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Origins of Bidirectional Replication of Epstein–Barr Virus: Models for Understanding Mammalian Origins of DNA Synthesis

Jindong Wang* and Bill Sugden*

University of Wisconsin-Madison, Madison, Wisconsin 53706

Abstract Epstein–Barr virus (EBV), provides unique advantages to understand origins of replication in higher eukaryotes. EBV establishes itself efficiently in infected B lymphocytes, where it exists as a 165 kb, circular chromosome which is duplicated once per cell cycle (Adams [1987] J Virol 61:1743–1746). Five to twenty copies of the EBV chromosome are usually present in each cell, increasing the signal/noise ratio for mapping and analyzing its replication origins. Remarkably only one viral protein is required for the synthesis and partitioning of the viral chromosomes: EBV nuclear antigen-1, or EBNA1. EBV uses distinct origins related to the ARS1 origin of *Saccharomyces cerevisiae* and to that of the dihydrofolate reductase (DHFR) locus in Chinese hamster ovary (CHO) cells [Bogan et al., 2000]. We shall review the properties and the regulation of these two kinds of origins in EBV and relate them to their cellular cousins. J. Cell. Biochem. 94: 247–256, 2005. © 2004 Wiley-Liss, Inc.

Key words: EBV; origin; bidirectional replication; metazoan

The replication mechanisms employed in prokaryotes, small DNA tumor viruses, and *Saccharomyces cerevisiae* closely follow the paradigm laid out by Jacob and Brenner [1963]. The replication origins of these micro-organisims span a few hundred base pairs and have a modular structure, consisting of one or multiple, specific, binding sites for initiators and several auxillary *cis*-elements. The initiators function to recognize the origin specifically, assemble the replication machinery around the origin and help the initial unwinding of DNA. In *Escherichia coli*, three proteins are used to carry out these functions: DnaA binds multiple sites in *oriC*, the origin of bidirectional replication (OBR) of the *E. coli* genome, and then recruits DnaC, the helicase loader and the helicase, DnaB, facilitating local assembly of replication machinery including DNA polymerases, primases, and single-strand DNA (ssDNA) binding proteins, which then unwind the DNA double helix and initiate bidirectional replication at oriC [Baker and Bell, 1998].

Several of these initiator functions have been assumed by separate multi-protein complexes in the budding yeast, *Saccharomyces cerevisiae*: a six member protein complex, origin recognition complex (ORC), binds the ARS consensus sequence (ACS) and serves as the platform for the assembly of other components of the pre-replicative complex (pre-RC); CDC6 and CDT1, in conjunction with ORC, act as the helicase loader; and the MCM complex (minichromosome maintenance), a six-member ring-like complex, likely serves as the helicase [Bogan et al., 2000; Bell and Dutta, 2002]. The assembly of this pre-RC serves both to identify a site at which DNA synthesis can initiate and to regulate synthesis to occur once each cell cycle. Once synthesis has initiated, multiple mechanisms ensure that an origin is not used again during the same S phase [Yanow et al., 2001; Mendez et al., 2002; Wuarin et al., 2002]. In

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*Correspondence to: Jindong Wang, McArdle Laboratory of Cancer Research, University of Wisconsin-Madison, Madison, WI 53706. E-mail: wang@oncology.wisc.edu

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apparent contrast to origin-binding initiators of prokaryotes and DNA viruses, the ORC complex binds to ACS with low specificity [Mizushima et al., 2000]. In a gel shift assay, when equal molar concentrations of protein and probe were used, ORC was found to bind to a mutant ARS origin lacking an ACS sequence. This non-specific DNA binding could be inhibited with the addition of Cdc6 [Mizushima et al., 2000]. Moreover, the binding of ORC is not the only determinant of origin activity. Replication origins are distributed at intervals between 20 and 60 kb across the yeast genome, with each of them having sequence-matches to the ACS [Wyrick et al., 2001]. However, the presence of the ACS is not sufficient for origin activity and analyses of sequences flanking the ACS have not identified candidate regulatory elements [Wyrick et al., 2001]. Thus, in addition to ORC-binding sites, other factors, such as local chromatin structure or some unknown yeast proteins, likely regulate the assembly of pre-RCs.

