Effect of Number and Position of EBNA-1 Binding Sites in Epstein-Barr Virus *oriP* on the Sites of Initiation, Barrier Formation, and Termination of Replication

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DNA replication intermediates of three plasmids containing all or part of a modified Epstein-Barr virus *cis*-acting plasmid maintenance region (*oriP*) were examined to further investigate *oriP* function. Replication intermediates were analyzed *in vivo* and *in vitro* by neutral-neutral two-dimensional gel electrophoresis. The major functional components of the wild-type *oriP* are a 140-bp dyad symmetry region (single dyad) and 20 tandem copies of a repeat with a 30-bp consensus sequence (family of repeats). A modified *oriP* was constructed by replacing the family of repeats with three tandem copies of the single dyad (D. A. Wysokenski and J. L. Yates, *J. Virol.* 63:2657–2666, 1989). Initiation was observed *in vivo* near the single dyad in the modified *oriP*, as seen in the wild-type *oriP* (T. A. Gahn and C. L. Schildkraut, *Cell* 58:527–535, 1989), but was not observed near the tandem dyads. A replication barrier and termination were observed near the tandem dyads and were similar to those observed at the family of repeats of the wild-type *oriP* (Gahn and Schildkraut, *Cell* 58:527–535, 1989). *In vitro* experiments indicate that the viral *trans*-acting factor EBNA-1 contributes to efficient barrier formation at the tandem dyads as observed in the family of repeats of the wild-type *oriP* (V. Dhar and C. L. Schildkraut, *Mol. Cell. Biol.* 11:6268–6278, 1991). The tandem dyads thus appear to function in a manner similar to the family of repeats. There are significant structural differences between the family of repeats and tandem dyads. The relationship between the number and relative positions of EBNA-1 binding sites in relation to the functions of the family of repeats and the dyad symmetry element is discussed.

Epstein-Barr virus (EBV), a member of the herpesvirus family, is a causative agent of mononucleosis, Burkitt's lymphoma, and nasopharyngeal carcinoma (for a review, see reference 14). The 172-kb linear DNA EBV genome contains one or two copies of the lytic origin of replication, *oriLyt* (9), and one copy of *oriP*, which was originally identified by its ability to permit plasmids to replicate autonomously (27). *oriP* contains the origin of replication functional in latent infection and is responsible for long-term viral maintenance. The wild-type *oriP* (Fig. 1) consists of two functional elements, a dyad symmetry region (single dyad) and a family of repeats, separated by approximately 960 bp (19). The single dyad consists of a set of four 16-bp palindromic Epstein-Barr nuclear antigen 1 (EBNA-1) binding sites plus flanking sequences contained within a 140-bp *EcoRV*-*HpaI* fragment (13, 19, 23). The family of repeats consists of 17 imperfect copies of a 30-bp consensus sequence plus three truncated copies, all 20 of which contain a 16-bp EBNA-1 binding site (13, 19).

The *oriP* region confers the ability to replicate autonomously to plasmids in B lymphocytes, provided that a single viral *trans*-acting factor, EBNA-1, is present (19, 23). DNA replication from *oriP* initiates at or near the single dyad (3, 8, 26) and proceeds bidirectionally. The replication fork that proceeds from the end of the tandem dyads closest to the family of repeats traverses the 960-bp spacer sequence between them to a site at or near the family of repeats, where it stalls. The other fork replicates the remainder of the

plasmid, and termination of replication occurs when this second fork meets the stalled fork at or near the family of repeats (8). The relative rates of movement of the two replication forks have not been measured. A replication barrier is observed at or near the family of repeats. A similar replication barrier and termination have been observed in latent replication of the 172-kb extrachromosomal viral genome (8). We define a replication barrier to be a region of DNA where replication fork progression is delayed, resulting in the accumulation of replicative intermediates. Termination of replication can occur at or near the site of a replication barrier; however, termination often occurs in regions where replication barriers have not been detected.

