MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 6268–6278
0270-7306/91/126268-11$02.00/0

Vol. 11, No. 12

Role of EBNA-1 in Arresting Replication Forks at the Epstein-Barr Virus *oriP* Family of Tandem Repeats

VEENA DHAR† AND CARL L. SCHILDKRAUT*

Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

Received 15 November 1990/Accepted 26 August 1991

The 20-member family of 30-bp tandem repeats located within the *oriP* region of Epstein-Barr virus (EBV) can act as a transcriptional enhancer in the presence of EBV nuclear antigen 1 (EBNA-1). A replication fork barrier and a termination site of plasmid replication in human B cells is also found within or near the EBV tandem repeats. Within each tandem repeat is a consensus binding sequence for the EBNA-1 protein that is required for extrachromosomal maintenance of *oriP*-containing plasmids. To investigate the factors that contribute to the arrest of replication forks and termination in the region of the family of repeats, we have used an in vitro replication system in which replication of EBV recombinant plasmids is initiated from the simian virus 40 (SV40) DNA replication origin in the presence of SV40 T antigen and soluble extracts prepared from human cells. The system can support bidirectional replication, initiating from the SV40 DNA origin with termination occurring in a region opposite the origin. Using two-dimensional agarose gel electrophoresis, we observed a barrier to replication forks in the presence of EBNA-1 in the region of the EBV repeats. Termination occurs at or near the tandem repeats in a manner similar to that observed in vivo (T. A. Gahn and C. L. Schildkraut, Cell 58:527–535, 1989). Reducing the number of repeats from 20 to 6 had little effect on the strength of the replication fork barrier. In the absence of EBNA-1, replication forks also arrested at the EBV repeats, but at a much lower efficiency. The addition of competitor DNA containing the EBV family of repeats can almost completely abolish the replication barrier produced in the presence of EBNA-1.

The replication of eukaryotic chromosomal DNA during the S phase of the cell cycle is regulated by a multistep process. The precise coordination between activation of multiple replication initiation sites, progression of the replication forks, and termination has important implications for ordered cell division. In prokaryotes such as *Escherichia coli*, *Bacillus subtilis*, and plasmid R6K, termination of replication forks occurs at specific DNA sequences. The function of these termination (*ter*) sites is dependent on the binding of a protein, which impedes the progression of replication forks (for a review, see reference 21). It is not yet known whether sequence-specific termination sites exist in eukaryotic chromosomes as well. The ability of a protein-DNA complex to terminate replication by arresting the progression of a replication fork can serve as a means to regulate the timing of replication during the S phase, the size of the replicon, and activation of a nearby origin in chromosomes.

In many ways, DNA viruses that replicate as minichromosomes in their hosts provide simple and well-characterized models with which to study replication in eukaryotes since their replication is dependent on host proteins and is subject to similar constraints of replication during the S phase of the cell cycle. Epstein-Barr virus (EBV) in its latent infective cycle is maintained as a circular, double-stranded, multicopy episomal plasmid (172 kb) in human B cells. It has been reported that the virus replicates once per cell cycle (1), in the nucleus of the host cell during the S phase (17). A *cis*-acting DNA segment (1.8 kb), *oriP*, is necessary to maintain the EBV plasmid in the B cells (40). The *oriP* segment contains two essential components: 20 copies of a tandemly repeated 30-bp sequence and, located about 1 kb from the repeats, a 120-bp dyad symmetry (DS) region (32). This DS region contains four truncated copies of the 30-bp repeat. Two of these copies plus additional flanking sequences form a 65-bp DS element. The functional limits of *oriP* have been defined (11), and evidence that the DS region contains the replicative origin of *oriP* has been presented (39). Recent studies demonstrate that replication initiates at or near the DS region in a plasmid containing *oriP* in B cells (16).

Only one viral protein, EBV nuclear antigen 1 (EBNA-1), is required for *oriP* function. EBNA-1 binds to a 12-bp palindromic consensus sequence within each member of the family of repeats and to the four copies of this consensus sequence located in the DS region (30). In the presence of EBNA-1, the family of repeats enhance the replication of plasmids that contain the DS region and also enhance the transcription of several latent genes (31). In this regard, *oriP* is similar to the replication origins of other DNA tumor viruses in which transcriptional enhancer elements form an important component of replication origins (13).

Gahn and Schildkraut (16) have analyzed replication intermediates (RIs) of p174, an 18-kb EBV plasmid, by two-dimensional (2D) gel electrophoresis. They have reported that DNA replication proceeds bidirectionally after initiating within or near the DS region. The fork that proceeds in the direction of the repeats is arrested until the other fork traverses the circular plasmid to meet the arrested fork at or near the repeats where termination occurs. Thus, plasmid p174 replicates predominantly in a unidirectional manner in human B cells.

Since EBNA-1 binds to each copy of the 20-member family of repeats, we have considered the possibility that its binding to the DNA arrests replication fork movement and causes termination. We have used an in vitro simian virus (SV40) DNA replication system followed by 2D gel electro-

* Corresponding author.
† Present address: Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, NY 10032.

phoretic analysis of the replication products to investigate the role played by EBNA-1 in barrier formation and termination. In vitro replication of SV40 origin/EBV repeat-containing plasmids mimics the replication of SV40 DNA (25, 34, 38), since it initiates from the SV40 origin sequences, proceeds bidirectionally, and terminates 180° opposite the origin.

Here we show that addition of purified EBNA-1 causes a shift in the site of termination, which now occurs at or near the EBV repeats. This shift is mediated by the arrest of replication forks at or near the repeats. The replication fork arrest and termination must depend on EBNA-1 binding to the repeats, since it is abolished by adding competitor DNA containing the EBV family of repeats but remains unchanged by the addition of nonspecific DNA. The EBV sequences, upstream or immediately downstream of the repeats, do not appear to be essential for this block. Thus, this assay should be useful for the dissection of the mechanism of termination and fork arrest by DNA-binding proteins in higher eukaryotes.

MATERIALS AND METHODS

Recombinant plasmids. Plasmid pEco3′Δ includes 360 bp of the SV40 DNA replication origin region (360 bp surrounding the 65-bp minimal core origin [12]) and 1,680 bp (from the *Eco*RI site at position 7315 to the *Eco*RV site at position 8992; nucleotide numbering as in reference 3) of the *oriP* region from the EBV B95-8 *Bam*HI C fragment and was kindly provided by S. Lupton and A. J. Levine (27). Plasmid p20neo is similar to pEco3′Δ except that the EBV *oriP* region is the 1.8-kb *Eco*RI-to-*Sst*II fragment, from positions 7315 to 9515 (11). Plasmids p15neo, p12neo, p6neo, and p2neo are equivalent to p20neo except that they contain deletions into the 5′ side of the EBV repeat region that progressively reduce the number of repeats from 20 to 2. These plasmids and p20neo were provided by T. S. Chittenden and A. J. Levine. p0neo was derived from p20neo by deletion of the *Eco*RI-to-*Mlu*I segment of EBV (7315 to 8316), resulting in a plasmid that lacks the EBV family of repeats. All of the plasmid DNAs were isolated by the Triton-lysozyme method and purified on CsCl gradients.

In vitro DNA replication. The in vitro replication reactions were similar to those described previously (25, 34, 38). For the reactions in the presence of EBNA-1, potassium glutamate was added. A total reaction volume of 50 μl included 30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5), 25 mM potassium glutamate, 12.5 μg of bovine serum albumin (BSA) per ml, 8 mM $MgCl_2$, 0.5 mM dithiothreitol, 4 mM ATP, 0.3 μg of supercoiled template DNA (pEco3′Δ or pneo series), 100 μM each dATP, dGTP, and dTTP, 200 μM each CTP, GTP, and UTP, [α-^{32}P]dCTP (specific activity, 1×10^3 to 3×10^3 cpm/pmol; Amersham), 40 mM creatine phosphate, 40 mM phosphocreatine kinase, 0.6 μg of SV40 T antigen, and 300 μg of human cell (HeLa, 293, or Raji) cytosol. Purified SV40 T antigen and HeLa cytosol, kindly provided by J. Borowiec and J. Hurwitz, were prepared as described previously (38). Reaction mixtures were incubated at 37°C for 30 to 60 min, and reactions were terminated by adjusting the reaction mixtures to 10 mM EDTA–0.5% sodium dodecyl sulfate–yeast tRNA (20 μg/ml). Proteins were digested with proteinase K (100 μg/ml) at 37°C for 60 min. Samples were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and twice with chloroform-isoamyl alcohol (24:1), and unincorporated ^{32}P-labeled nucleotides were removed by Sephadex G-50 chromatography. DNA was precipitated with 2 volumes of cold ethanol. The amount of DNA synthesis was measured by the incorporation of dCMP into trichloroacetic acid-insoluble material.