Homologues of most of the components of pre-RC in the budding yeast have been identified and demonstrated to be essential for genome duplication in metazoans. The replication steps carried out by the pre-RC have also been shown to be conserved, for example, in studies with extracts of *Xenopus* eggs [Blow, 2001]. However, the processes of selecting sites at which DNA synthesis initiates have evolved increased complexity in metazoans. The nature of metazoan origins of DNA replication is elusive: they can be large, ranging from a few to tens of kilobase pairs long; they lack consensus sequences, none having been identified for the 20 or so known mammalian replication origins; and the replicator and the origin are separable in some cases [Gilbert, 2001]. *Drosophila* and human ORCs apparently bind DNA with too little specificity alone to dictate sites for origins of replication [Vashee et al., 2003; Remus et al., 2004]. In at least one case extracts of *Xenopus* eggs, where the concentration of ORC proteins is high, any DNA sequence can apparently be replicated [Blow et al., 2001]. At least two mechanisms could resolve this conundrum. First, there may exist one or multiple factors in addition to the binding of ORC that negatively or positively influence the firing of origins. For example, the *Drosophila* homologues of E2F1 (dE2F1) and Rb (Rbf) have been found to interact with DmORC and restrict amplification at the chorion gene locus [Bosco et al., 2001]. Secondly, it has been postulated that some unknown factors, acting in *cis* or *trans*, could direct ORC to bind specifically to the replication origins. EBV exemplifies this last model.

DS AND REP*: EBNA1-DEPENDENT REPLICATION ORIGINS

Two viral elements have been identified by screening overlapping fragments of EBV DNA for ones that can support the maintenance and synthesis of a plasmid DNA in human cells: oriP, or origin of plasmid replication, is the *cis*-acting element encompassing a 1.8 kb region in the BamHI C fragment of EBV genome; EBNA1, a latent viral protein, is the required viral *trans*-acting factor consisting of 641 amino acids [Yates et al., 1984; Reisman et al., 1985; Reisman and Sugden, 1986]. EBNA1 and oriP comprise the minimal EBV plasmid replicon. This subviral replicon is licensed, and maintained in the nucleus with the rate of loss being 2%–4% per cell generation [Yates and Guan, 1991; Kirchmaier and Sugden, 1995].

OriP is composed of two elements: a 20-member family of 30 bp repeats, FR, and about 1 kb away, a 120 bp region containing a 65 bp dyad symmetry element, DS. Each member of FR contains one 18 bp, high affinity, EBNA1-binding site. DS contains four EBNA1-binding sites with lower affinity than the sites in FR. FR and DS support extrachromosomal replication independently of their spacing or their relative orientation [Reisman et al., 1985; Kirchmaier and Sugden, 1995]. The replication origin function of oriP-containing plasmid relies entirely on DS. Tandem copies of DS can partially replace FR and still support plasmid replication in both short-term and long-term assays, whereas FR can not substitute for DS [Wysokenski and Yates, 1989; Leight et al., 2001]. In addition, plasmids containing only DS replicate at much higher levels in cells expressing EBNA1 than FR-only plasmids [Aiyar et al., 1998; Yates et al., 2000]. Hence, DS carries in *cis* enough information to support the initiation of DNA synthesis. Analyses using two dimensional gel electrophoresis (2D gel) of the replication intermediates of oriP-containing plasmids have mapped the position from which the replication forks originate to be at or near DS with a resolution of roughly 1 kb [Gahn and Schildkraut, 1989].

The four EBNA1-binding sites of DS can be viewed as two pairs, and each pair alone