The present study included an analysis of replication intermediates associated with initiation, barrier formation, and termination in plasmids containing a modified *oriP* (26) in which the family of repeats is replaced by three tandem copies of the dyad symmetry element (tandem dyads [Fig. 1]). Replication intermediates were analyzed both *in vivo* and *in vitro*. *In vivo* experiments were performed on plasmid p425, which contains a modified *oriP*, maintained in a Raji cell line (Raji p425b). *In vitro* experiments were performed on two plasmids, pEco3d and pSst3d, which contain the tandem dyads and a complete modified *oriP*, respectively. Replication initiated in these plasmids at the simian virus 40 (SV40) origin of replication in T-antigen-dependent reactions. Both sets of experiments were performed by the neutral-neutral two-dimensional (2-D) gel technique of Brewer and Fangman (2). Comparisons of replication intermediates from modified and wild-type *oriP* sequences are made and a model of *oriP* function is presented below.

***In vivo* experiments.** Raji p425b cells were obtained from J. L. Yates (26) and were maintained in 5% CO₂-95% air in

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FIG. 1. Maps of wild-type *oriP* and modified *oriP*. Both wild-type *oriP* and modified *oriP* act as minimal *cis*-acting elements that allow maintenance and replication of recombinant plasmids in cells harboring either the complete EBV episome or, at a minimum, EBNA-1-encoding sequences from the EBV genome. The wild-type *oriP* is composed of a family of repeats consisting of 20 imperfect copies of a 30-bp consensus sequence, 3 of which are truncated, and a dyad symmetry element. Each of the 20 copies of the repeated sequence of the family of repeats contains a single EBNA-1 binding site. The dyad symmetry element contains four EBNA-1 binding sites. The modified *oriP* was constructed by replacing the family of repeats with three tandem copies of a 159-bp element, each consisting of a single 139-bp dyad symmetry region from the wild-type *oriP* plus 20-bp of polylinker sequence (26). Symbols: □, EBNA-1 binding site from family of repeats; ▤, EBNA-1 binding site from dyad symmetry region.

RPMI 1640–10% fetal calf serum supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). The p425 plasmid was maintained under selection in 300 µg of hygromycin B (Calbiochem) per ml. Cell density was maintained at 1×10^5 to 4×10^5 cells per ml. The Raji p425b cell line maintains a doubling time of approximately 32 h under these conditions. DNA was extracted as described elsewhere (8), except that cell lysis was performed at pH 9.0 with 0.15 M Na₂EDTA. DNA was digested with restriction endonucleases by using the assay buffer conditions of New England Biolabs. Digests were enriched for replicative forms by benzoyl-naphthyl-DEAE cellulose chromatography as described elsewhere (8). All gel electrophoresis, Southern transfer, hybridization and autoradiography procedures were performed as described elsewhere (8). All hybridizations were performed with probes derived from pHyg, since p425 was constructed by assembling a copy of the modified *oriP* in the pHyg vector (26). pHyg detects all three *EcoRI* segments of p425 (Fig. 2) and does not cross-hybridize to the Raji genome or the complete EBV episome present in the Raji p425b cell line.

In vitro experiments. Plasmids pEco3d and pSst3d were constructed from pEco3'Δ and pSst3'Δ (provided by S. Lupton and A. J. Levine [16]) and the tandem dyad sequences of p425 (plasmid provided by J. L. Yates [26]). pEco3'Δ and pSst3'Δ consist of approximately 4.1 kb of vector sequence containing the SV40 origin of replication and EBV sequences consisting of an *oriP* family of repeats and a complete wild-type *oriP*, respectively. pEco3d and pSst3d were constructed from them by replacing the family of repeats with one copy of the tandem dyads and flanking sequences from p425, producing constructs containing only the tandem dyads (pEco3d) or the complete modified *oriP* (pSst3d) (Fig. 3 and 4). The family of repeats was excised from pEco3'Δ and pSst3'Δ as a 999-bp *EcoRI*-*MluI* segment. The tandem dyads were excised from p425 as a 1,252-bp