2D gel analysis of replication products. To analyze the replication intermediates, purified DNA (2×10^4 to 3×10^4 cpm), synthesized in vitro, was digested in a 20-μl reaction with appropriate restriction enzymes and subjected to 2D gel electrophoretic analysis as described by Brewer and Fangman (8). The first dimension was 0.4% agarose in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) and was electrophoresed for 20 h at 1 V/cm in the same buffer. The second dimension was 1% agarose and was electrophoresed for 5 to 6 h at 5 V/cm in TBE with ethidium bromide (0.3 μg/ml) at 4°C. The gel was fixed in 7% trichloroacetic acid for 30 min, dried, and exposed to XAR-5 film (Kodak) at −70°C with an intensifying screen for 1 to 4 days.

Nitrocellulose filter binding assay. Gel-purified DNA fragments (1.0-kb *Eco*RI-*Mlu*I segment containing 20 30-bp EBV repeats and isolated from plasmid pEco3′Δ) were end labeled with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. EBNA-1 derivative, kindly provided by L. Frapier and M. O'Donnell, Cornell University Medical College, was purified from insect cells following overexpression obtained by using a baculovirus expression vector (15). EBNA-1/DNA binding was carried out at room temperature for 10 min in a reaction mixture (20 μl) containing 75 mM NaCl, 100 mM potassium glutamate, 40 mM HEPES-KOH (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg of BSA per ml, 0.2 μg of EBNA-1, and 10 to 20 fmol of [^{32}P]DNA fragments. The specificity of binding was determined by the amount of protein-bound DNA retained on the nitrocellulose filter. When EBNA-1-bound templates were used for replication assays, about 0.3 μg of the supercoiled plasmid DNA was incubated with 0.2 μg of the protein, as described above, and used directly for the replication assay.

RESULTS

Mode of replication of EBV recombinant plasmids in vitro. The replication fork barrier that was observed previously at the EBV *oriP* family of repeats in human B cells was further investigated by using an in vitro cell-free replication assay. The circular, double-stranded template DNA, plasmid pEco3′Δ (6.3 kb), contains the SV40 origin of DNA replication and the 1.68-kb *Eco*RI-to-*Eco*RV region from the *oriP* segment of EBV including the 20-member family of 30-bp repeats (Fig. 1A). Previous studies have shown that plasmids containing the SV40 DNA origin can replicate efficiently in vitro in the presence of SV40 T antigen and a soluble cytoplasmic extract from primate cells (25, 34, 38). In the in vitro system, SV40 DNA origin sequences serve as the replication initiation site of plasmid pEco3′Δ in the presence of purified SV40 T antigen. A HeLa cell extract was used as a source of the replication proteins. Supercoiled plasmid pEco3′Δ DNAs were incubated in reaction mixtures containing [α-^{32}P]dCTP at 37°C for 30 min as described in Materials and Methods. In the absence of T antigen, or when a plasmid (pBR322) lacking the SV40 origin was used, the radioactivity incorporated was reduced to 10% of the incorporation for plasmid pEco3′Δ. This finding indicates that the replication of plasmid pEco3′Δ is dependent on the presence of T antigen. The direction of DNA synthesis was monitored by digestion of the ^{32}P-labeled DNA with appropriate restriction enzymes and electrophoresis on agarose gels. The gels were then autoradiographed, and the radioactive signal from

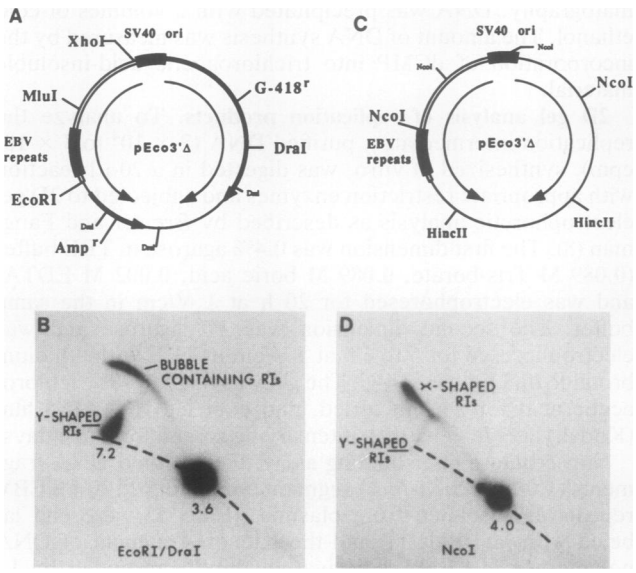

FIG. 1. 2D gel analysis of RIs from plasmid pEco3′Δ synthesized in an in vitro replication system. (A and C) Maps of plasmid pEco3′Δ (6,368 bp). The shaded rectangle indicates the EBV family of repeats consisting of 20 tandem copies of a 30-bp sequence, the solid rectangle indicates the SV40 DNA replication origin, the double line indicates the EBV *oriP*-derived sequences, and the single line indicates the prokaryotic sequences. The arrows inside the diagrams indicate the direction of replication from the SV40 DNA origin. (A) The direction of transcription of the Amp^r and G418^r genes is shown by thick arrows on the plasmid. The *Dra*I site at the boundary of the 3.6-kb *Eco*RI-*Dra*I segment containing the SV40 DNA origin is shown in large letters. The *Dra*I sites labeled in small letters indicate where *Dra*I cleaves the rest of the plasmid DNA into small segments that are not observed in the 2D gel. (C) Similarly, the *Nco*I sites at the boundary of the 4-kb segment, located opposite the SV40 DNA origin, are shown in large letters, and the rest of the plasmid DNA is cut with *Nco*I (at sites indicated by small letters) into small segments which are not observed in the 2D gel. Plasmid pEco3′Δ DNAs were incubated in reaction mixtures at 37°C for 60 min in the presence of [α-^{32}P]dCTP as described in Materials and Methods (potassium glutamate and BSA were not present in these reaction mixtures). DNA was purified, cleaved with restriction endonucleases *Eco*RI and *Dra*I (B) and *Nco*I (D), and electrophoresed on 2D gels. Gels were fixed in 7% trichloroacetic acid, dried, and exposed to X-ray films. (B) The radioactive signal from the linear, fully replicated, 3.6-kb *Eco*RI-*Dra*I segments that contain the SV40 DNA origin is shown in a 2D gel autoradiogram. An arc representing bubble-containing molecules is indicated. A signal at about 7.2 kb represents the nearly fully replicated, Y-shaped molecules. The dashed line indicates the arc of linear nonreplicating molecules. (D) *Nco*I-digested DNA shows a signal from the 4-kb linear segments located opposite the SV40 origin. A line indicative of double-Y-shaped (><) terminating molecules is observed. A faint Y arc representing the Y-shaped molecules in which the replication fork progresses from one end to the other is indicated. In both the bubble pattern (B) and double-Y-shaped pattern (D), the intensity of the signal increases in the direction of higher-molecular-weight molecules due to increased incorporation of radioactivity in the molecules as replication proceeds.

each DNA segment was quantitated by densitometric scanning. It was found that the signal (when corrected for the size of the segment) was highest for the segments nearest the SV40 DNA origin sequences and decreased gradually in segments distal to the origin (data not shown). These results were consistent with replication initiating at SV40 *ori*, proceeding bidirectionally and terminating in the segment opposite the origin.

Replication fork movement was further examined by using a 2D gel electrophoresis system (6, 8). In this system, RIs, because of their larger mass and their shape, are retarded in their mobility relative to their linear counterparts (8). Furthermore, since different RIs migrate in 2D gels according to their shape and mass in a characteristic and predictable manner, the technique allows identification of an RI containing a replication origin from a Y-shaped RI that contains a replication fork proceeding from one end to the other. Electron microscopy has been used to demonstrate that the average length of the forks on Y-shaped RIs increases with increasing position along the Y arc, with molecules having three equal branches being located at the inflection point (10). Double-Y forms derived from convergent forks from opposite ends of a segment, indicative of termination of molecules, are also distinguished.

