Single-Stranded Structures Are Present within Plasmids Containing the Epstein-Barr Virus Latent Origin of Replication

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The Epstein-Barr virus (EBV) latent origin of plasmid replication (*oriP*) contains two essential regions, a family of repeats with 20 imperfect copies of a 30-bp sequence and a dyad symmetry element with four similar 30-bp repeats. Each of the repeats has an internal palindromic sequence and can bind EBNA 1, a protein that together with *oriP* constitutes the only viral element necessary for EBV maintenance and replication. Using single-strand-specific nucleases, we have probed plasmids containing *oriP*-derived sequences for the presence of secondary structural elements. Multiple single-stranded structures were detected within the *oriP* region. Of the two essential elements of *oriP*, the family of repeats seemed to extrude these structures at a much higher frequency than did sequences within the dyad symmetry region. Though negative supercoiling was found to stabilize the single-stranded structures, they showed significant stability even after linearization of the *oriP* plasmids. Two major single-stranded structures detected involved approximately 12 bp of DNA. These loci could be transiently unwound regions that form because of negative supercoiling and the high A+T content of this region of DNA, or they could be cruciform structures extruded within the palindromic sequences of *oriP* that may be important sites for protein-DNA interactions in the EBV *oriP*.

Epstein-Barr virus (EBV), a human herpesvirus, infects B lymphocytes and transforms them into continuously growing cell lines. In most of these cells, the virus is latent. The 172-kb double-stranded DNA genome is carried as a multi-copy, supercoiled plasmid, and only a small number of viral genes are transcribed (reviewed in references 21 and 30). Replication of the EBV genome during latency occurs in synchrony with host cell DNA synthesis (1, 15) and requires only two viral elements. One of these is a *cis*-acting sequence called *oriP* (for origin of plasmid replication), which is found on a 1.8-kb subfragment of the EBV genome (51) (Fig. 1A). Sequence analysis of *oriP* revealed the presence of two unusual regions. One region, containing approximately 20 tandem, albeit imperfect copies of a 30-bp sequence, is called the family of repeats (FR). Located about 1 kb away is the second region, which contains a 65-bp dyad symmetry (DS) area within four additional copies of the 30-bp repeat (2). Deletion analysis indicated that both regions were necessary for *oriP* function (27, 38), though neither their relative orientations nor the distances separating them seemed to significantly affect their activity (38). The actual origin of replication seems to be within the DS element, while the FR region contains the termination site for replication (12).

The second viral element required for EBV replication in addition to *oriP* is EBNA 1, which acts in *trans* (27, 52). This protein is encoded in the *Bam*HI K fragment of the genome, far from the *oriP* region (9, 48). Studies using a bacterial fusion protein containing the carboxy-terminal one-third of EBNA 1 and partially purified EBNA 1 from lymphocytes showed that this protein could bind to each of the two essential loci of *oriP* (18, 37). DNase footprinting demonstrated that binding of EBNA 1 to linearized, labeled substrate DNA protected 24 to 25 bp of each of the 30-bp elements within the FR element as well as each of the four repeat elements in the DS region. A consensus sequence of

the EBNA 1 binding sites reveals an internal 12-bp palindrome, over which the DNase-protected region centers (37) (Fig. 1B). In addition to roles in replication, EBNA 1 and *oriP* seem to be involved in transcription during latent infection. Presence of the FR element in *cis* and EBNA 1 in *trans* has been shown to enhance transcription from heterologous promoters (38). Furthermore, an EBV promoter downstream of *oriP* which is the starting point for transcription of at least two of the known latent gene products, including EBNA 1 itself, can be transactivated by EBNA 1 (46). Thus, by virtue of its interaction with EBNA 1, *oriP* influences the biology of EBV in many ways.

The EBV origin of replication used in latent infection is particularly interesting with respect to potential secondary structures. Inverted repeat sequences are known to be able to form cruciform structures by intrastrand base pairing (14, 36). Palindromes can also bind with identical palindromes located nearby, and because the FR region contains 20 tandem repeats of an essentially 30-bp palindromic sequence (Fig. 1B), a very large number of alternative secondary structures is possible (20). The DS element too may form several alternative secondary structures (20, 37), and all of these would occur within sequences that are known to have the capacity to bind EBNA 1 (18, 37). In addition to the formation of such relatively stable structures, the *oriP* region, because of its high A+T content, might be expected to form transiently unwound, less stable structures under the influence of negative supercoiling. These too could occur within sequences that bind EBNA 1. A detailed understanding of the interaction between *oriP* and EBNA 1 and of the molecular basis for the functions that this interaction influences must therefore include a knowledge of the structures assumed by sequences within the *oriP* region. Although such structures were predicted (20, 37), no experimental data as to whether or not they exist have been available. We show here that incubation of supercoiled plasmids containing *oriP* sequences with single-strand-specific nucleases results in the appearance of multiple nuclease cleavage sites. Restriction

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FIG. 1. EBV *oriP* region and consensus sequence of the 30-bp repeats. The two essential elements of *oriP* are the FR and the DS elements (A), shown darkly stippled. Several restriction sites as reference points are indicated, as are the boundaries of the FR and the DS regions, all numbered according to their positions on the B95-8 EBV genome sequence (2). (B) Consensus sequence of the 30-bp repeats in *oriP*. The internal 12-bp palindrome is indicated.

mapping showed that many of these sites are localized within regions containing elements of *oriP*. The data are consistent with the presence of single-stranded structures within the EBV *oriP* in vitro.