EcoRI-*MluI* segment for insertion into pEco3'Δ and pSst3'Δ. There are 392 bp flanking the tandem dyads on the side bounded by the *EcoRI* site (14 bp of EBV sequences and 378 bp of vector sequences) and 383 bp (EBV sequences) flanking them on the side bounded by the *MluI* site in this segment. There are minor differences in the spacer regions between the single dyads and the tandem dyads in p425 and pSst3d. Plasmid DNA was replicated in vitro as described by Dhar and Schildkraut (4, 15, 21, 25) and then digested and electrophoresed as in the in vivo experiments. The gels were washed for 30 min in 7% trichloroacetic acid, vacuum dried, and exposed to film (Kodak XAR-5). The HeLa cytosol and T antigen (produced in a baculovirus expression system as described in reference 25) used in this study were provided by J. Borowiec and J. Hurwitz. EBNA-1 produced in a baculovirus expression system (bEBNA-1) and lacking the glycine-alanine repeat region was provided by L. Frappier and M. O'Donnell (6).

Replication initiation occurs primarily at or near the single dyad and is not observed at the tandem dyads in the modified *oriP*. DNA prepared from the Raji p425b cell line was digested with restriction endonucleases, enriched for replicative intermediates by benzoyl-naphthyl-DEAE cellulose chromatography, electrophoresed by the 2-D gel method of Brewer and Fangman (2), transferred to nitrocellulose membranes, hybridized with pHyg-derived probes, and autoradiographed. It was first necessary to determine that replication does in fact initiate in the modified *oriP* sequences in p425. *EcoRI* digestion of p425 yields three segments of 0.4, 2.3, and 3.8 kb. The 3.8-kb segment contains all of the EBV-derived sequences in the plasmid. The single dyad symmetry element is near the center of the 3.8-kb *EcoRI* segment. After 2-D gel electrophoresis, replicative intermediates from the two larger segments were detected by hybridization with the complete pHyg plasmid as a probe (Fig. 2A). Replication intermediates containing symmetrical bubbles were observed for the 3.8-kb segment, in a manner consistent with replication initiation near the center of the EBV sequences. When replication forks proceeding from an initiation site within a DNA segment reach the ends of the segment at or near the same time, a series of molecules with symmetrically located internal bubbles are produced. These molecules produce an arc that does not return to the arc of nonreplicated molecules (Fig. 2 and reference 2). This is referred to as a symmetrical bubble pattern. Replication bubbles were not observed in the 2.3-kb segment, which consists of vector sequences, indicating that replication of this segment initiated at an external site (Fig. 2A).

Having determined that replication initiates in the *EcoRI* segment containing the modified *oriP* in p425, we next performed experiments to further identify replication initiation sites. The first segment of the modified *oriP* to be examined contained the tandem dyads separated from the single dyad. Raji p425b DNA was digested with *NcoI* and *PstI*, analyzed by 2-D gel electrophoresis, and hybridized with the 2.3-kb *EcoRI* pHyg fragment as a probe. *NcoI*-*PstI* double digests cleave p425 to produce a 2.2-kb segment plus several smaller segments whose replication intermediates are not observed under the gel conditions used. The 2.2-kb segment contains the tandem dyads within its middle third but does not contain the single dyad. Molecules containing internal bubbles were not observed in the 2.2-kb segment of p425, even in long autoradiographic exposures (Fig. 2B). This indicates that initiation of replication was not detected within the tandem dyads.