Plasmid pEco3′Δ DNA, synthesized in vitro, was digested with restriction endonucleases and analyzed by 2D gel electrophoresis as described in Materials and Methods. The 3.6-kb *Eco*RI-*Dra*I DNA segments containing the SV40 DNA origin produced an arc pattern consistent with molecules containing a bubble (Fig. 1A and B). In addition, nearly fully replicated Y-shaped molecules, about twice the mass of a linear 3.6-kb segment, were observed. Taken together, these results indicate that the replication initiation site is asymmetrically located within the 3.6-kb *Eco*RI-*Dra*I segment. SV40 origin-containing segments as small as 2.3 kb were found to produce a similar arc representing bubble-containing molecules (e.g., the 2.3-kb *Mlu*I-*Nru*I DNA segments in Fig. 4B and D). On the other hand, the DNA segments located opposite the SV40 origin, e.g., the 4-kb *Nco*I (Fig. 1C and D) or 1.68-kb *Hin*cII (Fig. 1C; data not shown) segments, generated nearly a straight line starting at an angle of about 45° from the signal of the linear monomer (4-kb) segment. This pattern corresponds to a series of molecules containing two opposing forks and is consistent with the presence of a termination site within these segments (8). These results showed that in our in vitro replication assay, replication forks are progressing bidirectionally from the SV40 DNA origin and terminating opposite the origin. In Fig. 1D, in addition to the pattern expected of terminating molecules, a faint Y arc representing Y-shaped molecules was detected, indicative of replication that proceeds as a single fork from one end of the 4-kb *Nco*I segment to the other. These observations indicate that in a small proportion of the molecules, replication fails to terminate opposite the SV40 DNA origin.

We also found that termination of bidirectional replication forks did not occur at a unique site opposite the origin but instead appeared to involve a region at least 2 kb in length. This region was more defined when in vitro-synthesized DNA was digested with *Xho*I and *Pvu*I to generate two DNA segments, a 2.3-kb segment containing the EBV repeats and a 4-kb segment with the SV40 *ori* near the *Xho*I end and the termination site near the *Pvu*I end (Fig. 2A). Whereas the 2.3-kb segments produced a complete Y arc, those from the 4-kb segments produced a Y arc and a small triangular pattern arising out of this arc (Fig. 2B). A Y arc from the 2.3- or 4-kb segments is predicted, consistent with the SV40 DNA origin being very close to the *Xho*I-generated end. As the bubble grows and passes the *Xho*I site, the small bubble containing molecules change to Y-shaped molecules. The Y arc derived from the 4-kb segments does not reach the 2*X* mass (8 kb) value because the Y-shaped molecules finish

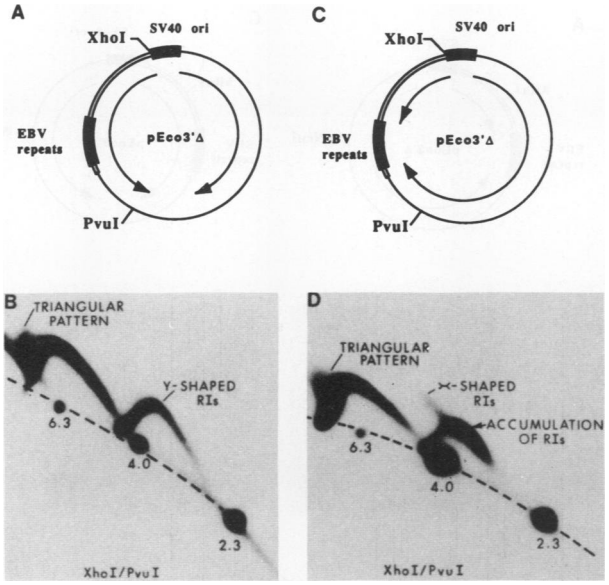

FIG. 2. Evidence that EBNA-1 binding to the EBV repeats blocks replication forks and terminates replication. (A and C) *Xho*I-*Pvu*I maps of plasmid pEco3′Δ. See the legend to Fig. 1A for an explanation of symbols. pEco3′Δ plasmid DNA was incubated without (B) or with (D) EBNA-1 added to the reaction mixtures and incubated for 30 min at 37°C as described in Materials and Methods. DNA was purified, digested with *Xho*I and *Pvu*I (positions shown in the maps in panels A and C), and subjected to 2D gel electrophoretic analysis. Strong radioactive signals at 2.3- and 4-kb represent the positions of completely replicated linear DNA segments. The weak signal at 6.3 kb is the linear plasmid DNA resulting from incomplete digestion by *Xho*I and *Pvu*I. An arc representing Y-shaped molecules is shown for both the 2.3- and 4-kb segments. The 4-kb segments also produce a triangular pattern representing double-Y-shaped terminating molecules (see text). In the presence of EBNA-1 (D), 2.3-kb segments generated a strong signal in the middle of the Y arc, indicative of an accumulation of Y-shaped molecules. Double-Y-shaped (><) or terminating molecules with two opposing forks are also produced by these segments.

replication as double-Y molecules which generate a small triangle. This triangular pattern was more intense in the DNA that was synthesized in a replication reaction incubated for 60 min (see Fig. 6A), presumably because of the presence of more terminating molecules. While a similar triangular pattern may be generated by branch migration of the replication forks in some instances (36), in the present system it is consistent with the presence of highly asymmetric molecules in which termination occurs at many sites within a region encompassing as much as 2 kb (see Fig. 6C). Recent analysis of the unidirectional replication of pBR322 by 2D gel electrophoresis indicated that when the termination site was placed at different positions within a particular segment, the resultant X-shaped molecules migrated at different positions arising out of the arc of Y-shaped RIs (28). Thus, the triangular pattern can result when there is a series of sites at which termination occurs in different molecules of pEco3′Δ DNA. These multiple termination sites may result from bidirectional replication that does not always initiate within the genetically defined SV40 origin core region. Alternatively, two replication forks that proceed bidirectionally from the SV40 DNA origin may not always proceed at an equal rate and terminate wherever the two forks meet (23, 35). On longer exposures of 2D gel autoradiograms, very faint triangular patterns, described above, were also detected in several segments spanning the length of the plasmid (data not shown). These observations suggest that in a small proportion of the molecules, replication terminates at sites other than the region opposite the SV40 DNA origin.

EBNA-1 causes replication fork arrest and termination at the EBV repeats. We examined the role of EBNA-1 in blocking the replication fork at the EBV repeats by analyzing the DNA synthesized in vitro, in the presence and absence of EBNA-1. Purified EBNA-1 derivative (see Discussion), overproduced in insect cells by using a baculovirus expression vector, binds specifically to the 1-kb *Eco*RI-*Mlu*I DNA segment that contains the 20 EBV tandem repeats (Fig. 1A). The specificity of the binding of the EBNA-1 derivative to the EBV repeats was confirmed by a filter binding assay as described in Materials and Methods (data not shown). Under similar conditions, this protein failed to bind to the remaining pEco3′Δ sequences, including the SV40 DNA origin.

Supercoiled plasmid pEco3′Δ DNA was preincubated with purified EBNA-1, and the protein-bound template was incubated for 30 min in the reaction mixture as described in Materials and Methods. The replication products were digested with *Xho*I and *Pvu*I and analyzed on 2D gels. In the absence of EBNA-1, the 2.3-kb DNA segments that contain the repeats at the center produced a Y arc consistent with a single replication fork proceeding through the segment (Fig. 2B). However, in the presence of EBNA-1, an intense signal near the inflection point of the Y arc was produced by the 2.3-kb segments (Fig. 2D). This is consistent with an accumulation of molecules in which nearly one-fourth to one-half of the 2.3-kb DNA segment has replicated. Since accumulation of half-replicated molecules represents arrested molecules, and since the EBV repeats are located in the middle of the 2.3-kb segment, these data indicate that EBNA-1 binding to the EBV family of repeats arrests replication forks. The accumulation of about one-fourth-replicated molecules suggests that some of the replication forks are arrested before they actually reach the EBV repeats. Since similar analysis of the DNA that was synthesized in a 60-min reaction shows only the accumulation of about half-replicated molecules, these results suggest that the EBV repeats are the major site of the replication fork barrier (see Fig. 6B). In the presence of EBNA-1, 2.3-kb DNA segments also produced terminating molecules and a complete Y arc, suggesting that the fork arrest leads to termination of some replication forks while other forks proceed through the segments as simple Y's and do not terminate at the repeats. A triangular pattern of terminating molecules was produced by the 4-kb segments, suggesting that the forks that failed to terminate at or near the repeats now terminate in the region opposite the SV40 origin. These results are strikingly similar to what was observed previously with EBV plasmids in human B cells (16).