supports extrachromosomal replication in long-term assays albeit at a reduced efficiency compared to wild type DS [Harrison et al., 1994; Shirakata and Hirai, 1998; Yates et al., 2000; Koons et al., 2001]. Within each pair, there is an exact 21 bp center-to-center spacing between EBNA1-binding sites [Rawlins et al., 1985]. Because 21 bp spans two full turns of the B-form of the DNA helix, EBNA1 proteins should bind DS on the same face of the DNA. The origin activity of DS has been shown to be dependent strictly on this spacing. Insertion or deletion of 1–2 bp between a pair of EBNA1-binding sites abolishes replication of the oriP plasmid [Bashaw and Yates, 2001]. When 10 bp is inserted to make the EBNA1-binding sites three helical turns apart but still in phase, the replication function of DS is also eliminated [Harrison et al., 1994]. Notably, the spacing between neighboring EBNA1-binding sites in FR is 30 bp and FR does not support initiation of DNA synthesis [Reisman et al., 1985; Aiyar et al., 1998; Yates et al., 2000]. These results demonstrate that the exact 21 bp-spacing is necessary for DS to support replication, and the same phasing of the two EBNA1-binding sites is not sufficient for its origin function. A precise spatial arrangement of initiator-binding sites also exists in related viral plasmid origins (Fig. 1A). The homologue of oriP of the herpesvirus papio (HVP), a close relative of EBV, functions in the presence of EBV's EBNA1, and the only conservation evident between them is this 21 bp center-to-center spacing [Loeb et al., 1990]. The plasmid origin of another related herpesvirus, Kaposi's sarcoma associated herpesvirus (KSHV), the terminal repeat (TR), contains a pair of binding sites for latency associated nuclear antigen (LANA), the replication initiator protein of KSHV with 22 bp center-to-center distance [Garber et al., 2002]. This spatial requirement is also reminiscent of the SV40 origin. The core origin of SV40 is arranged as two inverted pairs of T-antigen-binding pentanucleotides, and insertion of a single nucleotide in their center has been found to reduce replication dramatically [Cohen et al., 1984]. T-antigen is SV40's initiator which as a hexamer acts as a helicase [Stahl et al., 1986]. Interestingly a pair of pentanucleotides positioned about 10 bp apart in a head to head orientation was found to be the minimal requirement for the stable formation of the double hexamer of T-antigen on the core origin [Joo et al., 1997] (Fig. 1B). By analogy with the SV40 origin, a critical spacing requirement for EBNA1 to specify an origin of DNA synthesis may reflect its nucleating the formation of a cellular replication complex that acts bidirectionally, too.

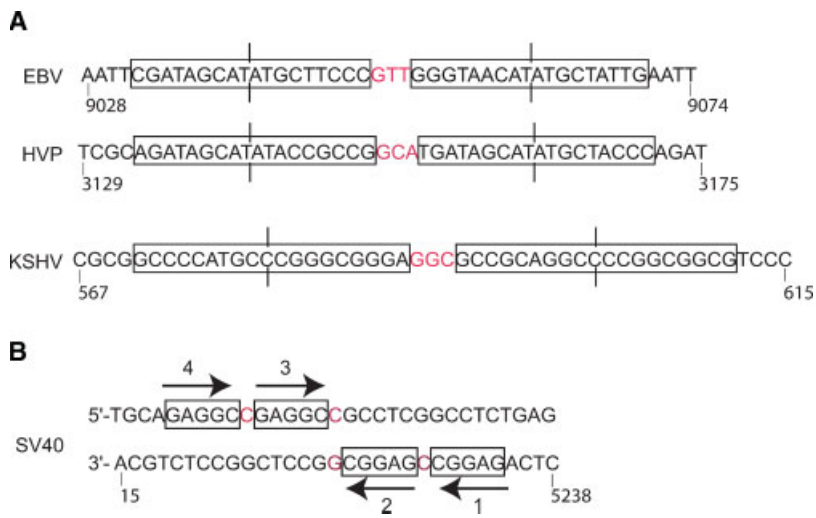

Fig. 1. DNA sequences of binding sites for viral binding proteins required for bidirectional replication. **A**: The pairs of binding sites for EBNA1 of Epstein–Barr virus (EBV) and herpesvirus papio (HVP), and latency associated nuclear antigen (LANA) of Kaposi's sarcoma associated herpesvirus (KSHV), the symmetric axis of each site and its coordinates in the viral genome are shown [Baer et al., 1984; Loeb et al., 1990; Garber et al., 2002]. The nucleotides located between the pairs are in red. Note that the center-to-center spacing of these pairs of viral protein binding sites are two full turns of the B-form DNA. **B**: Shown are the SV40 T antigen binding sites in site II, the main recognition sites in SV40's origin [Joo et al., 1997]. It is composed of four GAGGC palindromes with a single nucleotide in between them. Insertion of one nucleotide in the middle has been shown to dramatically reduce its origin activity [Cohen et al., 1984].