The next experiment to determine sites of replication

FIG. 2. In vivo replication of p425. (A) Replication in vivo initiates and terminates in the modified *oriP*. Raji p425b DNA was digested with *EcoRI* and probed with the complete pHyg plasmid, which detects segments of 2.3 and 3.8 kb. Replication initiation and termination were detected in the 3.8-kb segment, which contains the modified *oriP*, but were not observed in the 2.3-kb vector segment. The accompanying map of p425 indicates the positions of the tandem dyads, single dyad, and *EcoRI* restriction sites. EBV sequences flanking these elements are represented by double lines, and vector sequences are represented by a single solid line. In the diagram of the 2-D gel patterns, the positions of the 2.3- and 3.8-kb unreplicated segments are indicated by filled circles, and the position of unreplicated DNA species is shown by a dashed line. The straight line indicates the migration of molecules containing two opposing replication forks (termination), and the arc that does not return to the dashed line indicates the migration of molecules containing internal bubbles (initiation). Similar results were obtained with two additional independent preparations of DNA (data not shown). (B) Replication terminates at but does not initiate at the tandem dyads in vivo. Raji p425b DNA was digested with *NcoI* and *PstI* and probed with the 2.3-kb *EcoRI* pHyg segment, which detects a 2.2-kb *NcoI-PstI* segment containing the tandem dyads but not the single dyad. A replication barrier and termination, but not replication initiation, were observed in this segment. The tandem dyads are located in the middle third of the 2.2-kb *NcoI-PstI* segment, where initiation patterns are most readily detected, so a symmetrical bubble pattern should have been detected if replication initiated at or near the dyad symmetry regions of the tandem dyads. Two exposures of this hybridization are presented. At the top is a short photographic exposure of a 6-day autoradiograph to indicate the relative amounts of types of replicative intermediates that have accumulated, and below is a long photographic exposure of a 14-day autoradiograph that indicates an absence of replication initiation at detectable levels in this segment. The accompanying map of p425 indicates the positions of the tandem dyads, the single dyad, and *NcoI* and *PstI* restriction sites. Similar results were obtained in a second experiment with a separate preparation of DNA (data not shown). (C) Replication initiates predominantly at or near the single dyad in vivo. Raji p425b DNA was digested with *EcoRI* and *XbaI* and probed with the 2.2-kb *EcoRI* pHyg segment, which detects a 3.5-kb *EcoRI-XbaI* segment containing the single dyad but lacking most of the sequence of the tandem dyads. A bubble pattern of the type associated with a symmetrically positioned site of replication initiation (2) was found in this segment. The single dyad symmetry element of the modified *oriP* is in the middle third of the 3.5-kb *EcoRI-XbaI* segment, so this result indicates that initiation of replication occurs at or near the single dyad and possibly at other sites within this segment. Note that one dyad of the tandem dyads is also contained in this segment. Termination at the tandem dyads, as indicated by an accumulation of signal at the descending end of the arc of Y forms, is observed when this single dyad symmetry element is retained at one end of the segment. A symmetrical termination-like pattern was also observed at this segment, as discussed in the text. Note that there is a faint broad triangular smear bounded by the arc of Y forms and the symmetrical termination pattern extending from near the inflection point. The accompanying map of p425 indicates the positions of the tandem dyads, single dyad, and *EcoRI* and *XbaI* restriction sites.

initiation from specific segments of the modified *oriP* consisted of analysis of replication patterns of a 3.5-kb fragment containing the single dyad in the middle third and one of the three dyad symmetry regions of the tandem dyads at one end. Any molecules containing replication bubbles initiating at or near this terminal dyad should produce only the initial ascending portion of a bubble rather than a complete bubble arc, while replication initiation from the area of the single dyad should produce a symmetrical bubble pattern. Raji p425b DNA was digested with *EcoRI* and *XbaI*, yielding the 3.5-kb segment plus smaller segments, and examined by 2-D gel electrophoresis and hybridization with the 2.2-kb *EcoRI* pHyg segment as a probe. Replicative intermediates from the 3.5-kb p425 *EcoRI-XbaI* segment were detected. The symmetrical bubble pattern observed (Fig. 2C) suggests that replication initiated predominantly from a site in this segment at or near the single dyad. This finding is further strengthened by the observations that bubble patterns were not detected in the region of the tandem dyads, even in long autoradiographic exposures (Fig. 2B), or in the replication of the 2.3-kb *EcoRI* segment (Fig. 2A).