MATERIALS AND METHODS

Enzymes. Endonuclease T7 and nuclease P1 were from Pharmacia-LKB Biotechnology (Piscataway, N.J.); nuclease S1 was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Restriction enzymes were purchased from New England BioLabs (Beverly, Mass.) or Boehringer Mannheim and were used according to the manufacturer's specifications.

Plasmids. The EBV origin-containing plasmid pHEBO was a gift from B. Sugden (University of Wisconsin) (45). Other recombinant clones were constructed in our laboratory and have been described previously except for pSV2neo: C_{6,0} (14a) and pSV2CAT: C_{HET} (gift of J. L. Kolman). Plasmids were propagated in appropriate bacterial hosts and purified from *Escherichia coli* by standard techniques (28).

Probing single-stranded regions in plasmid DNAs. The presence of single-stranded regions in covalently closed, supercoiled plasmid DNAs was studied by using T7 endonuclease and nuclease P1. Typically, 200 ng of plasmid DNA was incubated with 6 U of T7 or 0.01 U of P1 at 37°C for 60 min. Endonuclease T7 reaction buffer contained 50 mM Tris hydrochloride (pH 8.0), 20 mM NaCl, 6 mM MgCl₂, 5 mM dithiothreitol, and 100 µg of bovine serum albumin per ml; nuclease P1 buffer contained 10 mM Tris hydrochloride (pH 7.5), 40 mM NaCl, and 0.4 mM ZnCl₂. Reactions were stopped by phenol-chloroform extraction, and products were concentrated by ethanol precipitation. Nuclease cleavage sites were mapped with respect to certain restriction enzyme sites by cutting the resuspended DNA with the enzyme of choice. After removal of protein, the products were analyzed by agarose gel electrophoresis and subsequent Southern blotting using radiolabeled probes.

The effect of negative supercoiling on the stability of the single-stranded structures was studied by comparing plasmids cleaved with endonuclease T7 and subsequently with a restriction enzyme with plasmids cleaved first with the restriction enzyme and then with the endonuclease. Both the endonuclease and restriction enzyme reactions were carried

out at 37°C for 60 min. DNA was ethanol precipitated on dry ice for 15 min between the two reactions, and protein was removed before agarose gel electrophoresis.

Estimates of the sizes of single-stranded structures were obtained by first digesting plasmid DNAs with endonuclease T7; after phenol-chloroform extraction, aliquots of the products were further digested with 3 U of nuclease S1 at 37°C for 30 min. Mapping was again done with respect to known restriction enzyme cleavage sites. To accurately separate the small fragments obtained from this procedure, reaction products were subjected to electrophoresis in 3.0% NuSieve GTG agarose gels (FMC Bioproducts; Rockland, Maine) and detected by Southern blotting.

Agarose gel electrophoresis and Southern blotting. The products of experiments studying plasmid DNA structure were subjected to electrophoresis in 0.8 to 1.2% agarose gels under standard conditions (28). *Hind*III-digested lambda bacteriophage DNA (New England BioLabs) was subjected to electrophoresis along with the samples to provide molecular size markers. Agarose gels were transferred to nitrocellulose (BA85; Schleicher & Schuell, Keene, N.H.) or Nytran (Schleicher & Schuell) nylon filters and hybridized by a modification of the method of Southern (44) in the presence of deionized formamide (28). Radiolabeled DNA probes were prepared by nick translation (40) and purified from unincorporated radioactive dCTP on Pharmacia NICK-T Sephadex G-50 columns. Specific activities of greater than 10⁸ cpm/µg of substrate DNA were regularly obtained.

RESULTS

Plasmids containing *oriP* sequences extrude multiple single-stranded structures. T7 endonuclease, the product of the bacteriophage T7 gene 3, is a single-strand-specific nuclease that also has a very high affinity for branched structures in DNA such as cruciforms (8, 34). These elements are recognized by the enzyme and subsequently cleaved, generating linear DNA molecules from the supercoiled substrate. Positions of the cuts can then be mapped with respect to known restriction enzyme cleavage sites (35). This approach was applied to study the plasmids pSV2neo: Bam C, pSV2neo: C_{6,0}, and pHEBO, which contain *oriP* sequences, and, as a control, pSV2neo, which contains only vector sequences.

pSV2neo DNA, as well as the other plasmid preparations, is a mixture of covalently closed supercoiled form I DNA with nicked form II and some linear form III species (Fig. 2A, lane pSV2neo /). Cleavage of this plasmid with endonuclease T7 slightly increased the amount of linear DNA present (lane pSV2neo T7), but subsequent digestion with the restriction enzyme *Hind*III reveals two novel bands (lane pSV2neo T7/H3, open arrowheads). One distinct band migrated in a position corresponding to about 4,370 bp, while the other, a weak band clearly visible on the autoradiographs but poorly reproduced in the prints, migrated at a position corresponding to about 1,470 bp. The sum of the base pairs of these two bands is about equal to the number of base pairs in the intact 5,729-bp plasmid, thus suggesting a single cleavage site. This finding, along with data obtained by using other restriction enzymes, allowed mapping of the cleavage site to an area between the origin of replication and the ampicillin resistance gene (see Fig. 2B for a diagrammatic summary of the cleavage data). This part of pSV2neo is derived from the plasmid pBR322, which contains a sequence known to be able to form a cruciform (35) and thus serves as a good positive control for the activity of the endonuclease.