In addition to EBNA1-binding sites, DS also contains three copies of a nonamer sequence, TTAGGGTTA, which matches the consensus binding sites of telomere-binding proteins. Indeed, DNA affinity chromotography has demonstrated that telomere-associated proteins, such as telomere repeat binding factor (TRF)-1 and -2, bind these nonamers in the presence of EBNA1 [Deng et al., 2002]. The deletion of all three nonamers decreases the replication activity of DS by two-fold in long-term assays; changing the nonamers into G/C rich sequences also has a modest effect on the replication of each pair of DS [Yates et al., 2000; Koons et al., 2001; Deng et al., 2003]. Mutations in copies of this nonamer sequence do not appear to affect replication of intact oriP [Yates et al., 2000], and thus their role in the replication of oriP plasmids remains enigmatic.

Approximately 300 bp downstream of DS, a second small replicator, Rep*, was identified serendipitously in an extensive genetic screen of the sequences flanking oriP for elements that mediate EBNA1's transcriptional activity [Kirchmaier and Sugden, 1998]. Rep* replicates at 40% of the level of DS, and tandem copies of this viral element enhance its replication ability, permitting it to be studied in detail. Inspection of its sequence initially did not identify any EBNA1-binding sites. However, we have unexpectedly found that two pairs of EBNA1-dimers bind Rep* with a 21 bp spacing, and this pair of EBNA1-binding sites is both necessary and sufficient for its replicator function (Wang, Lindner, Leight, and Sugden, unpublished results). These data, in combination with the known properties of DS, indicate that the minimal requirement for recruitment of cellular replication machinery to synthesize this class of viral plasmid replicons is EBNA1 and a pair of properly spaced EBNA1-binding sites.

EBNA1: A VIRAL RECRUITER OF ORC?

EBNA1 regulates transcription as well as replication of EBV's genome through its interaction with oriP. It has a modular structure as do many DNA-binding proteins (Fig. 2). Its 641 amino acids can be divided into three parts: the N-terminus that contains amino acids 1–391; the joining region from amino acid 391 to 458; and the C-terminal DNA binding and dimerization domain from amino acids 459 to 641. Two thirds of its N-terminus is composed of glycine–glycine–alanine repeats. These repeats support an escape from the host's immune surveillance by inhibiting ubiquitin/proteasome-dependent degradation, thereby preventing the presentation of EBNA1-derived peptides by the MHC class I complex to cytotoxic T cells [Levitskaya et al., 1995]. A large deletion of these Gly–Gly–Ala repeats does not affect EBNA1's function to support transcription and replication of EBV-infected B cells [Leight and Sugden, 2000]. Much of our knowledge, in fact, of EBNA1's N-terminus has come from the mutational analysis of a derivative of EBNA1 from which 90% of the gly–gly–ala repeats have been deleted.

The C-terminus of EBNA1 dimerizes and binds to an 18 bp DNA binding sequence. The structure of the DNA-binding domain (DBD) of EBNA1 is strikingly similar to that of the DNA-binding domain of E2 of bovine papillomavirus-1, even though they share no sequence similarity. Both of them represent a bona fide structural motif for the recognition of DNA: an eight-stranded antiparallel β-barrel made up of four strands from each subunit of the dimer [Bochkarev et al., 1996]. Consistent with EBNA1's lacking a helicase activity [Frappier and O'Donnell, 1991; Middleton and Sugden, 1992], binding of EBNA1 to sites in DS only causes local distortion of the DNA but no unwinding as shown by in vivo footprinting and permanganate oxidation [Frappier and O'Donnell, 1992; Hsieh et al., 1993]. Similar to BPV1's E2, the DNA-binding domain of EBNA1 bends its binding site by approximately 45° [Hegde, 1995; Bashaw and Yates, 2001]. The structure of E2 [Hegde, 1995] indicates that its bound DNA bends continuously towards the

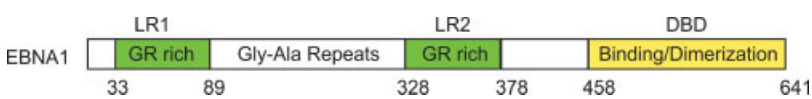

Fig. 2. Schematic structure of EBNA1. LR, linking region; GR, Glycine–Arginine; DBD, DNA-binding and dimerization. The amino-terminal half of EBNA1 contains two linking regions that can independently link DNA [Mackey et al., 1995]. Half of LR1 and 80% of LR2 consist of GR repeats, which have recently been shown to bind AT rich sequences preferentially [Sears et al., 2004]. The carboxy-terminus of EBNA1 encodes sequences required for site-specific DNA binding and for dimerization [Rawlins et al., 1985].

minor groove to complement the convex surface of E2's DBD; it is likely that EBNA1 bends DNA similarly in solution though its crystallized form apparently lacks this bend [Bochkarev et al., 1996].