In addition to segments containing a bubble, replicative intermediates indicative of a single fork originating from an external initiation site progressing through the 3.5-kb *EcoRI-XbaI* segment of p425 were detected (Fig. 2C). In a previous study of wild-type *oriP* done by Gahn and Schildkraut (8), a strong bubble arc and a weak signal indicative of molecules containing a single fork (Y arc) were observed. In contrast to the previous study, the bubble arc with the modified *oriP* is weaker than the Y arcs. We considered four possible mechanisms for the presence of molecules containing one replication fork. First, at least some of this signal could be due to breakage of replication bubbles initiating at the single dyad in the middle of this segment. Second, it is possible that replication initiates within a broader region within this segment than within the corresponding segment containing the wild-type *oriP* in the previous study (8). An example of this phenomenon is found in the amplified CHO dihydrofolate reductase domain, which initiates replication from a broad initiation zone (24). This has also been observed in the replication of ribosomal DNA plasmids in *Xenopus laevis* eggs and extracts (12). Third, since a replication barrier (see below) is present near the *XbaI* end of the 3.5 kb *EcoRI-XbaI* segment, the replication fork moving in that direction would be expected to proceed more slowly than the other fork. For this reason, the replication fork proceeding toward the *EcoRI* site would be expected to reach the end of the segment first, converting the replicative intermediates to Y forms, even when the site of replication initiation is symmetrically positioned in the segment. Fourth, the Y arcs could be due to replication through inactive origin sequences, as was observed in replication of oligomeric forms of pBR322 (17) and bovine papillomavirus (20). This is unlikely, since multimers occurred, at most, in a relatively small fraction of p425 molecules in vivo, as demonstrated by analysis of the 2-D gel pattern of intact Raji p425b DNA (data not shown). It is likely that several of the mechanisms discussed above contribute to the presence of Y forms in this segment.

Replication barriers occur at or near the tandem dyads, and termination occurs in a broad zone within the 3.8-kb *EcoRI* fragment. A set of in vivo and in vitro experiments was carried out to determine whether the modified *oriP* contained replication barriers and termination sites similar to those observed in the wild-type *oriP* (4, 8). The first experiments were studies of in vivo replication intermediates. A barrier was observed at the modified *oriP* when Raji p425b

DNA was digested with *EcoRI* and analyzed by 2-D gel electrophoresis as described above (Fig. 2A). This barrier is indicated by higher signal intensity near the descending end of the arc of replication intermediates from the 3.8-kb segment. A higher-intensity signal was also observed near the inflection point of the arc in Fig. 2A, consistent with the presence of a barrier in the central portion of the segment, near the single dyad, in at least some p425 molecules in Raji p425b. A higher-intensity signal was not consistently observed at the inflection point in Fig. 2C in the proximity of the single dyad, so it is not possible to definitively conclude whether a replication barrier is present at or near the single dyad. However, the accumulation of replicative intermediates indicative of a replication barrier at the tandem dyads was consistently observed in three different DNA preparations (data not shown).

Termination of replication was observed at or near the tandem dyads in vivo in these experiments (Fig. 2A) as a signal near the end of the descending arc. A signal was also observed near the inflection point of the arc, consistent with a pattern characteristic of more symmetrically positioned termination in this segment. We considered five explanations for this observation. First, termination could be expected in this region if there were a subpopulation of dimeric p425 molecules replicating from a single functional origin, as observed in bovine papillomavirus type 1 (20) and pBR322 (17). There are, however, few if any dimeric copies of p425 in Raji p425b, as discussed above. Second, termination might occur at or near the single dyad if replication were to initiate from the region of the tandem dyads in a subpopulation of the p425 molecules. Evidence presented above indicates that at most, only a small percentage of p425 initiates replication in this manner. Third, it is possible that replication sometimes initiates at sites other than *oriP* and terminates at the single dyad. 2-D gel analysis of p425 indicates that initiation of replication is not detectable in the vector sequences. Fourth, it is possible that termination occurs over a broad contiguous region, primarily in localized sites at or near the tandem dyads and single dyad but also elsewhere within the 3.8-kb *EcoRI* fragment. This is consistent with the observation of a broad triangular smear extending from the descending arc of Y forms and bounded by the pattern of termination from the tandem dyads. Termination has been observed in broad zones in *Saccharomyces cerevisiae* (29) and in ribosomal DNA plasmid replication in *X. laevis* eggs and egg extracts (12). Fifth, it is possible that at least some of this symmetrical pattern is due to branch migration (5) and thus is an artifact. It is possible that the termination pattern observed is the cumulative effect of low-level occurrence of more than one of these phenomena.

