replication from *oriLyt* (Table 1). EBV has no homolog of UL9, and HSV has no homolog of Z; the two proteins are unrelated functionally and phylogenetically.

The lytic replication origins of EBV and HSV do not share common recognizable sequence elements and appear, on initial inspection, to be organized quite differently. The EBV genome carries two lytic replication origins, *oriLyt*, which are determined by two essentially identical copies of a 1055-bp "duplicated segment," located approximately opposite each other on the circularized viral chromosome (Fig. 1A). EBV *oriLyt* is composed of multiple contributing elements (Fig. 3) (Hammerschmidt and Sugden 1988; Schepers et al. 1993b). These include two essential, or *core*, components, separated by about 400 bp, and several

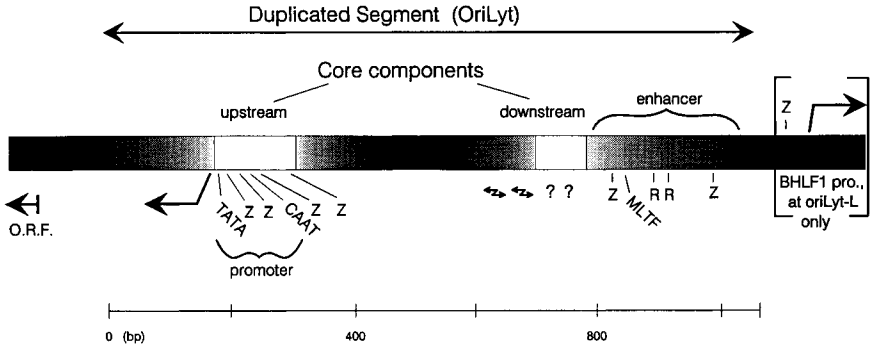

Figure 3 Functional components of *oriLyt*. *oriLyt* is contained within the 1055-bp duplicated segment, which is present at two locations on the EBV genome. The "upstream" and "downstream" core components of *oriLyt* are represented by the indicated white rectangles; the auxiliary regions are shaded. Bent arrows indicate the position and direction of transcription initiation. Binding sites for Z, R, MLTF, and for presumptive cellular factors (?) are indicated; a TATA-like element (GATAAAA) and a CAAT transcriptional element are also indicated. Zig-zag arrows indicate inverted repeats. The arrow at the lower left indicates the initial coding regions for BHLF1 and LF3 (also called *Not*I-repeat protein and *Pst*-repeat protein, respectively), which are synthesized from mRNA initiated from either the left or the right copy of *oriLyt*, respectively, on the EBV genome. Rightward transcription away from *oriLyt* occurs only for the left copy of *oriLyt* (Parker et al. 1990). The upstream core component was originally represented so as to exclude the two Z-binding sites most distal from the transcription start site (Schepers et al. 1993b). However, recent data indicate that the third and fourth ZREs support replication as effectively as the first two (Schepers et al. 1996), so the entire promoter region is presented here as a functional unit.

auxiliary components that contribute to activity but are not essential under all circumstances (see DePamphilis, this volume). The core components of EBV *oriLyt* lack significant activity in the absence of the auxiliary components, and the auxiliary effect of flanking DNA extends at least for several hundred base pairs to each side, making the functional boundaries of *oriLyt* rather ambiguous. One core component, at the left or "upstream" part of *oriLyt*, is a strong early promoter that is activated by Z, which binds to the promoter at four sites (Rooney et al. 1989; Leiberman et al. 1990); replication from *oriLyt* requires that Z interact with these same four sites (Schepers et al. 1993a,b). The transcriptional enhancer at the right end of *oriLyt* performs an auxiliary role that can be replaced by a heterologous enhancer (Hammerschmidt and Sugden

1988). The other core component of *oriLyt,* the "downstream" component, is a region less than 90 bp in length that is critical for replication but seems to play no role in transcription, consistent with its functioning directly in the initiation reaction. The factors that functionally interact with this critical region have not been identified, but the genetic studies mentioned above imply that they must be provided by the cell.