In addition to its DNA-binding activity, EBNA1 has been found to have linking and chromosome-binding activity. Linking activity refers to the ability of EBNA1 to link DNAs it independently binds in gel shift assays or by its ability to loop out the intervening DNA between FR and DS as visualized in electron microscopy [Frappier and O'Donnell, 1991; Su et al., 1991; Middleton and Sugden, 1992]. The chromosome-binding activity refers to the ability of EBNA1 to associate with mitotic chromosomes as seen by fluorescence microscopy [Marechal et al., 1999]. These two biochemical activities are mediated by overlapping domains of EBNA1, LR1 (Linking region 1, amino acids 40–89) and LR2 (Linking region 2, amino acids 325–376) (Fig. 2). Half of LR1 and 80% of LR2 consist of glycine–arginine repeats which have recently been shown to bind AT-rich sequences preferentially [Sears et al., 2004]. This "AT-hook" activity of the N-terminus of EBNA1 may underlie its linking and chromosome-binding activities.

EBNA1 LIKELY FACILITATES ORC'S BINDING TO DS

Because EBNA1 has neither an ATPase nor a helicase activity, it has been postulated that EBNA1 supports DNA synthesis at DS by recruiting at least a cellular helicase to unwind DNA as an early step of replication [Leight and Sugden, 2000]. Several groups have reported that not only does a cellular helicase associate with DS but also ORC, the "loading pad" for the assembly of the pre-RC, localizes site-specifically to regions at or near DS [Chaudhuri et al., 2001; Schepers et al., 2001]. By using chromatin immunoprecipitation coupled with quantitative PCR, it has been shown that at least Orc 1, 2, 3, and 4 are associated with DS in the presence of EBNA1. Moreover, oriP was found not to replicate in a cell line expressing a mutant ORC2 [Dhar et al., 2001]. These observations are exciting in that oriP behaves like a cellular replication origin, its only viral idiosyncrasy being the need for EBNA1. Perhaps, EBNA1 is a viral mimic of the long-sought cell factor that fosters ORC's recognition of origins in the mammalian genome.

A fascinating question has now emerged: "How does EBNA1 recruit ORC to DS?" Much evidence indicates that the answer will be complex. First, EBNA1 by itself does not bind ORC. Numerous attempts to search for cellular partners that associate with EBNA1 have failed to identify any components of ORC. These efforts include both biochemical and genetic approaches: yeast two hybrid assays; yeast one hybrid assays using DS; and protein affinity purification using purified EBNA1 as a ligand [Fischer et al., 1997; Kim et al., 1997; Wang et al., 1997; Aiyar et al., 1998; Shire et al., 1999; Ito et al., 2000; Holowaty et al., 2003]. Second, EBNA1 bound to DS does not appear to bind ORC. DS has been used in affinity chromatography to purify EBNA1 and other proteins from extracts of EBNA1-positive cells. These experiments identified some telomere associated factors as binding DS in an EBNA1-dependent manner but did not detect binding of ORC components [Deng et al., 2002]. Third, the contributions of EBNA1 to DNA synthesis appear complex and this complexity may underlie the recruitment of ORC. The amino-terminus of EBNA1 contributes one or more essential functions to DNA synthesis because its carboxy-terminal DNA binding and dimerization domain acts as a dominant negative mutant for EBNA1 in short-term assays [Kirchmaier and Sugden, 1997]. Substitution of EBNA1's amino-terminus with histone H1 or high mobility group protein HMG-I(Y) restores EBNA1's wild type function yet these are common, chromatin constituents [Hung et al., 2001]. Possibly, even more confusingly, histone H1, has been found to inhibit the assembly of pre-RC in extracts of *Xenopus* eggs [Lu et al., 1998].

