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A Putative Origin of Replication of Plasmids Derived from Epstein-Barr Virus Is Composed of Two *cis*-Acting Components

DAVID REISMAN, JOHN YATES, AND BILL SUGDEN*
McArdle Laboratory, University of Wisconsin, Madison, Wisconsin 53706

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A genetic element of Epstein-Barr virus, *oriP*, when present on recombinant plasmids allows those plasmids to replicate and to be maintained in cells that express the Epstein-Barr virus-encoded nuclear antigen EBNA-1. Here we define the DNA sequences required for *oriP* activity. Two noncontiguous regions of *oriP* are required in *cis* for activity. One consists of approximately 20 tandem, imperfect copies of a 30-base-pair (bp) sequence. The other required region, approximately 1,000 bp away, is at most 114 bp in length and contains a 65-bp region of dyad symmetry. When present together on a plasmid, these two components supported plasmid replication even when the distance between them was varied or their relative orientation was altered, or both. When present alone on a plasmid that expresses a selectable marker, the family of 30-bp repeats efficiently conferred a transient drug-resistant phenotype in human 143 cells that is dependent on the presence of EBNA-1. This result leads us to suggest that EBNA-1 interacts with the 30-bp repeated sequence to activate *oriP*. To test whether the 30-bp repeats might cause the increased transient expression of drug resistance by enhancing transcription, the family of 30-bp repeats was tested for the ability to activate the simian virus 40 early promoter present in plasmid $pA_{10}CAT_2$ (Laimins, et al., Proc. Natl. Acad. Sci. U.S.A. 79:6453–6457). In this assay, the 30-bp repeats could activate the simian virus 40 early promoter in Raji cells, an EBNA-positive Burkitt's lymphoma cell line, but not detectably an EBNA-positive 143 cells in which *oriP* also functions.

Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus which is the causative agent of infectious mononucleosis and is associated with two human cancers, Burkitt's lymphoma and nasopharyngeal carcinoma (for a review see reference 43). B-lymphoid cells that have been transformed in vitro by EBV, or lymphoid cells derived from Burkitt's lymphoma biopsies, generally express the nuclear antigen EBNA (27) and contain multiple copies of the EBV genome (usually 1 to 100 copies per cell). Most or all of the EBV DNA is present as supercoiled DNA plasmids of approximately 172,000 base pairs (bp) (20). These multiple copies appear to arise soon after infection, by amplification of the viral DNA relative to cell DNA (36).

For the most part, the study of DNA replication and the structure of origins of DNA replication in higher eucaryotic cells has been limited to lytic DNA viruses such as herpes simplex virus type 1 (HSV-1), simian virus 40 (SV40), polyomavirus, and the adenoviruses (3, 4, 25, 29, 33). Recently, however, studies of DNA replication have been extended to include stable extrachromosomal genetic elements such as those derived from bovine papillomavirus type 1 (23) and EBV (41). A *cis*-acting element, designated *oriP*, that allows replication and maintenance of recombinant plasmids in cells harboring either the EBV genome or the EBNA-1-encoding sequence from EBV has been isolated from the EBV genome (41, 42). In the presence of EBNA-1, *oriP* permits plasmid replication in a variety of mammalian cells that EBV cannot infect in culture (42).

To determine the DNA sequence requirements for *oriP*, we generated and tested deletion derivatives of *oriP*. Our analysis revealed two *cis*-acting regions within *oriP* that are required for its activity. These regions are composed of a 20-member family of 30-bp tandem repeats and a 114-bp sequence that contains a 65-bp dyad symmetry (2; G. S. Hudson, A. T. Bankier, S. C. Satchwell, and B. Barrell, submitted for publication).

* Corresponding author.