FIG. 2. Mapping of endonuclease T7-sensitive sites in plasmid DNAs. (A) The *oriP*-containing plasmids pSV2neo: Bam C, pSV2neo: C_{6.0}, and pHEBO were studied, along with pSV2neo, which contains no EBV sequences. A 200-ng sample of plasmid DNA consisting of a mixture of supercoiled (S), linear (L), and nicked (N) forms was used in each reaction. Plasmid DNAs were incubated either in the absence of enzymes (/), cleaved only with endonuclease T7 (T7), cleaved only with *Hind*III (H3), or cleaved with endonuclease T7 and then with *Hind*III (T7/H3). Endonuclease T7 digestions used 6 U of enzyme and were carried out for 1 h at 37°C. *Hind*III restriction endonuclease cleaves each plasmid at a single site. Products of the reactions were resolved by agarose gel electrophoresis and detected by Southern blotting (44). Sizes of the fragments generated were determined by linear regression analysis, using *Hind*III-digested λ DNA fragments as molecular size markers (indicated on the left in kilobases). DNA fragments within each T7/H3 lane are marked according to the position of the T7 cleavage

Cleavage of pSV2neo: Bam C DNA with T7 and then with *Hind*III again identified the 1,470-bp band. Therefore, this nuclease-sensitive structure is also present in a plasmid containing inserted EBV DNA. Many new cleavage products, however, were detected. There were two major bands of about 7,410 and 7,040 bp which had approximately equal intensities and were therefore of about equal molarity (Fig. 2A, lane pSV2neo: Bam C T7/H3, large filled arrowheads). The sizes of these two fragments add up to approximately the size of the pSV2neo: Bam C plasmid itself, which suggests that a single cleavage was responsible for generating these fragments; this site could be located within a region corresponding to the EBV *oriP*, whose FR element is located 6,800 to 7,400 bp away from the *Hind*III site on the plasmid (Fig. 2B). In addition to these major bands, there were four weaker bands of 10,600, 5,900, 4,280, and 3,740 bp. Two of these bands, those of 5,900 and 3,740 bp, were also present in pSV2neo: Bam C digested only with T7 (lane pSV2neo: Bam C T7, double open arrowheads). Since these fragments are bounded by T7 cleavage sites, they are not useful in mapping. They do, however, suggest that the endonuclease can cleave the plasmid DNA at a minimum of two sites in addition to cleavage within the region containing the pBR322-derived fragment and that the T7 cleavages that generated them occurred in the cloned EBV DNA. The bands of 10,600 and 4,280 bp (small filled arrowheads), which were approximately equimolar and added up to the size of the entire pSV2neo: Bam C plasmid, seemed to be generated by a single endonuclease T7 cleavage (Fig. 2B). This recognition site for T7 would be located downstream of the *oriP* sequences in a region that contains an EBV promoter known to function during latent infection (5).

pSV2neo: C_{6.0} contains a 6.0-kb *Eco*RI-*Bam*HI subfragment of the region found in pSV2neo: Bam C (14a). Cleavage with T7 followed by *Hind*III resulted in the appearance of two major bands of about equal intensity with migration rates corresponding to about 7,490 and 3,480 bp, respectively (Fig. 2A, lane pSV2neo: C_{6.0} T7/H3, large filled arrowheads), suggesting that they are present in equimolar amounts. The sizes of these two fragments added up approximately to the size of the intact plasmid DNA, which suggests that cleavage by endonuclease T7 at one site within the plasmid was responsible for generating both fragments (Fig. 2B). Several minor components were also visible, with those corresponding to 6,900 and 4,280 bp being the most

site. Bands marked with an open arrowhead result from T7 cleavage within vector sequences, those marked with a large filled arrowhead are major cleavage sites within the EBV *oriP*-derived sequences, small filled arrowheads indicate minor cleavage sites within other regions of the EBV-derived DNA, and double open arrowheads indicate fragments bounded at both ends by T7 cleavage sites. The 1,570-bp bands in pSV2neo, pSV2neo: Bam C, and pSV2neo: C_{6.0} T7/H3 digests were visible on autoradiographs but did not reproduce well in the prints. (B) Major endonuclease T7 cleavage sites on plasmids are presented in diagrammatic form and are oriented with respect to the single *Hind*III site on each of the plasmids, which have been drawn as if they had been linearized at this point. The sizes of the fragments that resulted after digestion with T7 and *Hind*III are indicated in base pairs. Bold numbers and lines indicate cleavage within EBV-derived sequences, light numbers and lines indicate cleavage within vector-derived sequences. Blackened boxes represent the EBV *oriP*-derived sequences, darkly stippled regions indicate cloned EBV sequences that flank the *oriP*, lightly stippled boxes represent antibiotic resistance genes, and blank boxes represent the pBR322-derived procaryotic replication origin.