The HSV lytic origin, ori_S, has a single core component, to which the UL9 protein binds, flanked by divergent transcriptional control regions which serve auxiliary functions for replication (Challberg, this volume). EBV *oriLyt* contains two core components, but since one core component is a promoter, this might be viewed as a variation of the design used by HSV, with the "real" (downstream) core of EBV simply having greater dependence on its auxiliary components. The core of HSV ori_S in fact shows more autonomy from its auxiliary components than do the two EBV core components combined, since the HSV core can function as a cloned fragment as small as 150 bp, and the combined EBV core components cannot function alone. Studies to be described below, however, have revealed that the *oriLyt* promoter, or upstream core component, contributes to replication through interactions with Z in a more specific way than is typical of auxiliary components of replication origins.

A Specific Replication Function for Z; The Possible Involvement, or Entanglement, of Other Transcription Factors with *oriLyt*

The *oriLyt* promoter is quite efficient once activated by Z, yielding the two most abundant EBV early mRNAs, via transcription outward from each copy of *oriLyt* (Hummel and Kieff 1982; Freese et al. 1983). The fact that this strong promoter colocalizes with an essential component of *oriLyt* raises two questions. First, what promoter elements, if any, support replication activity? Second, do transcription factors aid replication directly through interactions among proteins, or indirectly by excluding nucleosomes from the region, or might transcription itself be part of the mechanism? (For general discussions of the involvement of transcriptional control elements with replication, see DePamphilis; van der Vliet; both this volume.)

Z is the only transcription factor that is known to be essential for the replication function of the *oriLyt* promoter (Schepers et al. 1993a). Replication activity was seen to be diminished, but not abolished, by deleting small regions of this core component (Schepers et al. 1993b). The results can be explained partly by the fact that all four Z-binding

sites are not required for replication, although all apparently contribute to it; abolishing either the two Z-binding sites closest to the transcriptional start site or the two farthest away by substitution mutations reduced replication activity to 20–30% of the wild-type level (Schepers et al. 1996). However, deleting from the TATA element through the CAAT element, or simultaneously deleting these two elements while leaving three of the four Z-binding sites, had the effect of completely abolishing replication activity (Schepers et al. 1993b). The study of these and other mutants indicates that the Z-binding sites alone might not be sufficient for the replication function of this region of *oriLyt* and that other promoter elements may contribute. Z has been shown to promote the stable association of transcription factors TFIID and TFIIA with the *oriLyt* promoter and to bind directly to TATA-binding protein (TBP), a component of TFIID (Lieberman and Berk 1991, 1994), so the *oriLyt* promoter might contribute to replication through a Z-containing transcriptional preinitiation complex.

The results of "domain-swapping" experiments involving Z suggest that Z contributes to replication in a way that other transcription factors cannot (Schepers et al. 1993a). The Z protein has two separable domains: an activation domain, which binds to TFIID (Lieberman and Berk 1991; Giot et al. 1991; Flemington et al. 1992), and a DNA-binding/dimerization domain, which is structurally related to the equivalent region of the AP1 family of cellular transcription factors (Z binds well to AP1 sites, but AP1 does not recognize ZREs; Farrell et al. 1989; Urier et al. 1989; Leiberman et al. 1990). Using altered versions of *oriLyt*, in which all Z-binding sites had been replaced with binding sites for either GAL4 (of yeast) or E2 (of BPV), Schepers et al. asked whether the activation domain from Z or from unrelated transcriptional activators would stimulate replication from *oriLyt*. (It was not possible simply to replace the activation domain of Z with other activation domains because wild-type Z is needed for induction of EBV replicative gene expression.) The result was that the *trans*-activation domain of Z could activate replication if bound to *oriLyt* through the binding domains of either GAL4 or E2, although the efficiency was much lower than with wild-type Z bound to *oriLyt* in the natural way. However, the transcriptional activation domains of several unrelated transcription factors, including c-fos, were completely inactive for support of replication, although they activated the *oriLyt* promoter effectively. Because the *trans*-activating domain of Z was unique in being able to activate replication, it is reasonable to suspect that replication and *trans*-activation might be distinct functions specified by the same region of the Z polypeptide.