There was a greater difference between the relative strengths of the termination pattern and the descending arc in the replication patterns observed in vivo at the tandem dyads (Fig. 2B, light exposure) than in those observed at the wild-type *oriP* at the family of repeats (8). We considered two possible reasons for this difference. The observed signal differences might reflect differences in the relative efficiencies of the two sequences as termination sites. It is also possible that the tandem dyads are sometimes less efficient than the wild-type family of repeats as barriers.

When Raji p425b DNA was digested with *NcoI* and *PstI*, a barrier was found at or near the tandem dyads (Fig. 2B, light exposure). In this segment, a barrier is observed as two regions of higher signal intensity adjacent to the inflection point of the arc. A similar signal of two regions of higher intensity was observed at the family of repeats both in vivo

and in vitro (4, 8). When Raji p425b DNA was cleaved with *EcoRI* and probed with the complete pHyg plasmid, a barrier and termination were observed in the replication intermediates from the 3.8-kb *EcoRI* segment of p425 (Fig. 2A). No termination was observed in the 2.3-kb *EcoRI* segment in these hybridizations. The center of this segment is approximately 180° opposite the site where replication initiates in p425, indicating that replication proceeds in an asymmetric bidirectional manner. If there were neither a barrier nor termination at the tandem dyads in a subset of the population of p425 molecules large enough to be detectable by autoradiography, termination would be observed in the middle of the 2.3-kb segment. If there were a barrier site in or near the tandem dyads but no site-specific termination, asymmetrically positioned termination would be expected in the 2.3-kb segment unless the replication fork traversing the tandem dyads proceeded sufficiently slowly that it did not enter the 2.3-kb segment. In this instance, termination would not be observed in the 2.3-kb segment, and a more asymmetric termination pattern would be observed in the 3.8-kb *EcoRI* segment (Fig. 2A).

A strong signal resulting from termination was observed near the descending end of the simple Y component of the replication pattern of the 3.5-kb *EcoRI*-*XbaI* segment (Fig. 2C) when Raji p425b DNA was digested with *EcoRI* and *XbaI* and analyzed by 2-D gel electrophoresis as described above, with the 2.2-kb *EcoRI* pHyg segment as a probe. One of the three dyad symmetry regions of the tandem dyads is located, as noted above, at one end of this segment. This indicates that termination occurs at or near the tandem dyads in at least a significant subpopulation of p425 molecules. A more symmetrically positioned pattern associated with termination at or near the single dyad with a triangular smear extending from it was also observed in this segment, as in the case of the 3.8-kb *EcoRI* segment (Fig. 2A) discussed above. This is consistent with termination occurring in a broad zone.

In vitro experiments were next performed to determine whether barrier formation at the tandem dyads was dependent on EBNA-1, as was observed for the family of repeats of the wild-type *oriP* (4). pEco3d and pSst3d were replicated in vitro in the presence and in the absence of bEBNA-1 and were examined for replication barriers at the modified *oriP* sequences. pEco3d and pSst3d were replicated from the SV40 origin of replication in T-antigen-dependent reactions, digested with *PvuI* and *XhoI*, electrophoresed by the neutral-neutral 2-D gel method of Brewer and Fangman (2), and autoradiographed. In experiments in which bEBNA-1 was absent, weak accumulation of replicative intermediates at a barrier was observed in pEco3d (Fig. 3A), and replication proceeded through the tandem dyads in both pEco3d (Fig. 3A) and pSst3d (Fig. 3B). However, accumulation of replicative intermediates was found to occur at a higher level in the presence of bEBNA-1 than in the absence of bEBNA-1. This produced stronger barriers to fork migration through the tandem dyads (Fig. 4). Evidence that replication forks originating at the SV40 origin do not (in the majority of molecules) proceed past the triple dyad is provided by the incomplete arcs produced by the replication intermediates of the 2.6- and 3.1-kb insert segments (Fig. 4). These arcs do not extend down to the arc of nonreplicated molecules since branched molecules with longer forks are not present. Absence of the single dyad does not alter the barrier or fork arrest at the tandem dyads.