FIG. 3. Mapping of nuclease P1-sensitive sites in pSV2neo and pSV2neo: C_{6.0} in the presence and absence of EBNA 1. (A) Plasmids pSV2neo and pSV2neo: C_{6.0} in the presence (+EBNA 1) or absence (-EBNA 1) of 100 ng of bacterially expressed (33), immunoaffinity-purified EBNA 1 were exposed to 0.01 U of nuclease P1. Products were deproteinized, either electrophoresed without further modification (/) or digested with various restriction enzymes (R.E.) as indicated, and analyzed by Southern blotting (44). Sizes of the detected fragments were calculated by linear regression analysis, using the *Hind*III-digested λ DNA fragments as standards (indicated at the left in kilobases). Because of a lower abundance of cleavage products, in order to visualize small fragments the Southern blot of this experiment had to be overexposed, and only the lower half is shown for clarity. Open arrowheads indicate fragments resulting from cleavage within vector sequences; closed arrowheads indicate fragments resulting from cleavage within *oriP*-derived sequences. The purification of EBNA 1, as well as further studies on its effect on plasmids bearing the EBV *oriP*, will be presented elsewhere. (B) Major nuclease P1 cleavage sites on pSV2neo: C_{6.0}

evident (small closed arrowheads). The sizes of these fragments also added up to approximately the size of the entire plasmid, which implies cleavage at a single site. This position maps to the same region identified above in pSV2neo: Bam C near the latent promoter 3' to *oriP*.

The plasmid pHEBO contains less of the *oriP* region than does either pSV2neo: Bam C or pSV2neo: C_{6.0} and because of its smaller size is a better target for mapping studies. Treatment with T7 and then *Hind*III generated a very strong band at 5,840 to 6,090 bp and a ladder of bands from about 1,140 to 1,460 bp (Fig. 2A, lane pHEBO T7/H3, closed arrowheads). These two sets of fragments were bounded by T7 and *Hind*III sites, and the total of their sizes agrees well with the complete length of pHEBO, 7,068 bp. These data, along with those presented subsequently, allowed mapping of the cleavage sites resulting in these fragments to an area clockwise to the *Hind*III site. This area, from about 880 to 1,480 bp, contains the FR element of *oriP*. Thus, these repeats, in the context of a supercoiled plasmid environment, can extrude single-stranded structures. Furthermore, several of the repeats have this capacity, since several bands are seen in this region, and it is likely that endonuclease T7 cuts at several structures being extruded by different repeats on different plasmids. This would explain the width of the band at about 6,470 bp, since it is probably made up of several DNA fragments varying in length by up to 600 bp. These fragments would be generated by endonuclease cleavage at different repeats within the FR element of *oriP*, followed by linearization with *Hind*III.

In addition to several of the repeats having the ability to extrude single-stranded structures, more than one such structure seemed to be extruded in at least some of the *oriP*-containing plasmids. Examination of each of the T7 digestion lanes showed that though linear DNA was the major product, several fragments appeared that were the products of T7 cleavage at more than one site on a plasmid. This could occur either by simultaneous attack of endonuclease at two positions or by attack at one position followed by attack at a second before reabsorption of the second region due to loss of supercoiling. pSV2neo, by comparison, did not seem to yield any such products of double T7 digestion.

Fine mapping of single-strand nuclease cleavage sites. Use of endonuclease T7 had demonstrated that *oriP*-containing plasmids contained regions of DNA susceptible to cleavage with this single-stranded nuclease. It was of interest to verify these results with a second nuclease that is often used to probe for single-stranded regions within DNA, nuclease P1 (7, 22). Cleavage of pSV2neo with nuclease P1 and *Hind*III generated a fragment of about 4,480 bp and three closely spaced fragments at 1,280 to 1,460 bp (Fig. 3A, lane pSV2neo -EBNA 1, open arrowheads). The combination of the sizes of the larger fragment with that of any of the smaller ones equaled approximately that of the pSV2neo plasmid and allowed mapping of this site into the area identified earlier with T7 that contains a cruciform derived from

identified in panel A are indicated in diagrammatic form and are oriented with respect to the cleavage site of each of the restriction enzymes used. The plasmid is drawn as if that restriction site were at the left of the diagram. Blackened boxes represent the EBV *oriP*-derived sequences, darkly stippled regions represent cloned EBV sequences that flank the *oriP*, lightly stippled boxes represent antibiotic resistance genes, and blank boxes represent the pBR322-derived procaryotic replication origin.