MATERIALS AND METHODS

Cell lines. D98/Raji is a somatic cell hybrid between the human epithelial cell line D98 and the EBV-genome-positive Burkitt's lymphoma line Raji. D98/Raji contains multiple copies of the EBV genome (8). Raji and D98/Raji are grown in RPMI 1640 culture medium containing 10% fetal bovine serum. Human 143 cells were derived from an osteosarcoma (10). 143/EBNA cells were previously designated 143/SVoB-H2.9 Cl.4 (42). They are a derivative of human 143 cells that carry the *Bam*HI-to-*Hin*dIII portion of the EBV *Bam*HI K restriction fragment stably integrated into chromosomal DNA and express the EBV-associated nuclear antigen EBNA-1, as determined by anti-complement indirect immunofluorescence (27, 37). Both 143 and 143/EBNA were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Recombinant plasmids. Plasmid pKan2 contains the kanamycin resistance gene of the bacterial transposon Tn*5* under the transcriptional control of the HSV-1 thymidine kinase promoter and confers G418 resistance to mammalian cells (41). Plasmid pHyg is identical to pKan2, except that it expresses resistance to the antibiotic hygromycin B (35). pΔSstII3 was generated by digestion of the *oriP*-carrying plasmid pBamCΔJ with *Sst*II, followed by ligation (41; Fig. 1). pΔBH was generated by digestion of pBamCΔJ with *Hpa*I plus *Bam*HI, filling in the termini with the Klenow fragment of *Escherichia coli* DNA polymerase I, and ligating with T4 DNA ligase. Deletions at the right boundary of *oriP* were generated by linearizing pBamCΔJ with *Hpa*I, treating it with BAL 31 exonuclease, and digesting it with *Bam*HI to remove the residual EBV DNA to the right of the *Hpa*I site, thus generating plasmids with one common end at the left at the *Eco*RI site and variable endpoints at the right. The ends of the DNA were repaired with the Klenow fragment of *E.*

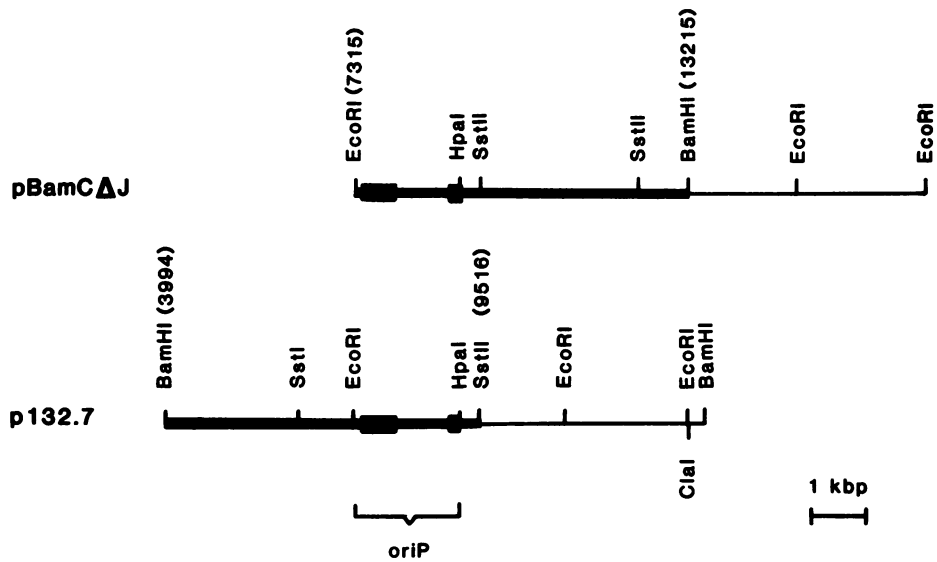


FIG. 1. Restriction maps of pBamCΔJ and p132.7 (41). Thick lines represent EBV DNA, and thin lines represent vector pKan2 DNA. The numbers above some restriction sites represent the location of the site, in nucleotides, on the EBV map (2). *oriP* maps to the region between the *Eco*RI site at 7,315 nucleotides and the *Hpa*I site (41) and is indicated by the bracket. The large black box and the small black box within *oriP* represent the 20-member family of 30-bp direct, imperfect repeats and the region containing a 65-bp dyad symmetry, respectively. pBamCΔJ was used to generate BAL 31 deletions at the right end of *oriP* from the *Hpa*I site, and p132.7 was used to generate BAL 31 deletions at the left end of *oriP* from the *Sst*I site.