Replication of plasmids containing wild-type and modified *oriP* sequences. The replication of wild-type and modified

FIG. 3. Strong barriers are not observed when pEco3d and pSst3d replicate in vitro in the absence of bEBNA-1. Autoradiographs of *PvuI*-*XhoI* digests of pEco3d (A) and pSst3d (B) replicated in the absence of bEBNA-1 are shown. The *PvuI*-*XhoI* segments containing the EBV sequences of pEco3d and pSst3d are 2.6 and 3.1 kb long, respectively. Both constructs also contain a 4.1-kb *PvuI*-*XhoI* segment consisting of the SV40 origin of replication and other vector sequences. A weak signal indicating an accumulation of replication intermediates was observed in the 2.6-kb segment in pEco3d. Replication proceeded through the tandem dyads in both pEco3d and pSst3d. Positions of modified *oriP* elements, the SV40 origin of replication (*ori*), and restriction sites used are shown on the accompanying maps. The tandem dyads, the single dyad, and the positions of unreplicated DNA species are indicated as described in the legend to Fig. 2. Sequences flanking the tandem dyads, excised from p425 with the tandem dyads and ligated to pEco3'Δ and pSst3'Δ sequences to produce pEco3d and pSst3d, respectively, are represented by thick solid regions. In pSst3d, EBV sequences extend from the *XhoI* site through the single dyad to 14 bp past the tandem dyads.

oriP sequences has been compared by three experimental approaches. The first approach determined whether the modified *oriP* confers autonomous replication to plasmids maintained in B lymphocytes. Plasmids containing the wild-type *oriP* replicate autonomously in B-cell lines if EBNA-1 is present (28). Wysokenski and Yates (26) found that the modified *oriP* supports autonomous replication. They found EBNA-1 to be a necessary *trans*-acting factor for autonomous replication from the modified *oriP*. The second approach analyzed replication intermediates by 2-D gel electrophoresis of in vivo-replicated DNA. Gahn and Schildkraut (8) found that replication initiates at or near the single dyad and terminates at or near the family of repeats in a plasmid, p174, containing the wild-type *oriP* and in the extrachromosomal EBV episome present in the 721 cell line. They also found a replication barrier at the family of repeats

FIG. 4. Strong barriers are observed when pEco3d and pSst3d replicate in vitro in the presence of bEBNA-1. Autoradiographs of *PvuII*-*XhoI* digests of pEco3d (A) and pSst3d (B) replicated in the presence of bEBNA-1 are shown. Stronger barriers are observed here than in the case of replication in the absence of bEBNA-1, and replication did not proceed through the tandem dyads. Conditions for restriction digestion and electrophoresis were the same as those in the experiments whose results are shown in Fig. 3 (see text for description). Positions of modified *oriP* elements, the SV40 origin of replication, unreplicated DNA species, and restriction sites used are shown on the accompanying maps as in Fig. 3. Dashed arcs represent nonreplicated molecules.

in p174. The functional limits of the wild-type *oriP* have been defined (3). In the present study, we found that replication initiated at or near the single dyad, but we did not observe initiation at or near the tandem dyads (Fig. 2B, long exposure). We observed termination at or near the tandem dyads and also at or near the single dyad in p425. We have also observed a replication barrier at the tandem dyads in p425. The third approach was to study in vitro replication of plasmids containing *oriP* sequences in constructs containing the SV40 origin of replication. Dhar and Schildkraut (4) found that in the presence of bEBNA-1, 20 tandem copies of the EBNA-1 consensus binding sequence of the wild-type *oriP* family of repeats act as a strong barrier to replication in vitro. A barrier of decreasing strength was observed when the family of repeats was truncated to 15, 12, or 6 copies of the EBNA-1 consensus binding sequence (4). Replication barriers were also observed in the present study when plasmids containing modified *oriP* sequences were replicated in vitro under the conditions described by Dhar and Schildkraut (4). Thus, the modified *oriP* was found to replicate in a manner similar to that of the wild-type *oriP* by all three approaches.