pBR322 sequences. Endonuclease T7 was also seen to generate three lower-molecular-weight products when it digested plasmids containing this region of DNA (see Fig. 5, lane pSV2CAT T7/H3). Cleavage of pSV2neo: C_{6.0} with nuclease P1 and then one of five different restriction enzymes resulted in the generation of a set of intense bands at different positions, depending on the enzyme used (Fig. 3A, lane pSV2neo: C_{6.0} -EBNA 1, closed arrowheads). Linear regression analysis indicated that the nuclease P1-sensitive sites were located 3,160 to 3,830 bp from the *Hind*III site, 550 to 850 bp from the *Eco*RI site, 5,120 to 6,080 bp from the *Bam*HI site, 900 to 1,440 bp from the *Eco*RV site, and 2,360 to 2,900 bp from the *Kpn*I site (see Fig. 3B for a diagrammatic summary of these data). All of these sites map into a region of DNA that contains the FR element of *oriP* and confirm the data presented above for the T7 single-strand specific endonuclease. Figure 3 also shows the effect of preincubating the plasmids with bacterially expressed (33), purified EBNA 1. The presence of EBNA 1 seemed to enhance cleavage by nuclease P1 within the same sequences that were recognized in unbound pSV2neo: C_{6.0} plasmid DNA, but it seemed to have little effect on pSV2neo, which does not contain high-affinity EBNA 1 binding sites. Further studies to determine the effect of binding of EBNA 1 to plasmids containing the EBV *oriP* are under way and will be presented elsewhere.

To more closely map the endonuclease T7-sensitive sites, pSV2neo: C_{6.0} was examined with additional restriction enzymes (Fig. 4A). Plasmid DNA was cleaved with T7 and then with a combination of two restriction enzymes. Cleavage with *Eco*RI and *Eco*RV selects against visualization of products formed by T7 cutting at the dyad because the *Eco*RV digestion would give fragments of 160 bp or less that would not be detected in this gel system. Three major fragments are clearly visible. Two of these (open arrowheads), those of about 9,300 and 1,350 bp, were derived from cleavage of pSV2neo: C_{6.0} only with *Eco*RI and *Eco*RV, as seen in an adjacent control lane (pSV2neo: C_{6.0} R1/RV) in which products of a similar reaction with the same restriction enzymes but in the absence of T7 were analyzed. The third major component of the reaction detected was a set of bands ranging in size from about 1,020 to 1,150 bp (filled arrowhead). Since these were seen only after treatment of the T7 digest with *Eco*RI and *Eco*RV nucleases, the cleav-

ages producing these bands occur 1,020 to 1,150 bp from one of these two enzyme sites.

To determine more precisely the locations of the sites cleaved by endonuclease T7 to generate this last set of fragments, a parallel set of reactions was cleaved with *Eco*RV and *Kpn*I. These once again revealed a set of fragments ranging in size from 1,020 to 1,150 bp (Fig. 4A, lane pSV2neo: C_{6.0} RV/K1, filled arrowhead). Were these T7 cleavage sites located about 1,100 bp from the *Eco*RI site, cleavage with *Eco*RV and *Kpn*I would have increased their sizes by about 1,700 bp, but no such fragments of approximately 2,800 bp are visible. The T7 cleavages, therefore, occurred 1,020 to 1,150 bp away from the *Eco*RV site and map into the right end of the FR element of *oriP* (see Fig. 4B for a diagrammatic summary of the data). This element is present about 960 to 1,540 bp away from the *Eco*RV site in pSV2neo: C_{6.0}.

FIG. 4. Fine mapping of endonuclease T7-sensitive sites in pSV2neo: C_{6.0}. (A) pSV2neo: C_{6.0} plasmid DNA was either incubated in the absence of enzyme (/), cleaved only with endonuclease T7 (T7), cleaved with endonuclease T7 and then with restriction enzymes *Eco*RI and *Eco*RV (T7/R1/RV), cleaved with endonuclease T7 and then with restriction enzymes *Eco*RV and *Kpn*I (T7/RV/K1), cleaved only with restriction enzymes *Eco*RI and *Eco*RV (R1/RV), or cleaved only with restriction enzymes *Eco*RV and *Kpn*I (RV/K1). Open arrowheads indicate DNA fragments generated by restriction enzyme cleavage of pSV2neo: C_{6.0}; filled arrowheads indicate bands resulting from T7 cleavage within cloned EBV sequences. Radiolabeled pSV2neo: Bam C was used as the probe. (B) Endonuclease T7 cleavage sites and the fragments generated by restriction enzyme cleavage are shown in diagrammatic form. The top diagram represents the results obtained with the *Eco*RV and *Kpn*I enzymes; the bottom diagram represents results from the *Eco*RI-*Eco*RV trials. Numbers in light type indicate the sizes of fragments generated when pSV2neo: C_{6.0} was digested with the restriction enzymes alone; numbers in bold type indicate the sizes of fragments generated by endonuclease T7 cleavage followed by the respective restriction enzymes.

Single-strand nuclease cleavage of plasmids lacking the DS element. To determine whether the FR elements present alone on a plasmid without the DS area could extrude single-stranded regions of DNA, the plasmid pSV2CAT: C_{HET} was examined. This construct contains a cloned 7,196-bp fragment derived from a heterogeneous, rearranged strain of EBV and contains part of the repeat elements but is missing the dyad (21a). Cleavage of the parent plasmid, pSV2CAT, with endonuclease T7 followed by *Hind*III yielded a novel fragment of about 4,120 bp that was visible between the bands representing linear pSV2CAT plasmid DNA and residual uncleaved supercoiled plasmid (Fig. 5A, lane pSV2CAT T7/H3). Three additional fragments of about 1,420 bp were also generated which were visible on the original autoradiograph but poorly reproduced in the photographs. The fact that the sizes of these two sets of fragments (open arrowheads), within the limits of resolution of agarose gel electrophoresis, added up to approximately the size of the pSV2CAT plasmid itself implies that a single cleavage event by the T7 enzyme created them. This event most probably occurred at a site between the *amp* and *ori* genes of pSV2CAT (Fig. 5B) which, as for pSV2neo, is derived from pBR322 and contains a sequence known to be able to form a cruciform (35).