coli DNA polymerase I and ligated, and the products were used to transform *E. coli* DH1 (12). Deletions at the left boundary were made from p132.7 (41; Fig. 1). p132.7 was linearized with *Sst*I, treated with BAL 31, and digested with *Bam*HI to remove residual EBV DNA to the left of the *Sst*I site. The ends were repaired with the Klenow DNA polymerase and ligated. Deletion endpoints were estimated to within 50 bp from the sizes of appropriate restriction fragments on 2% agarose gels. Spontaneous deletions within ori_L of HSV-1 have been reported to occur during propagation of recombinant plasmids in *E. coli* (32, 40). Such possible deletions were searched for in those *oriP* constructions that failed to replicate, by digesting the DNAs with an appropriate endonuclease and resolving the products on polyacrylamide gels which would detect a difference of as little as 10 bp.

Internal deletions were generated by digesting pΔSstII3 with *Eco*RV and *Hpa*I to obtain p148.2, with *Eco*RV and partially with *Nco*I to obtain p145.3, and partially with *Nco*I to obtain p142.15 (Fig. 2).

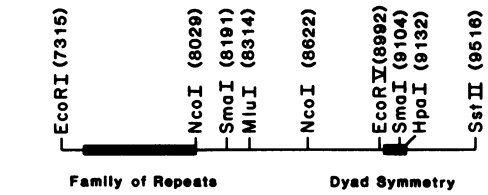
Transfections. DNA transfections were performed by the calcium phosphate coprecipitation method of Graham and van der Eb (11). Transfected cells were selected in medium containing 600 μg of G418 (GIBCO Laboratories) per ml, as previously described (42), or in medium containing 250 μg of hygromycin B (Calbiochem-Behring) per ml.

Isolation and analysis of plasmid DNA. At 2 to 4 weeks after transfection, G418- or hygromycin B-resistant colonies either were pooled and expanded or were isolated individually and expanded into cell lines. Low-molecular-weight DNA was isolated by the method of Hirt (14). Total cell DNA was isolated by lysing cells in 1% Sarkosyl (CIBA-GEIGY Corp.), digesting the lysates with 1 mg of self-digested pronase per ml, extracting with phenol-chloroform-isoamyl alcohol (25:24:1), and precipitating with ethanol. The amount of DNA was measured by the Hoechst dye fluorimetric assay (17). To detect plasmid sequences, DNA, either undigested or digested with restriction endonucleases, was electrophoresed through 0.8% agarose gels, transferred to nitrocellulose, and hybridized to nick-translated ^{32}P-labeled vector (pKan2 or pHyg) DNA (31). Filters were washed and subjected to autoradiography for 1 to 4 days.

Plasmid segregation assay. Clones of D98/Raji carrying plasmids were propagated for different lengths of time in the absence of the selective drug (G418). The cells were then trypsinized, diluted, plated in the absence of selection, and grown until the colonies contained 16 to 32 cells. The colonies were counted, and the culture medium was replaced with medium containing 600 μg of G418 per ml. The medium was replaced every 3 to 5 days, and the number of viable, G418-resistant colonies was counted after 2 weeks. In parallel, cells that had been propagated continuously in the presence of G418 were also treated as described above. Such cells yielded cloning efficiencies ranging from 63 to 100% after being reexposed to medium containing G418.