These findings have several implications. While the EBNA-1 binding sites are similar in the family of repeats and tandem dyads, the sequences between the EBNA-1 binding

sites in the two structures are different in their length and composition. In the family of repeats, adjacent EBNA-1 binding sites are separated by approximately 14 bp (1, 13, 19). In the tandem dyads, they are separated by segments of up to 74 bp, 20 bp of which are polylinker sequence (26). We have observed replication barriers and termination of replication at the wild-type *oriP* and modified *oriP* in vivo and in vitro in the presence of EBNA-1. Barriers and termination of replication were not observed in vitro at the single dyad with its four EBNA-1 binding sites, even in the presence of bEBNA-1. These results suggest that the number of EBNA-1 binding sites in the family of repeats determines barrier and termination function in *oriP* rather than the length or specific base pair composition of flanking sequences, further supporting the conclusions of Wysokinski and Yates (26).

Recent studies, in combination with the findings presented here, suggest a possible mechanism for the production of replication barriers and site-specific replication termination. Strong barriers and replication termination do not occur at *oriP* sequences in the presence of bEBNA-1 unless there is a minimum of approximately six contiguous EBNA-1 binding sites (4). The minimum number of family of repeat elements required to complement the single dyad so that it serves as a site of replication initiation appears to be similar to the minimum number required to produce a strong barrier and termination at the family of repeats (4, 26). These functions are also EBNA-1 dependent. This suggests that EBNA-1 binding might have a single effect that accounts for all three functions. One mechanism for this would be that a conformational change occurs when sufficient EBNA-1 binding sites are occupied close to each other, as might be expected from cooperativity effects between DNA binding proteins. Frappier and O'Donnell (7) and Su et al. (22) have recently demonstrated a conformational change at the family of repeats due to bEBNA-1 binding, using electron microscopy to analyze binding of bEBNA-1 to wild-type *oriP* sequences. The family of repeats folds into a compact, approximately spherical structure as a tight protein-DNA complex in the presence of bEBNA-1. If there is enough bEBNA-1 to form this complex and also to bind to the single dyad, a larger complex forms; in this complex, the bEBNA-1-bound family of repeats associates with the single dyad, causing looping out of the intervening DNA. Su et al. (22) suggest that replication from *oriP* is likely to be contingent on the formation of this larger protein-DNA complex. This larger complex is observed to occur at high frequency when there is sufficient bEBNA-1 to bind to the family of repeats and dyad symmetry element. Moreover, while both the family of repeats and the single dyad are needed for replication from the wild-type *oriP*, these elements were not observed to associate in a complex in the absence of bEBNA-1. The compact protein-DNA complex observed at the family of repeats could account for barrier formation and termination, since such a structure could be expected to affect both the rate of advance and the processivity of replication factors. Middleton and Sugden (18) have recently provided further evidence in support of this model with their findings that the family of repeats interacts with the dyad symmetry element in reactions dependent on bEBNA-1.

Differences in the distribution of EBNA-1 binding sites may help to maintain a functional distinction between the family of repeats and the single dyad. Reisman et al. (19) found that when 960 bp are deleted between the family of repeats and the dyad symmetry element of the wild-type *oriP*, the resulting sequence still confers efficient autono-

mous replication leading to long-term plasmid maintenance. This suggests that the single dyad symmetry region remains functionally distinct from the family of repeats even when they are in proximity. Two major differences between the single dyad and the family of repeats, other than the number of EBNA-1 binding sites, appear to be the length and composition of the sequences between the binding sites. Adjacent EBNA-1 binding sites are separated by approximately 14 bp in the family of repeats, while EBNA-1 binding sites are separated by 5, 19, and 8 bp in the single dyad (1, 19). In studies using the complete EBNA-1 protein, Hearing et al. (11) have provided further evidence supporting the hypothesis that internal spacing differences in the distribution of the EBNA-1 binding sites in the family of repeats and the single dyad make them functionally distinct.

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