EBNA-1-induced replication fork arrest and termination at the EBV repeats can be abolished by competitor DNA containing the EBV family of repeats. To test further the requirement for EBNA-1 in producing a replication fork barrier and termination, we included competitor DNA in the reaction. A 10-fold molar excess of DNA (relative to the concentration of the 1-kb *Eco*RI-*Mlu*I segment; Fig. 1A, in the plasmid) containing 20 copies of the EBV repeats efficiently competes for EBNA-1 binding in the reaction. Competitor and template pEco3′Δ DNAs were incubated with the EBNA-1 prior to the replication reaction. As a control, under similar conditions, template DNA was also incubated with EBNA-1

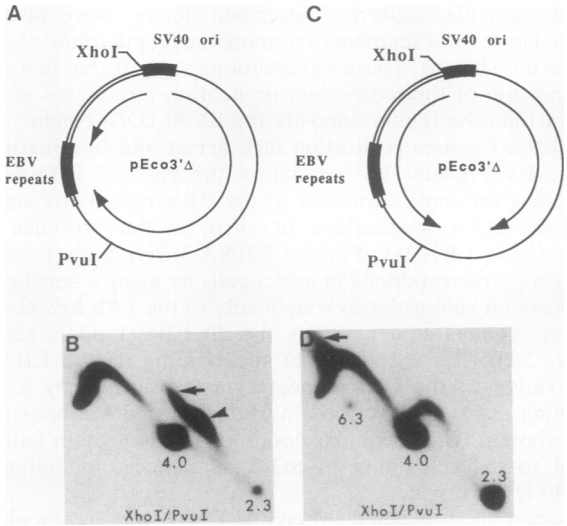

FIG. 3. Evidence that addition of competitor DNA abolishes the barrier and termination produced at the repeats due to EBNA-1 binding. (A and C) *Xho*I-*Pvu*I maps of plasmid pEco3′Δ. Purified EBNA-1 protein was allowed to bind to the supercoiled plasmid pEco3′Δ DNA in the absence (B) or presence (D) of an excess of competitor DNA as described in Materials and Methods. The competitor was a 1-kb *Eco*RI-*Mlu*I DNA segment isolated from plasmid pEco3′Δ DNA and contains 600 bp of the 20 copies of 30-bp EBV repeats (shown in Fig. 1A). DNA that was synthesized during a 60-min reaction was digested with *Xho*I and *Pvu*I and analyzed by electrophoresis on 2D gels. The radioactive signals at 2.3 and 4 kb represent the fully replicated linear DNA segments. (B) In the absence of competitor, 2.3-kb segments generate an accumulation of the Y-shaped molecules (arrowhead) and double-Y-shaped terminating molecules (arrow). A reduced signal at the position of the 2.3-kb molecules indicates a low proportion of the completely replicated linear molecules. (D) In the presence of competitor DNA, 2.3-kb segments produce a Y arc. The arrow indicates a triangular pattern, representing double-Y-shaped terminating molecules produced by RIs of the 4-kb segments.

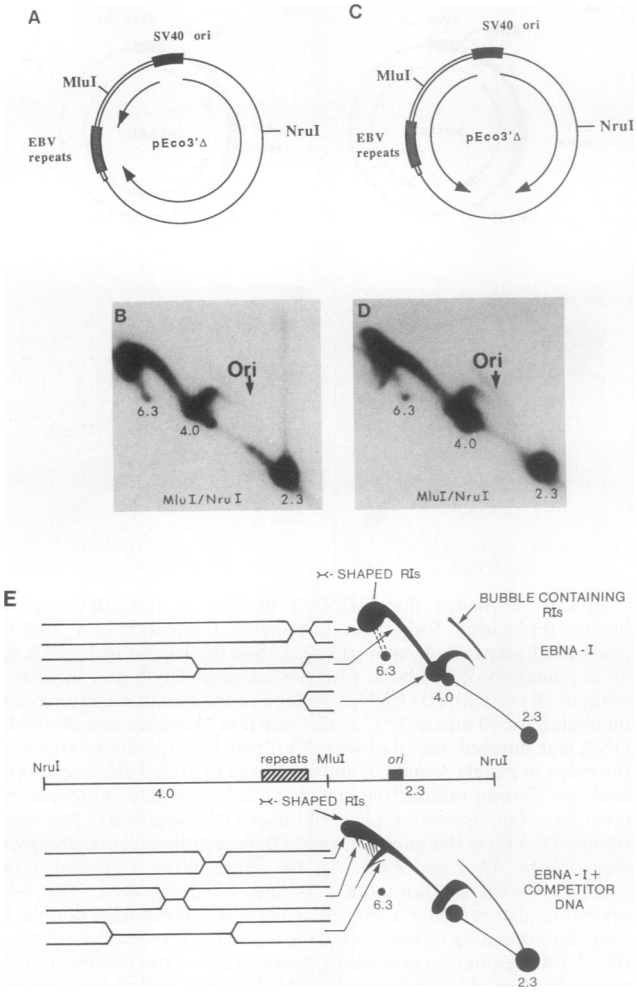

FIG. 4. Evidence that the competitor DNA eliminates the EBNA-1-mediated replication fork barrier at the EBV repeats and shifts the termination site of plasmid replication. (A and C) *Mlu*I-*Nru*I map of plasmid pEco3′Δ. See the legend to Fig. 1A for an explanation of symbols. EBNA-1 binding to plasmid pEco3′Δ DNA was carried out in the absence (B) or presence (D) of an excess of competitor DNA as described in the text and the legend to Fig. 3. DNAs were incubated in reaction mixtures at 37°C for 60 min; replication products were digested with *Mlu*I and *Nru*I and analyzed by electrophoresis on 2D gels. (B and D) 2D gel autoradiograms showing RIs generated from the 2.3- and 4-kb *Mlu*I-*Nru*I DNA segments. In the absence and presence of competitor, an arc representing bubble-containing molecules is produced by the 2.3-kb segments containing the SV40 DNA origin. The 4-kb segments generate a hooklike pattern in the absence of competitor (B) and a straight line pattern in the presence of competitor (D); both patterns represent double-Y-shaped terminating molecules. (E) Diagrammatic sketch showing how two different patterns representing terminating molecules would be produced by the 4-kb *Mlu*I-*Nru*I DNA segments on 2D gels. A hooklike pattern (shown above the linear *Mlu*I-*Nru*I map of plasmid pEco3′Δ) would be expected when one fork is stalled once it enters the restriction fragment. Replication of the segment would be terminated when an opposing fork from the other end of the segment approaches and meets the stalled fork. The process would produce a series of terminating molecules that would migrate at the positions on 2D gels shown by the arrows. On the other hand, when two opposing forks gradually progress and meet near the center of the segment, the result will be a linear pattern on 2D gels, representing nearly symmetric double-Y-shaped (><) terminating molecules (shown below the map). This would occur when there is no barrier to replication forks.

in the presence of nonspecific DNA. These DNAs were incubated in reaction mixtures at 37°C for 60 min as described in Materials and Methods, and the level of DNA synthesis was measured by the incorporation of [^{32}P]dCMP into acid-precipitable DNA. The addition of competitor as well as nonspecific DNAs reduced DNA synthesis approximately two- to threefold (data not shown).

The effect of competitor DNA on the EBNA-1-induced barrier was examined on 2D gels (Fig. 3 and 4). In the presence of EBNA-1, 2.3-kb *Xho*I-*Pvu*I DNA segments that contain the EBV repeats at the center produced a strong replication fork barrier and a line pattern representing double-Y-shaped terminating molecules (Fig. 3B). In this particular experiment, the 2.3-kb segments did not produce a complete Y arc, indicating that all of the replication forks were arrested and terminated at or near the repeats and that the barrier was absolute. This was confirmed further by the absence of a triangular pattern of terminating molecules in the 4-kb *Xho*I-*Pvu*I segments, which now produced only a Y arc corresponding to the forks progressing from one end of the segment to the other. In contrast, addition of competitor DNA virtually abolished the barrier as well as termination in the 2.3-kb *Xho*I-*Pvu*I DNA segments and yielded only a Y arc, indicating that replication forks pass through the segments without being stopped (Fig. 3D). A triangular pattern corresponding to the terminating molecules was now pro-

duced by the 4-kb *Xho*I-*Pvu*I segments, suggesting that the competitor DNA had abolished the barrier and shifted the termination site from the region of the repeats to the region opposite the SV40 origin. Addition of nonspecific DNA that does not bind EBNA-1 did not abolish the barrier, nor did it affect termination in the region of the repeats (data not shown). The simplest interpretation of these data is that an excess of DNA containing the EBV repeats has competed with the EBV repeats, present in plasmid pEco3′Δ, for EBNA-1 binding and relieved the block to replicating forks. Although the competitor DNA is 1 kb and includes about 400 bp of sequences flanking the repeats, footprinting experiments have established that EBNA-1 binds to a consensus sequence present only in the DNA of the repeats (30). Thus, our results indicate that formation of a replication barrier and generation of a termination site at or near the EBV repeats involves EBNA-1 binding to DNA of the EBV repeats.