When pSV2CAT: C_{HET} was exposed to endonuclease T7 followed by *Hind*III, two major fragments of approximately equal intensity were formed (Fig. 5A, lane pSV2CAT: C_{HET} T7/H3, closed arrowheads), suggesting a single cleavage site. The sizes of these fragments are 7,000 and 5,660 bp, respectively, and the cleavage site maps approximately to the middle of the C_{HET} fragment where the 30-bp repeats derived from *oriP* are located (Fig. 5B). Again faintly evident on the original autoradiograph is a band of about 1,420 bp, along with a band of about 11,000 bp (open arrowheads), suggesting that cleavage at the pBR322-derived cruciform can also occur. Cleavage within the sequences derived from the FR element of *oriP*, however, seemed much more pronounced.

Supercoiling stabilizes the single-stranded structures but relaxation is not instantaneous. The extrusion of double-stranded DNA into transient or stable single-stranded structures is often driven by negative supercoiling (17, 31). To determine whether this is the case for the *oriP* structures, pSV2neo: C_{6.0} either was cut with T7 and then with *Hind*III or was first cut with the restriction enzymes and then cut with the endonuclease (Fig. 6). Cleavage with T7 and then *Hind*III led to the appearance of three major bands having lengths of 7,480, 3,480, and 3,090 bp and a minor band of 4,370 bp with respect to the *Hind*III site. When the plasmid was first linearized with *Hind*III, however, subsequent use of T7 produced bands of the same size but of reduced intensity (pSV2neo: C_{6.0} T7/H3 versus H3/T7). Similarly, cleavage of the DNA with *Bam*HI before addition of endonuclease reduced subsequent T7 cleavage of cruciform structures (T7/B1 versus B1/T7). Calculations based on laser densitometric scanning of the original autoradiograph (data not shown) indicated that precleavage with *Bam*HI decreased subsequent T7 digestion by 1.8-fold, while predigestion with *Hind*III decreased T7 sensitivity by about 2.7-fold. Completion of linearization is demonstrated in the last two lanes of Fig. 6, in which pSV2neo: C_{6.0} cut either with *Bam*HI or *Hind*III was subjected to electrophoresis. Aliquots of these reaction mixtures were used for further analyses with endonuclease T7. Thus, loss of negative supercoiling led to the relaxation of many of the structures

FIG. 5. Sensitivity of *oriP* plasmids lacking the DS symmetry element to cleavage by endonuclease T7. (A) Plasmid pSV2CAT: C_{HET}, which contains only the FR element of *oriP*, and its control pSV2CAT, which contains no EBV DNA, were examined for endonuclease T7 sensitivity. As for Fig. 3, plasmids were incubated in the absence of enzyme (/), cleaved only with endonuclease T7 (T7), cleaved only with *Hind*III (H3), or cleaved with endonuclease T7 and then with *Hind*III (T7/H3). Supercoiled, linear, and nicked forms of pSV2CAT are indicated by S, L, and N. Filled arrowheads indicate fragments generated by endonuclease T7 cleavage in *oriP*-derived sequences; open arrowheads indicate fragments resulting from T7 cleavage within vector sequences. Radiolabeled pSV2neo: Bam C was used as the probe. (B) Major endonuclease T7 cleavage sites within pSV2CAT and pSV2CAT: C_{HET} with respect to the single *Hind*III site in each plasmid are presented in diagrammatic form. Plasmids are drawn as described in the legend to Fig. 2B. Light numbers and lines indicate cleavage within vector sequences; bold lines and numbers indicate cleavage within EBV *oriP*-derived sequences.

FIG. 6. Effect of supercoiling on stability of the single-stranded structures. Plasmid pSV2neo: $C_{6,0}$ was incubated in the absence of enzymes (/), cleaved only with endonuclease T7 (T7), cleaved first with endonuclease T7 and then with *Hind*III (T7/H3), cleaved first with *Hind*III and then with endonuclease T7 (H3/T7), cleaved first with endonuclease T7 and then with *Bam*HI (T7/B1), cleaved first with *Bam*HI and then with endonuclease T7 (B1/T7), or cleaved only with *Hind*III (H3) or *Bam*HI (B1). Reactions in which plasmid DNA was first linearized and then exposed to endonuclease T7 are aliquots of these cleaved with the restriction enzyme only. Filled arrowheads indicate fragments resulting from cleavage within *oriP*-derived sequences.

that endonuclease T7 recognized, but this process was not instantaneous.