Although EBNA1's recruitment of ORC to DS is likely complex, there is sufficient information to propose elements for a model of how it does so. The origin activity of DS depends on a precise positioning of a pair of EBNA1 dimers. We propose that EBNA1's bending of DNA on its binding pairs of sites in DS yields a structure that can be recognized by ORC in the context of the local chromatin formed around DS. Although direct evidence for this model is lacking, there is some support by analogy from studies of the assembly of transcriptional initiation complexes. The TATA-binding protein (TBP), for example, bends its TATA element in the core promoter towards the major groove to form a saddle-like structure onto which the

transcriptional preinitiation complex can form [Nikolov et al., 1995]. This bending is intrinsic to TBP and the TATA element as it is to EBNA1 and its origins.

A REPLICATION INITIATION ZONE IN EBV USED IN SOME CELL LINES

DS is not the only replication origin present in the latent EBV genome. In fact, DS appears not to function as an origin in the viral genome found in several Buritt's lymphoma-derived cell lines [Little and Schildkraut, 1995; Norio and Schildkraut, 2001]. In addition, a mutant of EBV from which DS was deleted could still replicate in a cell line, BL30, with an efficiency similar to that of wild type strain [Norio et al., 2000]. Finally, EBV can establish latent infection and be maintained extrachromosomally in several rodent cell lines that express the human CD21 and HLA-DR as entry receptors for EBV [Haan et al., 2001]. Because DS does not support plasmid replication in rodent cells [Yates et al., 1985], this observation indicates that replication origins other than DS in the EBV genome can be used in some circumstances.

One such origin of DNA synthesis in the Raji cell line has been studied by 2D gels and a DNA fiber technique [Little and Schildkraut, 1995; Norio and Schildkraut, 2001]. DNA synthesis initiates primarily within a region of 14 kb, termed "Raji ori," which lies 30 kb away from oriP (Fig. 3). The 2D gel analyses of Raji ori have identified multiple inefficient initiation sites in it [Little and Schildkraut, 1995]. Raji ori thus shares its size and complexity with mammalian initiation zones.

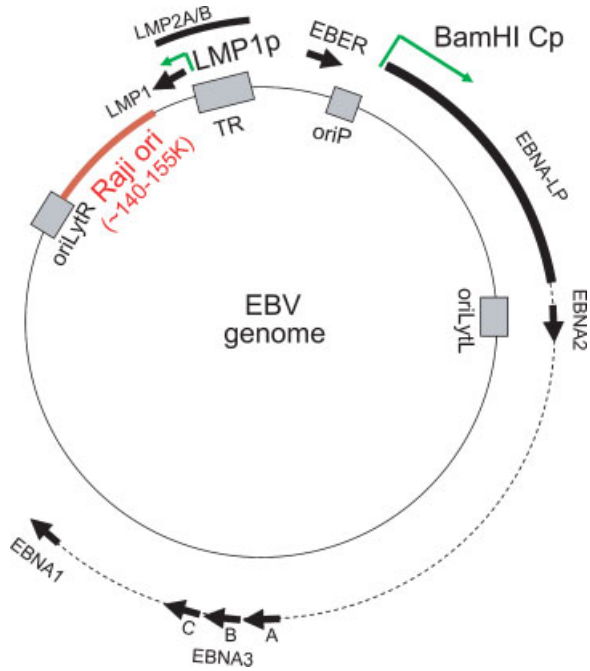

Fig. 3. Raji ori, which shares features with metazoan zones of replication, does not overlap with regions of the EBV genome that are actively transcribed in latently infected cells. The expressed viral genes, promoters frequently used and the abundant EBER transcripts during latent infection of EBV are depicted. EBNA1 binds to oriP, and enhances transcription from the LMP and BamHI C promoters, which are approximately 10 and 3 kb away from oriP, respectively [Sugden and Warren, 1989; Gahn and Sugden, 1995]. In the lytic phase of EBV's life cycle, DNA synthesis emanates from lytic origins, orilytL and orilytR (shown in black) using distinct, virus-encoded replication machinery. Bidirectional replication initiates in multiple domains throughout Raji ori (shown in red). The numbers in the parenthesis are coordinates for the DNA of B95-8 strain of EBV and approximate the region in the Raji strain of EBV.