Although addition of the competitor DNA reduced replication efficiency threefold, it did not affect the pattern of replication initiation from the SV40 origin. This was illustrated by the 2D gel pattern generated by *Mlu*I-*Nru*I digestion of the DNA. *Mlu*I-*Nru*I digestion yields two DNA segments, a 2.3-kb segment with the SV40 DNA origin and a 4-kb segment containing the EBV repeats located toward the *Mlu*I end (Fig. 4A and C). In the presence or absence of the competitor DNA, the 2.3-kb segments produced an arc representing bubble-containing molecules and almost fully replicated Y-shaped molecules. This pattern was consistent with an asymmetrically located SV40 DNA origin (Fig. 4B and D). However, the termination pattern produced by the 4-kb segments was different and depended on the presence or absence of competitor DNA. In the absence of the competitor, a hooklike pattern was generated by these segments (Fig. 4B). This pattern is consistent with the presence of double-Y-shaped terminating molecules in which one fork is stalled near the end of the segment (19, 28) (Fig. 4E). Replication of the molecule would be expected to terminate when a second fork approaches from the other end and meets the arrested fork. In the presence of the competitor DNA, the 2D gel pattern observed was nearly a straight line, consistent with the presence of terminating molecules in which two opposing replication forks meet in the approximate center of the segment (Fig. 4D). These results suggest that in the presence of competitor DNA, the forks proceed through the family of repeats and termination now occurs near the middle of the segment (Fig. 4E).

There are two replication fork arrest sites within the EBV repeats. On shorter exposures of the autoradiograms, similar to the one shown in Fig. 2D, two distinct spots (equal in intensity) of accumulated molecules could be distinguished within the signal near the center of the Y arc generated by the 2.3-kb *Xho*I-*Pvu*I segments (Fig. 5B). This indicates that there are two sites within or near the 600-bp EBV repeats, at which replication is arrested with equal probability. Two barriers at the repeats were also observed in the EBV plasmids in human B cells (16, 29a).

Replication of plasmid pEco3′Δ DNA proceeds bidirectionally from the SV40 DNA origin. Since the replication forks that progress in a counterclockwise direction from the SV40 origin would reach the repeats first, their arrest at two distinct sites (barriers) within the repeats would result in the accumulation of two populations of RIs. Termination of replication would occur when the clockwise fork moves around the molecule and meets the arrested fork at the repeats. To confirm this direction of replication fork arrest,

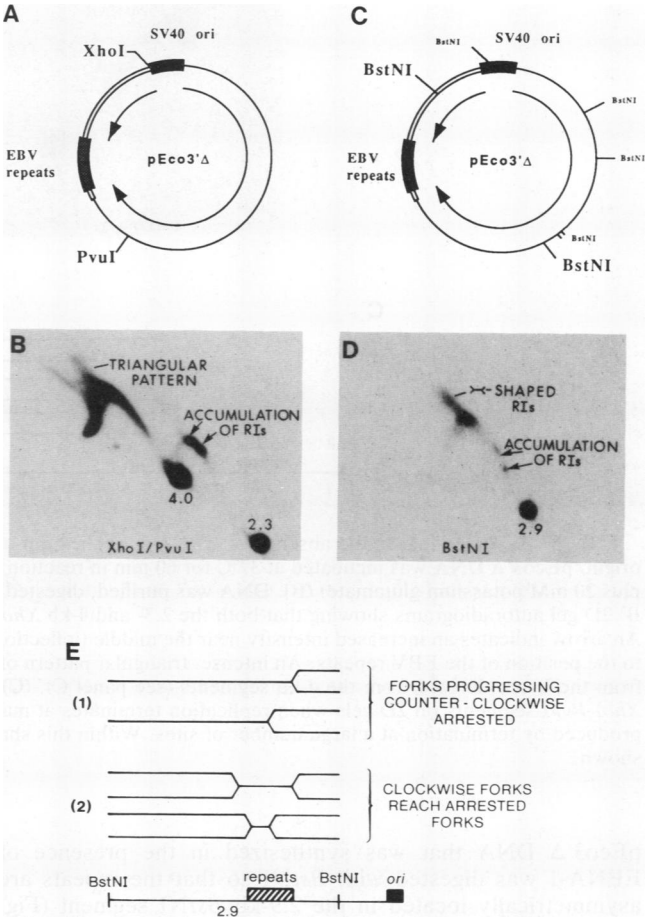

FIG. 5. Evidence that in the presence of EBNA-1, the EBV repeats arrest replication forks progressing counterclockwise from the SV40 DNA origin. (A and C) *Xho*I-*Pvu*I (A) and *Bst*NI (C) maps of plasmid pEco3′Δ. See the legend for Fig. 1A for an explanation of symbols. pEco3′Δ DNA synthesized in vitro in the presence of EBNA-1 was digested with *Xho*I and *Pvu*I (B) or *Bst*NI (D) and analyzed by electrophoresis on 2D gels. (B) The 2.3- and 4.0-kb linear *Xho*I-*Pvu*I DNA segments produce high-intensity spots. Two arrows indicate two accumulations of Y-shaped molecules near the middle (inflection point) of the Y arc derived from the 2.3-kb segments. (D) In the *Bst*NI-cut DNA, the intense signal at 2.9 kb corresponds to the position of the completely replicated linear molecules. An arc of Y-shaped molecules and a line of double-Y-shaped (><) terminating molecules is produced from these segments. In addition, two spots representing accumulations of Y-shaped molecules are detected in the middle of the ascending part of the Y arc. Their positions correspond to Y-shaped molecules of approximately 1.2*X* to 1.3*X* mass (see panel E). (E) Diagrammatic sketch showing different forms of RIs that would be expected to accumulate depending on the direction in which replication forks are arrested at the EBV repeats. Shown at the bottom is the map of the 2.9-kb *Bst*NI DNA segment with asymmetrically located EBV repeats. (1) If forks progressing in the counterclockwise direction from the SV40 origin were arrested at the repeats, Y-shaped molecules of about 1.2*X* to 1.3*X* mass would accumulate, where *X* is the mass of the linear 2.9-kb *Bst*NI segment; (2) replication of the molecule would be expected to terminate when an opposing fork from the other end approaches and meets the arrested fork.

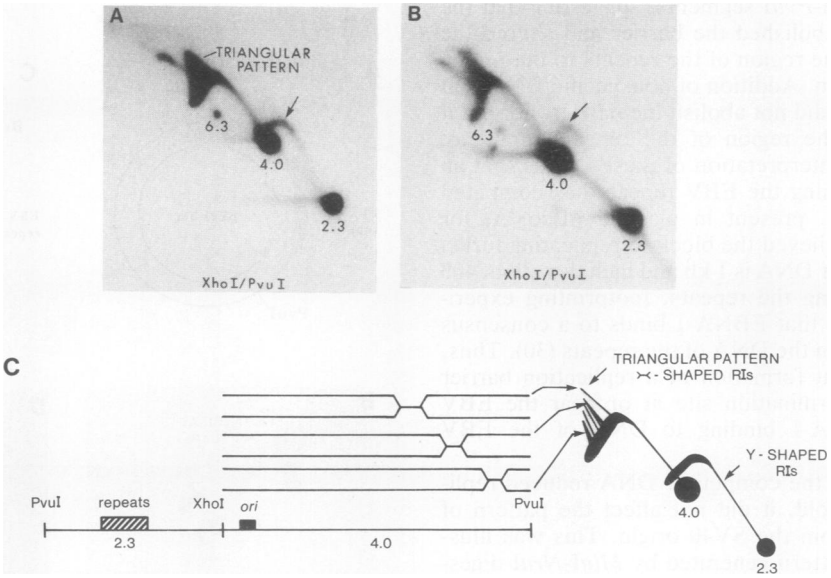

FIG. 6. Evidence that in the absence of EBNA-1, replication of plasmid pEco3′Δ terminates in a broad region opposite the SV40 DNA origin. pEco3′Δ DNA was incubated at 37°C for 60 min in reaction mixtures containing low salt (25 mM NaCl) (A) or high salt (50 mM NaCl plus 20 mM potassium glutamate) (B). DNA was purified, digested with *Xho*I and *Pvu*I, and analyzed by electrophoresis on 2D gels. (A and B) 2D gel autoradiograms showing that both the 2.3- and 4-kb *Xho*I-*Pvu*I DNA segments produce an arc representing Y-shaped molecules. An arrow indicates an increased intensity near the middle (inflection point) of the Y arc produced by the 2.3-kb segments which corresponds to the position of the EBV repeats. An intense, triangular pattern of radioactivity representing double-Y-shaped terminating molecules arises from the Y arc derived from the 4-kb segments (see panel C). (C) Diagram showing the triangular pattern (shaded) generated by the 4-kb *Xho*I-*Pvu*I segments on 2D gels when replication terminates at many sites near the end of this segment. The shaded area in the diagram is produced by termination at a large number of sites. Within this shaded area, the pattern produced by termination at three particular sites is shown.

pEco3′Δ DNA that was synthesized in the presence of EBNA-1 was digested with *Bst*NI so that the repeats are asymmetrically located in the 2.9-kb *Bst*NI segment (Fig. 5C). If replication forks proceeding only in the counterclockwise direction from the SV40 DNA origin were arrested at two sites within the repeats, we would expect an accumulation of Y-shaped molecules of about 1.2*X* to 1.3*X* mass, where *X* is the mass of the unreplicated 2.9-kb *Bst*NI molecule (Fig. 5E). If, however, forks moving in a counterclockwise direction proceeded through the repeats, then forks moving in a clockwise direction would encounter them before reaching the repeats themselves, in the region opposite the SV40 origin.