DNA replication assay. Plasmid DNAs (20 μg) were introduced into 1.2×10^8 Raji cells by electroporation (26, 35). After 24 h in culture, viable cells (8 to 14% of total) were separated from dead cells by sedimentation onto a cushion of Renografin-Ficoll. After an additional 72 h in culture, by which time the cell population had doubled twice, low-molecular-weight DNA was isolated by the method of Hirt. After two extractions with phenol-chloroform-isoamyl alcohol, the samples were dialyzed against 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA (TE buffer), treated with RNase A (50 μg/ml) for 1 h at room temperature, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. Precipitates were suspended in TE buffer, divided into two portions, and digested with *Dpn*I or *Dpn*I plus *Mbo*I. The digested DNAs were electrophoresed through 1% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pKan2 DNA (specific activity, ca. 5×10^8 cpm/μg). The filters were washed and subjected to autoradiography.

CAT assay. Regions of *oriP* DNA were cloned into the unique *Bgl*II site of the chloramphenicol acetyltransferase



	Plasmid	No. Colonies Counted at 2 Wk	4 Wk	Plasmid Copies per Cell
A	pΔSstII3	1000	C	5
	pΔBH	750	C	2
	pΔBal2	500	2	0
	p148.2	400	4	NT
	pΔBal12	1200	7	0
	pΔBal15	600	0	NT
	vector	5	5	0
B	pΔSstII3	250	C	5
	pΔBal15-11	350	C	5
	pΔBal14-2	15	7	0
	pΔBal17-1	10	2	0
	vector	10	1	0
C	pΔSstII3	2500	C	5
	p142.15	1500	C	1
	p145.3	2000	C	1
	vector	1	1	0

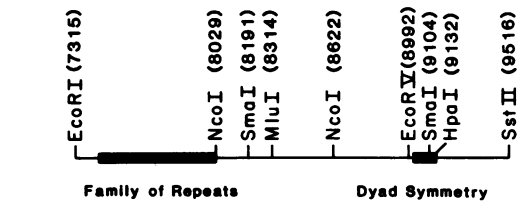
FIG. 2. Deletion analysis of *oriP*. The top line represents the DNA containing nucleotides 7315 to 9516 of EBV strain B95-8 (2) to which *oriP* was previously mapped (41). The box between the *Eco*RI and *Nco*I sites represents the 20-member family of repeats, and the box between the *Eco*RV and *Hpa*I sites represents the region of dyad symmetry (see Fig. 6 for a detailed illustration of this region). Below are the *oriP* deletions, with the dashed line representing the deleted sequences and the solid line representing the sequences present on the plasmid. The vector sequences are not shown. Deletions generated by digestion with restriction endonucleases have vertical lines at the deletion endpoints. Deletions generated by BAL 31 digestion do not contain restriction sites at their endpoints. The number of drug-resistant colonies was determined 2 and 4 weeks after transfection of plasmids into D98/Raji cells. The average number of plasmid molecules per cell was estimated after growing cells for 25 to 30 generations under selection and then harvesting either total or low-molecular-weight DNA. DNA from equal numbers of cells was analyzed by Southern transfer analysis. The intensities of plasmid bands were compared to intensities obtained from known amounts of plasmid DNA analyzed on the same gel. NT, Not tested; C, colonies had grown to confluence.

(CAT) expression vector $pA_{10}CAT_2$, kindly provided by Laimins and co-workers (18). Plasmid pSV2CAT contains the CAT gene downstream of the SV40 early promoter and includes the SV40 72-bp enhancer. pSV2CAT was kindly provided by Gorman and co-workers (9). Adherent cells at approximately 50% confluence in 100-mm tissue culture dishes were transfected with 10 μg of plasmid DNA by the calcium phosphate method of Graham and van der Eb (11). Plasmid DNAs were introduced into nonadherent B-lymphoid cells by electroporation (26, 35). After 72 h, cell extracts were prepared and assayed as described by Gorman et al. (9). Protein determinations were performed by the method of Lowry et al. (22).