It is clear from the domain-swapping experiments that efficient transcription from the *oriLyt* promoter is not a sufficient contribution from this component of *oriLyt* to support replication. Unfortunately, we still cannot conclude that transcription from this promoter is not a prerequisite for replication. For example, the GAL4-binding domain linked to the Z activation domain activated transcription from the *oriLyt* promoter only weakly, but replication was correspondingly weak (although it was specific) when compared to the wild-type situation. It seems simpler to assume that transcription and replication are independent processes with shared elements, but genetic manipulations that might allow efficient replication without transcription from the *oriLyt* promoter have not yet been achieved.

The enhancer at the right end of *oriLyt* is activated by Z and by another EBV early gene *trans*-activator, R (Chevallier-Greco et al. 1989; Cox et al. 1990). This region of *oriLyt* performs an auxiliary function for replication that can be replaced by the enhancer for the major immediate-early gene of human cytomegalovirus (Hammerschmidt and Sugden 1988; Schepers et al. 1993b). However, the results of the transfection studies mentioned above imply that R itself does not contribute significantly to replication from *oriLyt* (Fixman et al. 1995), and the Z-binding sites in this region do not contribute measurably to replication, either (Schepers et al. 1993b). A binding site for the ubiquitous transcription factor MLTF (USF) is largely responsible for constitutive activity of the *oriLyt* enhancer (Ryon et al. 1993), but the contribution of this site to replication has not been determined.

Little is known about the auxiliary functions of other sequences between the two core components of *oriLyt* or flanking them, except that sequences throughout *oriLyt* are about 90% identical between EBV and HVP, and thus among the most highly conserved regions of these viral genomes (Ryon et al. 1993). Deletion of a pair of inverted repeat sequences that exist to the left of the downstream core component (Fig. 3) reduced replication by 40% (Schepers et al. 1993b) or by 80% (Ryon et al. 1993) in two different assays.

Initiation of EBV Lytic Replication by Cellular Factors: A Common Mechanism for Herpesviruses?

Discovering what factors, presumably of cellular origin, functionally interact with the "downstream" core component will very probably be the key to learning how *oriLyt* is activated. The downstream component is

less than 100 bp long and contains a central 40-bp region that is highly sensitive to substitution mutations (Schepers et al. 1993b; Gruffat et al. 1995). This extreme sensitivity to substitution mutations contrasts with the additive nature of contributing elements at the *oriLyt* promoter and suggests an exactness of essential protein/DNA interactions. Deletions in this region either had no effect on transcription from the *oriLyt* promoter or caused a moderate increase in transcription, perhaps by disrupting association between proteins ordinarily bound to the downstream component and Z and transcription factors bound to the promoter (Schepers et al. 1993b).

Initiation of replication at HSV origins undoubtedly involves the UL9-encoded origin-binding protein, which has DNA helicase activity (Challberg, this volume). Since EBV encodes no equivalent function, it is natural to expect that a cellular helicase might be recruited by EBV to act at *oriLyt*. Despite these differences between the way the two viruses appear to initiate lytic replication, experiments performed by Fixman, Hayward, and Hayward indicate that the intermediates that are formed for initiation of replication by the two viruses are fundamentally similar. In a transfection assay for replication of an *oriLyt*-containing plasmid, providing the set of six replication proteins from HSV rather than from EBV gave full replication activity so long as the Z protein of EBV was provided (Fixman et al. 1995). This remarkable result would appear to indicate that HSV replication proteins do not need to recognize UL9 at the HSV origins and, because the HSV proteins probably cannot recognize Z, that the EBV replication proteins do not need to recognize Z at the EBV origins. The result suggests that the replication intermediates generated during initiation by Z and cellular proteins at the EBV lytic origins are similar or identical to those produced during initiation by UL9 (and cellular proteins?) at the HSV origins and that it is these replication intermediates that are recognized by the herpesvirus family replication proteins.

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