When the RIs from the 2.9-kb *Bst*NI segments were analyzed on the 2D gels, a Y arc, a line representing double-Y-shaped terminating molecules, and two spots near the middle of the ascending part of the Y arc were detected (Fig. 5D). Analysis by electron microscopy (10) has established that this region of the Y arc contains molecules that have replicated about 25% of their DNA. Thus, the two spots in Fig. 5D indicate the presence of molecules whose mass is about 1.2*X* and 1.3*X* the mass of an unreplicated *Bst*NI segment. The *Xho*I-*Pvu*I analysis of the same DNAs has already demonstrated that termination occurs at the EBV repeats in the 2.3-kb segments in some molecules and opposite the SV40 origin in the 4-kb segments in others (Fig. 5B). Therefore, the termination pattern produced by the 2.9-kb *Bst*NI segments represents a mixture of molecules terminating at either of these two sites. The presence, however, of two spots whose positions corresponded to the Y-shaped molecules of about 1.2*X* and 1.3*X* mass indicate that replication forks moving in a counterclockwise direction from the SV40 DNA origin have arrested at two distinct sites within or near the EBV repeats (Fig. 5E).

In the absence of EBNA-1, the EBV repeats can also arrest replication forks, but at reduced efficiency. The binding of EBNA-1 to the pEco3′Δ DNA was carried out at 75 mM NaCl plus 100 mM potassium glutamate for 10 min at room temperature; subsequent in vitro replication assays were at higher salt concentrations (35 to 50 mM NaCl plus 20 mM potassium glutamate) than normally used (20 to 25 mM NaCl) for SV40 DNA replication assays. To determine whether this increase in the salt concentration alone could produce the replication fork barrier at the EBV repeats, experiments were carried out in which the pEco3′Δ DNA template was preincubated for 10 min at room temperature with high salt (75 mM NaCl plus 100 mM potassium glutamate) and then diluted to 50 mM NaCl plus 20 mM potassium glutamate for the replication reaction. In parallel experiments, the template was simply allowed to replicate at high salt concentrations ranging from 30 to 50 mM NaCl plus 20 to 75 mM potassium glutamate. Replication products from both reactions were digested with *Xho*I and *Pvu*I and analyzed on 2D gels. We found that preincubation of the template with high salt without EBNA-1 or replication in the presence of high salt did not produce a detectable replication fork barrier or termination at the EBV repeats in a 30-min replication reaction, although EBNA-1 produced a strong barrier and termination at the EBV repeats during this period (Fig. 2D). If, however, this reaction was allowed to proceed for 60 min or longer, a faint barrier corresponding to the position of the repeats was detected in the absence of EBNA-1, regardless of the salt concentration (indicated by arrows in Fig. 6A and B). We observed a similar faint barrier when cell extracts

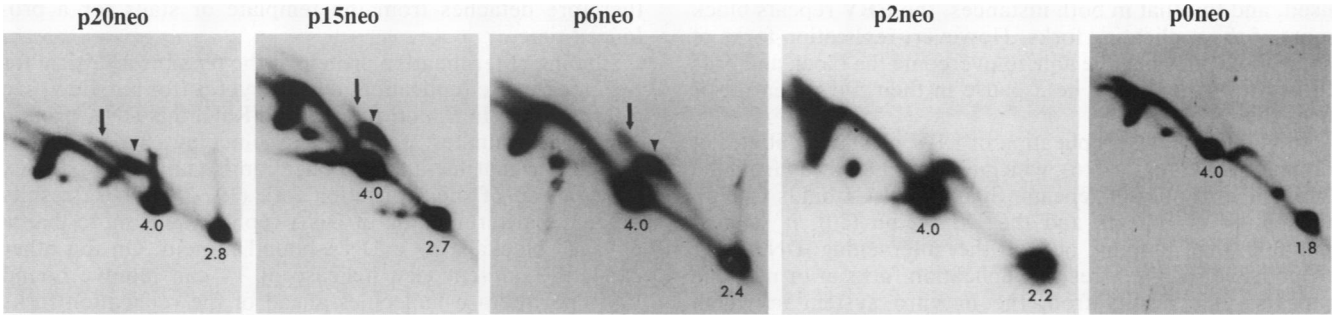

FIG. 7. Barrier to replication forks with a reduced number of repeats. Plasmids p20neo, p15neo, p6neo, p2neo, and p0neo DNAs, containing 20, 15, 6, 2, and 0 repeats, respectively, were incubated with EBNA-1, and protein-bound DNAs were incubated in reaction mixtures at 37°C for 60 min. Purified DNAs were digested with *Xho*I and *Pvu*I and electrophoresed on 2D gels. For each plasmid, the size of the DNA segment containing the region opposite the SV40 origin is 4.0 kb. In each case, a Y arc, indicative of a replication fork progressing from one end of the segment to the other, is detected. The sizes of the DNA segments (in kilobases) containing the repeats are 2.8 for p20neo, 2.7 for p15neo, 2.4 for p6neo, 2.2 for p2neo, and 1.8 for the corresponding segment in p0neo in which the repeats are absent. An arrow indicates termination of replication, and an arrowhead indicates an accumulation of RIs due to the replication fork barrier at the repeats in p20neo, p15neo, and p6neo. The Y arc from the smaller segment in p0neo emanates from the 1.8-kb linear segment, rather than the segment at about 2.2 kb, which may be the result of partial digestion.

prepared from two additional human cell lines (293 and Raji) were used for replication assays. One explanation for these results is that there are proteins present in human cell extracts that bind to the repeats and create a weak barrier. Alternatively, since each copy of the family of repeats contains a partial inverted sequence, it is possible that incubation at 37°C for 60 min or longer can induce an alteration in the secondary structure of the DNA of the repeats which then impedes the progression of replication forks.

Relationship between the number of the EBV repeats and the strength of the EBNA-1-mediated barrier. Each of the 20 tandem repeats contains a consensus sequence for binding of EBNA-1. We examined a series of plasmids which contained progressively lower numbers of repeats in order to determine whether replication forks encountering fewer molecules of EBNA-1 will be arrested. Except for p20neo, which contains all 20 repeats, the templates contain 15 (p15neo), 12 (p12neo), 6 (p6neo), 2 (p2neo), and 0 (p0neo) repeats (see Materials and Methods). These templates were allowed to replicate in the presence of EBNA-1, and DNA was digested with *Xho*I and *Pvu*I and analyzed on 2D gels (Fig. 7; data not shown for p12neo).

Several types of evidence for the presence of a replication barrier were observed. First, in the templates containing between 6 and 20 repeats, termination (arrows in Fig. 7) occurred primarily in the region at or near the repeats instead of opposite the SV40 origin of replication. Second, in the absence of any repeats or in the presence of only two repeats, termination occurred predominantly in the region opposite the SV40 origin of replication (indicated by the filled triangular region at the far left of the Y arc for the 4-kb segments in p2neo and p0neo [Fig. 7]). Conversely, in the presence of six or more repeats, termination is minimal in the region opposite the SV40 origin. Third, for the segments containing from 6 to 20 repeats compared with the segments containing 2 or no repeats, significantly fewer RIs are detected in the region through which the replication fork would progress if it were not blocked at or near the repeats. Fourth, an accumulation of RIs (arrowheads in Fig. 7) is evident in the region of the repeats for plasmid p20neo. This accumulation is not as clear for the other plasmids shown in Fig. 7, since the intensity of the pattern increases with increasing size of the RIs as a result of increased incorporation of [^{32}P]dCMP. Our results indicate that binding of the EBNA-1 protein to at least 6 repeats can create an effective barrier and that the presence of 20 repeats in tandem is not required for producing this barrier. Deletion of all 20 repeats in plasmid p0neo completely abolished barrier formation as well as termination in the 1.8-kb *Xho*I-*Pvu*I segments, although the EBV sequences near the 3′ end of the repeats are still present. Failure to detect any barrier in plasmid p0neo suggests that these EBV sequences are not sufficient for impeding the replication fork progression.