RESULTS

***oriP* contains two *cis*-acting components that are required for its activity.** We found previously that *oriP* is located within an 1,800-bp segment of the short unique region of the EBV genome (41). The leftward boundary of *oriP* was placed within the 800 bp to the right of the *Eco*RI site at position 7315, a region containing 20 tandem copies of a 30-bp sequence (see the map in Fig. 2). The rightward boundary of *oriP* was placed within 200 bp of the left of the *Sma*I site at position 9104, a region containing a 65-bp dyad symmetry. The deletion analysis (Fig. 2) shows that both the family of 30-bp repeats and the region of dyad symmetry are necessary for *oriP* activity.

The plasmids depicted in Fig. 2 carry a gene for G418 resistance and deleted derivatives of *oriP* generated by digestion with BAL 31 nuclease or restriction endonucleases. These deleted derivatives were examined for *oriP* activity by testing their ability to yield a high number of stable G418-resistant colonies and to be maintained as plasmids when transfected into D98/Raji cells. Plasmids that carry a functional *oriP* such as pΔSstII3 and pΔBH consistently yielded at least a 100-fold increase in the number of stable G418-resistant colonies by 4 weeks after transfection when compared with vector pKan2. After expanding the colonies or pools of colonies for 25 to 30 cell generations, generally 1 to 10 copies of the input plasmid per cell were



Plasmid	No. Colonies	Plasmid Copies per Cell
A		
pΔBH	400	2
pΔBHλ2	500	NT
pΔBHλ6	580	10
pΔBHλ14	400	5
pΔBHλ46	0 [a]	NT
vector	1	0
B		
pΔSstII3	500	5
pI5I.2	525	1
pI5I.5	525	1
pΔBal I7-I	2	0
vector	I	0

FIG. 3. Analysis of the spatial dependence of the two *cis*-acting components of *oriP*. (A) Structure of plasmids containing bacteriophage lambda insertions into the *Eco*RV site of *oriP*. (B) Analysis of the orientation dependence of the two *cis*-acting components of *oriP*. The arrows represent the orientation of the insert. The heavy black line represents an insertion of pBR322 sequences from between the *Cla*I and *Bam*HI sites of pBR322 (see the text). The colonies were counted at 4 weeks after transfection. [a], Although no stable drug-resistant colonies were obtained, 230 abortive colonies were observed after 2 weeks of culture under selection. All other notations are described in the legend to Fig. 2.

recovered from Hirt extracts as detected by Southern transfer analysis. Plasmids that lack the region of dyad symmetry lack *oriP* activity (Fig. 2A). Plasmids p148.2 and pΔBal2 lack only 145 and 200 bp, respectively, to the left of the *Hpa*I site, but neither plasmid yielded increased levels of stable, G418-resistant colonies or was maintained as a plasmid in transfected cells. However, such plasmids lacking the region of dyad symmetry yielded as many surviving colonies after only 2 weeks of selection as did pΔSstII3 or pΔBH which contain *oriP* intact. Unlike the colonies obtained with pΔSstII3 or pΔBH which are stably resistant to G418, these colonies obtained with plasmids lacking the region of dyad symmetry died after further selection. As discussed below, this transient drug-resistant phenotype is dependent on the presence of the family of 30-bp repeats and expression of the EBNA-1 gene.

The only DNA to the left of the *Eco*RV site near the region of dyad symmetry that is required for *oriP* to function is the DNA within the family of 30-bp repeats (Fig. 2B and C). p145.3, which lacks the 960 bp between the *Eco*RV site and the *Nco*I site at position 8029, functioned as efficiently as did pΔBal 15-11, which lacks all EBV DNA to the left of the repeats as well as approximately half of the family of repeats. The 10 to 12 copies of the 30-bp repeat present on pΔBal 15-11 are sufficient for function. No *oriP* activity was observed for pΔBal 14-2, which lacks all copies of the 30-bp sequence.