Mammalian initiation zones span a few to tens of kilobase pairs of DNA, as does, for example, the 55 kbp dihydrofolate reductase (DHFR) locus in CHO cells. In this locus, most initiation events have been mapped to a 55 kbp intergenic spacer which is bordered upstream by the DHFR gene and downstream by a gene of unknown function. Different analyses of origins of DNA synthesis within this 55 kbp region using different techniques have yielded quite different pictures of it. Three primary initiation sites were identified in this region when it was subjected to origin-mapping techniques based on the abundance of nascently synthesized DNA; oriβ, oriβ', and oriγ [Vassilev et al., 1990; Kobayashi et al., 1998]. Because these three sites were estimated to account for more than 80% of the initiation events in this region, it was concluded that the DHFR initiation zone consists of several specific initiation sites for DNA synthesis. However, when replication intermediates in this 55 kbp locus were "visualized" by 2D gels, replication bubbles indicative of origins were detected in at least 20 sites with varying intensities throughout this zone [Dijkwel et al., 2000, 2002]. The intensities of the replication bubble structures surrounding oriβ, oriβ', and oriγ were only slightly greater than elsewhere within the 55 kbp region. It is particularly striking that a deletion of all three primary initiation sites identified by nascent strand abundance analyses showed no effect on replication timing or efficiency of replication of the DHFR locus while still supporting initiation

of DNA synthesis as measured by 2D gels [Mesner et al., 2003]. These disparate findings indicate that whatever elements specify the sites at which DNA synthesis initiates in mammalian zones of replication, they are redundant and may be independent of local DNA sequence. These apparently contradictory findings also demonstrate that metazoan origins of DNA replication are operationally defined; our understanding of them is necessarily limited by the experiments used to identify them.

A second ill-defined but tantalizing feature of mammalian replication zones is their relationship to sites of transcriptional initiation. Replication zones apparently rarely overlap sites of active transcription [Wei et al., 1998], a property shared with some mammalian viral origins of DNA synthesis. Viruses that rely on host cell replication machinery, such as simian virus 40, human papilloma viruses, and EBV with its oriP, have evolved with their origins of replication flanked but not overlapped by regions of active transcription. Raji ori also does not overlap regions of active transcription within EBV's genome in latently infected cells (Fig. 3). This feature may mean that either process interferes with the other.

The features of Raji ori shared with mammalian replication zones, their large size, multiple apparent sites of initiation of DNA synthesis, and lack of overlap with actively transcribed regions, indicate that a detailed analysis of Raji ori should help to illuminate the defining characteristics of metazoan zones of replication. These zones have been difficult to dissect genetically [Aladjem et al., 1998; Altman and Fanning, 2001; Mesner et al., 2003]; the genetic dissection of Raji ori may be more facile. Derivatives of EBV have been constructed that lack DS and use an equivalent of Raji ori [Norio et al., 2000]. EBV has also been cloned into an F-plasmid and now can be readily dissected genetically in its *E. coli* host, shuttled into mammalian cells, and there tested functionally [Delecluse et al., 1998]. The presence of FR in *cis* and EBNA1 in *trans* insures that EBV lacking DS but with Raji ori or its functional derivatives will be maintained extrachromosomally, thus obviating the uncertainties of integrating any given mammalian replication zone ectopically. Raji ori thus may prove a valuable tool with which to understand the puzzling complexities of mammalian origins of bidirectional replication.

TO FURTHER OUR UNDERSTANDING

Despite EBV's chromosome being only 165 kb, it uses two types of origins to support its duplication during its latency: the discrete and structurally simple DS; the delocalized and complex Raji ori replication zone. DS is amazingly efficient in initiating DNA synthesis by cellular factors given that it is only 120 bp in length. To compete against a vast excess of host genomic DNA for ORC, DS uses EBNA1 to nucleate the assembly of the multi-protein replication complex. Accumulated evidence indicates that the mechanism by which EBNA1 upon binding to a pair of precisely spaced binding sites, recruits ORC is complex and involves a network of protein–protein and protein–DNA interactions. Resolving this complexity likely will require more structural information about EBNA1 bound to an origin complexed with ORC; and/or an in vitro assay that reconstitutes the EBNA1-dependent replication of oriP. The presence of an initiation zone in several strains of EBV and their lack of currently identified specific sites for initiation of DNA synthesis is consistent with a model in which chromatin structure plays a determining role in defining initiation zones in mammalian cells and in EBV, too. To discover the control of the replication of these zones, we will need a detailed genetic dissection of their replication activity, and more knowledge about their chromatin structural information, such as their methylation and acetylation status in comparison to other parts of the EBV's genome.

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