DISCUSSION

As part of our effort to understand the mechanism of replication fork arrest and termination at the EBV family of repeats, we have analyzed the role of EBNA-1 protein binding in this process. This was done by analyzing the RIs that are synthesized in vitro, in a cell-free replication system, from SV40 origin/EBV *oriP* tandem repeat-containing plasmids. Replication of EBV plasmids in human B cells requires the presence of a single viral protein, EBNA-1, that binds to a consensus sequence present in each repeat unit (30). Our results show that in the presence of EBNA-1, the replication forks have arrested with high efficiency at the family of repeats. When additional copies of the family of repeats were added to compete in *trans* for this specific interaction of EBNA-1, the replication barrier and termination were barely detectable. This finding provides direct evidence that binding of the EBNA-1 protein to the repeats produces the barrier to replication fork progression. The ability of EBNA-1 to bind to the EBV repeats and arrest replication forks enabled us to relate results directly to previous work in which a replication fork barrier was also observed at the EBV repeats in human B cells (16).

There are strong similarities between the replication barrier and termination that were observed at the EBV repeats in human B cells (16) and in the present in vitro SV40 DNA replication assays. These results imply (i) that the assembly of template DNA into chromatin is not essential for blocking replication forks, (ii) that the EBV repeats in conjunction with EBNA-1 arrest replication forks and mediate replication termination, regardless of the replication origin that was

used, and (iii) that in both instances, the EBV repeats block most of the replicating forks. However, replication forks of some molecules may be able to overcome the block and pass through, or there may be a pause in their movement at or near the repeats.

In human B cells, replication of EBV plasmids initiates at or near the DS region (16), which is about 1 kb away from the 20-member family of repeats. Since EBNA-1 binds to both the family of repeats and the DS region (30), it seemed possible that looping out of the intervening DNA was responsible for the arrest of replication forks at or near the repeats. Our results from the in vitro system in which plasmid pEco3′Δ lacks the DS region and initiates replication from the SV40 origin clearly indicate that EBNA-1-mediated looping between the DS element and the repeats is not the cause of the barrier at or near the repeats. Our studies also indicate that the presence of 20 repeats in tandem is not necessary for replication fork arrest, since reducing the number of repeats from 20 to 6 still resulted in a replication barrier at the repeats.

In prokaryotes, the replication termination region contains two physically separate stop sites, one that can arrest forks approaching from the clockwise direction and one that can arrest forks approaching from the counterclockwise direction (for a review, see reference 21). In *E. coli*, each of these sites is present in duplicate. Our studies also revealed two stop sites within or near the EBV repeats. However, both of these sites are due to the arrest of replication forks progressing in only one direction. This finding, the explanation for which is presently unclear, may reflect the mode of binding of the EBNA-1 or the formation of a characteristic secondary structure of the repeats as a result of protein binding. These results are being investigated further.

A replication fork barrier has been reported in yeast ribosomal DNA molecules (9, 26). These reports and electron microscopic studies on *Drosophila* ribosomal DNA (29) and in vitro replication studies on *E. coli* phage fd DNA templates (4) led to the proposal that the act of transcription inhibited the movement of replication forks to avoid direct collision between the polymerases, creating barriers at the 3′ ends of the actively transcribed genes (7, 9). In latently infected human B cells, the most frequently transcribed EBV genes are the two small EBER genes whose 3′ ends lie about 300 bp from the family of repeats and are transcribed in the direction of the repeats (2, 33). It was therefore possible that the replication fork barrier observed in the region of the EBV family of repeats in the viral genome and in the EBV plasmid p174 was produced by the same in vivo mechanism (16). In our studies, we have used plasmid pEco3′Δ and the plasmids of the p20-2neo series that lack EBER genes. Moreover, the direction of transcription from the Amp^r and the $G148^r$ gene promoter in these plasmids is the same as the direction of replication from the SV40 origin (Fig. 1A). These experiments, carried out in vitro, indicate that at least in these plasmids, the replication barrier is not due to opposing RNA/DNA polymerase molecules.

The mechanism by which EBNA-1 binding can stop the progression of replication forks in the region of the repeats is currently unknown. There is increasing evidence that protein bound to DNA can cause termination of replication (for a review, see reference 21) and termination of transcription (22, 14). T4 DNA polymerase, which is required for replication, pauses at specific sites on double-stranded DNA templates in the presence of protein (5). It has been suggested that T4 polymerase holoenzyme becomes destabilized on regions of the template that are difficult to traverse and therefore detaches from the template or stalls for a prolonged time.

Binding of termination protein at the *ter* sites is critical for the block in the replication in prokaryotes (for a review, see reference 21). In *E. coli* and plasmid R6K, this DNA-protein complex terminates replication by blocking DnaB helicase, an enzyme that facilitates replication by unwinding duplex DNA ahead of the replication fork (19, 24). These results suggest that termination of DNA replication is more than a physical blockage by a DNA-bound protein. On the other hand, the *dda*-encoded helicase of T4 can remove bound RNA polymerase molecules ahead of the replication forks and thus eliminate the replication fork barrier caused by this protein binding (4). Replication of the plasmids from the SV40 origin is dependent on the binding of the SV40 T antigen that subsequently functions as a helicase, unwinding the DNA in advance of the replication fork while progressing in the 3′-to-5′ direction. In our studies, the T antigen acting as a helicase is unable to overcome the EBNA-1-mediated replication block at the repeats. Whether this EBNA-1/DNA complex also arrests replication forks by blocking T-antigen helicase or some other replication protein, in a manner analogous to that of the *ter*-protein complex that blocks the DnaB helicase in prokaryotes, remains to be determined. The EBNA-1 used in the present studies was synthesized in baculovirus-infected cells and does not exhibit ATPase activity, suggesting that this protein is not a helicase (15).

Another possibility is that binding of EBNA-1 can promote bending of the DNA or some other overall structural change in organization in the region of the repeats, preventing progress of the replication forks. Consistent with this interpretation is the fact that the replication block does not appear to depend on the DNA sequence alone. When EBNA-1 binding was abolished in competition experiments, barrier formation was almost completely eliminated, suggesting that a specific protein-DNA interaction is required for the barrier and termination.

A number of published studies with EBNA-1 have attempted to define the regions of the protein essential for the replication of EBV plasmids in human B cells (41; for a review, see reference 20). EBNA-1 produced in B cells is a 72-kDa protein consisting of 641 amino acids with a basic amino-terminal end and a carboxy terminus that is rich in acidic and basic amino acids. DNA binding specificity resides in the C-terminal third of EBNA-1 (30), and this region appears to be important for the replication of EBV plasmids (41). For our experiments, we used an EBNA-1 derivative which lacks the glycine-alanine domain and six amino acids from the N-terminal end (15). These two deletions (when examined separately) did not appear to affect the replication of EBV plasmids in human cells (41). We have now found that the replication fork barrier produced by this EBNA-1 derivative in the in vitro system is similar to that observed in human cells.

The ability of the EBV repeats to produce a faint replication fork barrier in the absence of EBNA-1 could have several explanations. One simple explanation is that the EBV family of repeats adopts a secondary structure that can retard the progression of replication forks. The structure of the EBV family of repeats has been defined in great detail (30). Each 30-bp repeat unit contains a partial 9-bp inverted repeat that has the potential to form a small 9-bp stem-loop structure. In single-stranded fd DNA, stable hairpinlike helices have been shown to act as barriers to DNA synthesis (18). It should be noted that a DNA region expected to form a very stable hairpin helix in single-stranded DNA does not

always result in pausing of DNA replication on a double-stranded DNA template (5).

Alternatively, since the EBV family of repeats can act as an enhancer element, it might serve as a binding site for a variety of other cellular proteins. It is possible, therefore, that some proteins present in the human cell cytosol can bind to the repeats and cause fork arrest. In this context, it is interesting that at least one cellular factor (anti-EBNA-1) from tetradecanoyl phorbol acetate-activated human B cells competes with EBNA-1 for binding to *oriP* (37). Whether cellular factors play a direct role in replication fork arrest and termination or an indirect role, such as being necessary for transcription or replication, is not clear at present. It is possible that EBNA-1 produces a strong barrier because the amount of protein present in human B cells or in an in vitro replication system is not limiting. Moreover, this protein binds very strongly to the DNA of the repeats. However, if the putative cellular protein was sufficiently weak at binding or the amount present in the cell extract was limiting, a weak barrier could be produced. This appears to be the case with replication termination at the *ter* site in prokaryotes, the *ter*-binding protein of which is expressed at low amounts in the cell. When, however, the *ter*-binding protein is supplied in excess in pure form, it enhances termination by a factor of 2 to 4, and a stronger barrier to replication forks is detected (19). The EBNA-1 binding to the repeats could also generate a strong barrier simply by sequestering more proteins from the cellular extract. In fact, from our studies it is not yet clear whether EBNA-1 by itself is sufficient to arrest replication fork movement. Experiments are in progress to determine whether EBNA-1 can arrest replication forks when added to an in vitro replication system reconstituted with purified proteins.