Two single-stranded structures extruded within pHEBO are approximately 12 bp long. Endonuclease T7 cuts cruciform structures at their bases, leaving one hairpin end on each side of the cleavage product. Since this hairpin is susceptible to S1 nuclease, use of this enzyme after T7 allows calculation of the number of bases involved in a cruciform (25). This approach was applied to plasmid pHEBO, a large preparation of which was digested with T7 and then with *Bam*HI, *Sal*I, or *Hpa*I. Aliquots of the digestions with *Sal*I and *Hpa*I were then treated with S1 nuclease, and the products were analyzed on a high-percentage agarose gel to resolve the smaller digestion products. Cleavage with T7 and *Bam*HI revealed three major endonuclease-sensitive sites located 1,460, 1,070, and 780 bp from the *Bam* site (Fig. 7, lane T7/B1, one open and two filled arrowheads, respectively). Cleavage with T7 and *Sal*I showed that the 1,460 site is located counterclockwise from the *Bam* site and is probably the structure identified in pBR322 from which this region in pHEBO is derived. The sites at 1,070 and 780 bp from *Bam*HI, however, are in the FR elements, since cleavage with *Sal*I reduced their size by approximately the predicted amount, which is 276 bp (compare lanes T7/B1 and T7/*Sal*I). Probing of similar blots with an *oriP*-specific radio-labeled DNA fragment identified only these last two sites (data not shown), further showing that these cleavages occur within *oriP*. Use of nuclease S1 after *Sal*I restriction accelerated the apparent migration of both fragments by about 12 bp (lane T7/*Sal*I/S1 versus lane T7/*Sal*I). This same estimate was obtained by comparing the sizes of pHEBO cut

FIG. 7. Determination of the approximate sizes of the single-stranded structures within *oriP* by nuclease S1 analysis. pHEBO plasmid DNA was digested with endonuclease T7 and then with restriction enzyme *Bam*HI (B1), *Sal*I (SAL1), or *Hpa*I (H1). Aliquots of the T7-*Sal*I and T7-*Hpa*I reaction mixtures were then digested with the single-strand-specific nuclease S1 (S1). *Hae*III-digested ϕ X174 and *Msp*I-digested pBR322 DNAs were electrophoresed in the gel to serve as molecular size standards. Filled arrowheads indicate fragments generated by cleavage of endonuclease T7 within *oriP*-derived sequences followed by *Bam*HI cleavage; the open arrowhead indicates the fragment generated by T7 cleavage within the pBR322-derived sequences.

with T7 and *Hpa*I with pHEBO cut with T7, *Hpa*I, and then S1. Similar calculations estimate that the cruciform in the pBR322-derived DNA, cleavage at which generated the 1,460-bp fragment in the pHEBO T7/B1 lane (open arrowhead), is about 27 bp long, which corresponds to the size predicted by sequence data (35).

DISCUSSION

Palindromic sequences are often found near functionally interesting regions of DNA, such as in replication origins that have been identified in many procaryotes, including *E. coli* and *Vibrio harveyi* (29, 54), and also in many mammalian viruses, including JC and herpes simplex viruses (10, 50). Because inverted repeats can form cruciforms (14, 36), these structures have long been thought to be possible loci for protein-DNA interactions important in replication. That cruciforms exist in vivo has been shown for *E. coli* with the use of an inducible T7 endonuclease expression system. Both bacterial chromosomal DNA and exogenous plasmid DNA were cleaved by this enzyme in vivo, and the plasmids were digested at the same sites in vivo as in vitro (34). Also, stabilization of cruciform structures with specific monoclonal antibodies has been shown to stimulate DNA synthesis in vivo (53). Thus, it seems likely that cruciforms detected in vitro will prove to have important functions in vivo. The presence of many repeated palindromic regions in the *oriP* region of the EBV genome suggested that cruciforms could be extruded by these sequences. Furthermore, these sequences are known to be able to bind the EBNA 1 protein (18, 37), which is necessary, along with *oriP*, for viral DNA replication during latency (27, 52). An understanding of the interaction of EBNA 1 with *oriP* and of how this interaction is important in EBV replication must therefore include a knowledge of any secondary structure present in the origin region.

Studies using endonuclease T7 and nuclease P1 presented here demonstrated that *oriP*-containing plasmids contain single-stranded structures in vitro at several places within the origin region. One possible interpretation of these results is that the single-stranded regions detected represent transiently unwound regions of DNA that form under the influence of negative supercoiling. Because the sequences comprising *oriP* are 60% A+T, whereas the rest of the EBV genome is only 40% A+T (2), this region of DNA would have a particularly high tendency to form such transiently melted areas. Such localized unwinding, however, may be more than just a by-product of the unusual sequence of this region and may indicate the presence of elements important in the function of *oriP*. Replication of the *E. coli* chromosomal DNA is initiated by the binding of the DnaA protein to the bacterial origin, *oriC*, which leads to a localized unwinding of DNA necessary for the assembly of a prepriming complex (6, 11, 19). This unwinding occurs within three repeated 13-bp sequences that are A+T rich (6). However, these sequences can form single-stranded regions when they are present on supercoiled plasmids even without the presence of bound DnaA protein, which has led to their being named DNA-unwinding elements (23). For plasmids containing *oriP* to replicate, they must be similarly unwound at the origin for the assembly of a complex capable of initiating replication of the two single strands of DNA. That supercoiled plasmids containing *oriP* extrude single-stranded structures within the sequences that would be expected to be unwound first in replication could indicate the presence of DNA-unwinding elements analogous to those identified in the *E. coli oriC* within the EBV *oriP*.