ACKNOWLEDGMENTS

We thank L. Frappier and M. O'Donnell for providing the EBNA-1 used in these studies and for helpful discussions. We also thank J. Borowiec and J. Hurwitz for providing T antigen. We are grateful to T. Kelly, B. Stillman, and B. Schvartzman for valuable suggestions and to B. Brewer and W. Fangman for critical reading of the manuscript. We thank I. Tcherepanova for carrying out the studies with plasmid p2neo.

These studies were supported in part by grant CD 312 from the American Cancer Society. Support was also provided by Cancer Center Core Grant NIH/NCI P30-CA13330.

REFERENCES

1. **Adams, A.** 1987. Replication of latent Epstein-Barr virus genomes in Raji cells. J. Virol. **61**:1743–1746.
2. **Arrand, J. R., and L. Rymo.** 1982. Characterization of the major Epstein-Barr virus-specific RNA in Burkitt's lymphoma-derived cells. J. Virol. **41**:376–389.
3. **Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatful, G. S. Hudson, S. D. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
4. **Bedinger, P., M. Hochstrasser, C. V. Jongeneel, and B. M. Alberts.** 1983. Properties of the T4 bacteriophage DNA replication apparatus: the dda DNA helicase is required to pass a bound RNA polymerase molecule. Cell **34**:115–123.
5. **Bedinger, P., M. Maureen, and B. M. Alberts.** 1989. Sequence-specific pausing during *in vitro* DNA replication on double-stranded DNA templates. J. Biol. Chem. **264**:16880–16886.
6. **Bell, L., and B. Byers.** 1983. Separation of branched from linear DNA by two-dimensional gel electrophoresis. Anal. Biochem. **130**:527–535.
7. **Brewer, B. J.** 1988. When polymerases collide: replication and the transcriptional organization of the *E. coli* chromosome. Cell **53**:679–686.
8. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins on ARS plasmids *S. cerevisiae*. Cell **51**:463–471.
9. **Brewer, B. J., and W. L. Fangman.** 1988. A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. Cell **55**:637–643.
10. **Brewer, B. J., E. P. Sena, and W. L. Fangman.** 1984. Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. Cancer Cells **6**:229–234.
11. **Chittenden, T., S. Lupton, and A. J. Levine.** 1989. Functional limits of *oriP*, the Epstein-Barr virus plasmid origin of replication. J. Virol. **63**:3016–3025.
12. **Deb, S., A. L. DeLucia, C.-P. Baur, A. Koff, and P. Tegtmeyer.** 1986. Domain structure of the simian virus 40 core origin of replication. Mol. Cell. Biol. **6**:1663–1670.
13. **DePamphilis, M. L.** 1988. Transcriptional elements as components of eukaryotic origin of replication. Cell **52**:635–638.
14. **Deuschle, U., R. A. Hipskind, and H. Bujard.** 1990. RNA polymerase II transcription blocked by *Escherichia coli* lac repressor. Science **248**:480–483.
15. **Frappier, L., and M. O'Donnell.** 1991. Overproduction, purification, and characterization of EBNA1, the origin binding protein of Epstein-Barr virus. J. Biol. Chem. **266**:7819–7826.
16. **Gahn, T. A., and C. L. Schildkraut.** 1989. The Epstein-Barr virus origin of plasmid replication, *oriP*, contains both the initiation and termination sites of DNA replication. Cell **58**:527–535.
17. **Hampar, B., A. Tanaka, M. Nonoyama, and J. G. Derge.** 1974. Replication of the resident repressed Epstein-Barr virus genome during the early S phase (S-1 period) of non-producer Raji cells. Proc. Natl. Acad. Sci. USA **71**:631–633.
18. **Huang, C. C., and J. E. Hearst.** 1980. Pauses at positions of secondary structure during *in vitro* replication of single-stranded fd bacteriophage DNA by T4 DNA polymerase. Anal. Biochem. **103**:127–139.
19. **Khatri, G. S., T. MacAllister, P. R. Sista, and D. Bastia.** 1989. The replication terminator protein of *E. coli* is a DNA sequence-specific contra-helicase. Cell **59**:667–674.
20. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr Virus and its replication, p. 1889–1920 *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, Ltd., New York.
21. **Kuempel, P. L., A. J. Pelletier, and T. M. Hill.** 1989. Tus and terminators: the arrest of replication in prokaryotes. Cell **59**: 581–583.
22. **Kuhn, A., I. Bartsch, and I. Grummt.** 1990. Specific interaction of the murine transcription termination factor TTF1 with class-1 RNA polymerases. Nature (London) **344**:559–562.
23. **Lai, C. J., and D. Nathans.** 1975. Non-specific termination of simian virus 40 DNA replication. J. Mol. Biol. **97**:113–118.
24. **Lee, E. H., A. Kornberg, M. Hidaka, T. Kobayashi, and T. Horiuchi.** 1989. *Escherichia coli* replication termination protein impedes the action of helicases. Proc. Natl. Acad. Sci. USA **86**:9104–9108.
25. **Li, J. J., and T. J. Kelly.** 1984. Simian virus 40 DNA replication *in vitro*. Proc. Natl. Acad. Sci. USA **81**:6973–6977.
26. **Linskens, M. H. K., and J. A. Huberman.** 1988. Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4927–4935.
27. **Lupton, S., and A. J. Levine.** 1985. Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. Mol. Cell. Biol. **5**:2533–2542.
28. **Martin-Parras, L., P. Hernandez, M. L. Martinez-Robles, and J. B. Schvartzman.** 1991. Unidirectional replication as visualized by two-dimensional agarose gel electrophoresis. J. Mol. Biol. **220**:843–853.
29. **McKnight, S. L., M. Bustin, and O. L. Miller, Jr.** 1978. Electron microscopic analysis of chromosome metabolism in the *Drosophila melanogaster* embryo. Cold Spring Harbor Symp. Quant. Biol. **42**:741–754.

29a. **Platt, T., and C. Schildkraut.** Unpublished data.

30. **Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward.** 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42**:859–868.
31. **Reisman, D., and B. Sugden.** 1986. *trans* activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell. Biol. **6**:3838–3846.
32. **Reisman, D., J. Yates, and B. Sugden.** 1985. A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two *cis*-acting components. Mol. Cell. Biol. **5**:1822–1832.
33. **Rosa, M. D., E. Gottlieb, M. R. Lerner, and J. A. Steitz.** 1981. Striking similarities are exhibited by two small Epstein-Barr virus-encoded ribonucleic acids and adenovirus-associated ribonucleic acids VAI and VAII. Mol. Cell. Biol. **1**:785–796.
34. **Stillman, B. W., and Y. Gluzman.** 1985. Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. Mol. Cell. Biol. **5**:2051–2060.
35. **Tack, L. C., and G. N. Proctor.** 1987. Two major replicating simian virus 40 chromosome classes. J. Biol. Chem. **262**:6339–6349.
36. **Vaughn, J. P., P. A. Dijkwel, and J. L. Hamlin.** 1990. Replication initiates in a broad zone in the amplified *CHO* dihydrofolate reductase domain. Cell **61**:1075–1087.
37. **Wen, L.-T., A. Tanaka, and M. Nonoyama.** 1989. Induction of anti-EBNA-1 protein by 12-*O*-tetradecanoyl phorbol-13-acetate treatment of human lymphoblastoid cells. J. Virol. **63**:3315–3322.
38. **Wobbe, C. R., F. B. Dean, L. Weissbach, and J. Hurwitz.** 1985. *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. Proc. Natl. Acad. Sci. USA **84**:1834–1834.
39. **Wysokenski, D. A., and J. L. Yates.** 1989. Multiple EBNA 1 binding sites are required to form an EBNA 1-dependent enhancer and to activate a minimal replication origin within *oriP* of Epstein-Barr virus. J. Virol. **63**:2657–2666.
40. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81**:3806–3810.
41. **Yates, J. L., and S. M. Camiolo.** 1988. Dissection of DNA replication and enhancer activation functions of Epstein-Barr virus nuclear antigen 1. Cancer Cells **6**:197–205.