A second possible interpretation of the finding that supercoiled plasmids containing *oriP* are sensitive at several sites to cleavage by single-stranded nucleases is that these sites represent cruciforms extruded within the palindromic sequences of *oriP*. This interpretation is supported by the kinetic data presented in Fig. 6. If the single-stranded structures detected were transiently formed melted regions of DNA, linearization of the plasmids would tend to lead to an almost complete loss of these regions. This is not the case with the *oriP* structures, however, since although precleavage of the supercoiled plasmids with a restriction enzyme does reduce the degree of subsequent susceptibility to endonuclease T7, it does not abolish it. Thus, the structures being detected have a significant half-life even in the absence of negative supercoiling, arguing for the presence of a more stable structure such as a cruciform. Kinetic studies have shown that both cruciform extrusion and reabsorption are slow processes (13) having half-lives of several hours even at 50°C with cruciforms of about 700 bp.

oriP sequences have been predicted to be able to form many structures (20, 37). The DS region, for example, has been predicted to have two possible alternative structures, one involving 64 bp in a hairpin structure with three mismatches and a second, larger structure involving 116 bp in a stem plus loop with more extensive mismatches (37). Our data indicate that endonuclease cleavage within the DS region is minimal and that at least in vitro the major secondary structural features sensitive to cleavage occur in the FR element. Only after exposure of *oriP* plasmids to 250 mM KCl, which stimulates extrusion of certain types of cruciforms (47), can any cleavage within the dyad be detected, at which point four sensitive sites occur, probably corresponding to the four 30-bp-like repeats that are found there (data not shown). It is possible that, in analogy with the interpretation of recent results obtained with the lambda

bacteriophage (43), replication of EBV drives the extrusion of cruciforms within the dyad region. In the plasmid DNA itself, however, such extrusion seems to be rare.

The FR element of *oriP*, because of its highly repetitive and palindromic nature, has been predicted to have a large number of possible alternative structures. Mathematical analysis of the EBV sequence localized two exact palindromes within this region, both 18 bp in length (20). Our data are consistent with the possibility that there are two major single-stranded structures. Most of the cleavage patterns visible in Fig. 2 to 4, 6, and 7 show two major bands, along with several minor bands representative of endonuclease cleavage. This is most apparent in Fig. 7, in which three major T7 recognition sites are identified, two within the repeats and a third representing a pBR322-derived structure (35). On the basis of S1 nuclease experiments, these two major structures seem to involve only about 12 bp of DNA and not the 18 bp predicted. Though molecules with hairpins at their ends may migrate anomalously during electrophoresis, similar calculations indicated that the pBR322-derived cruciform was about 27 bp long, which is indeed the length predicted by sequence data (35). Interestingly, several 30-bp repeats within the 20-copy family contain internal 12-bp perfect palindromes. It seems likely that these are the sequences that are extruded into cruciforms and that within the plasmid DNA itself there is little if any interaction between adjacent repeats.

The in vitro studies on *oriP* reported here are the first to examine the secondary structure of this region of DNA. EBV plasmids in vivo, in latently infected cells, are present as negatively supercoiled plasmids (26, 32), and this would tend to stabilize any single-stranded structures present. However, these plasmids are thought to be packaged into a nucleosomal structure very similar to that of the host chromosomal material (42), and such packaging might be expected to interfere with or even prevent cruciform extrusion. Nonetheless, it is interesting that the 3' DS region of *oriP* was recently shown to exclude nucleosomes in reconstitution experiments with linear DNA fragments containing the EBV *oriP* (41). The structures detected in *oriP* may constitute sites for interactions important in the replication of the EBV genome during latency. Notably, the FR element has been recently found to contain the termination site for EBV replication, and two major stop sites were in fact detected (12). It is tempting to speculate that these two sites may have some relationship with the two major single-stranded regions that we have detected in this study. Perhaps the presence of a cruciform in the FR element provides a termination signal for replication of the origin-bearing DNA. EBNA 1, the origin-binding protein, may have some role to play in conjunction with cruciforms. Formation of a cruciform structure at the simian virus 40 replication origin in vitro abolishes binding of large T antigen (49), and if such is the case for EBNA 1, its inability to bind the cruciforms may in some way disrupt the progress of a replication complex passing through *oriP*. Indeed, we find in related studies that binding of EBNA 1 adjacent to a single-stranded structure may stabilize this conformation. Alternatively, since EBNA 1 has been reported to have single-stranded DNA-binding capacity (16), it may be able to bind cruciforms, and such binding may cause the terminations of replication within the repeats. Just such a cruciform-binding protein, which may have roles in both DNA transcription and replication, has recently been isolated from rat liver extracts (4). Further studies will be necessary to distinguish between these possibilities or to establish an alternative theory for the importance of the

single-stranded *oriP* structures in the functions of this region of DNA.

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