Since it previously was found (41) that the DNA to the right of the *Sma*I site at position 9104 is dispensible for *oriP* activity, we conclude that the maximal limits for the sequences that are required for *oriP* activity are: (i) the 114 bp between the *Sma*I site and the *Eco*RV site containing the dyad symmetry and (ii) at most, 10 to 12 copies of the 30-bp repeats. Although the DNA between these two components of *oriP* is not required for function, plasmids lacking the DNA between the *Nco*I sites (p142.15 and p145.3; Fig. 2C) were found at fewer copies per cell (approximately one molecule per cell compared with five molecules of pΔSstII3 per cell). Also, pΔBH, lacking sequences to the right of the *Hpa*I site, was maintained at approximately half the number of plasmid molecules per cell compared with pΔSstII3 (Fig. 2 and 3). Further experiments are required to determine whether particular sequences within these regions influence plasmid replication.

We did measure the plasmid segregation rate or percent loss per generation in the absence of selection of two plasmids that have DNA deleted between the two required components of *oriP* (Table 1). Given the spread in our measurements and the few number of cell clones studied, it is not possible to conclude whether the rates of loss differ. If

TABLE 1. Stability of *oriP* plasmids in D98/Raji cells

Clones of D98/Raji carrying[a]:	Population doublings in the absence of G418	% of cells that are G418[r]	% Loss per generation[b]
pBamC cl 2[c]	33	40	2.8
pBamC cl 3	33	41	2.7
pBamC cl 4	33	15	5.7
pΔSstII3 cl 1	10	38	5.3
pΔSstII3 cl 2	20	19	5.9
pΔSstII3 cl 3	20	29	4.7
p142.15 cl 1	20	12	8.4
p142.15 cl 2	20	27	4.5
p145.3 cl 1	20	16	8.6

[a] pBamC contains all of the *Bam*HI C fragment of EBV (41). See Fig. 2 for the structures of pΔSstII3, p142.15, and p145.3. Clones (cl) carrying pBamC contained 4 to 10 copies of the plasmid per cell (41); clones carrying pΔSstII3, p142.15, and p145.3 contained an average of 5, 1, and 1 copies of plasmid per cell, respectively.

[b] The segregation rate or percent loss per generation, k, was calculated assuming a first-order rate of loss, by the equation $2.303 \log (No/N) = kT$, where No is the percentage of cells that were G418 resistant after continuous propagation in the presence of G418 and N is the percentage of cells that were G418 resistant after propagation for T generations in the absence of G418.

[c] pBamC data were taken from Table 2 of reference 43 and are included for the purpose of comparison to the data generated in the present study. For all cases, the number of clones in the absence of G418 ranged from 125 to 320.

the deleted sequences do affect the rate of loss of plasmids, they do not do so in a dramatic fashion.

Spatial requirements of the two components of *oriP*. Since 960 bp between the region of dyad symmetry and the family of repeats can be deleted without eliminating *oriP* activity (Fig. 2C, p145.3), a precise distance between the two components is not essential for *oriP* activity. We asked two additional questions regarding the spatial requirements of the two components: (i) whether *oriP* activity is lost when the distance between the family of repeats and the region of dyad symmetry is increased, and (ii) whether *oriP* activity is lost when one element is inverted relative to the second.

To address the first question, bacteriophage lambda DNA was digested with *Eco*RV and inserted into the unique *Eco*RV site in the *oriP*-containing plasmid pΔBH. DNA inserted at the *Eco*RV site will increase the distance between the repeats and the region of dyad symmetry. Four insertion derivatives were isolated and tested for *oriP* activity in D98/Raji cells. Insertion of an additional 268, 588, or 1,377 nucleotides had no effect on *oriP* activity, since these plasmids continued to yield a high number of stable G418-resistant transfectants and to be maintained as plasmids (Fig. 3A). There is apparently a limit as to how far apart these components can be on a plasmid and still function, since the insertion of 4,613 bp between the *cis*-acting components of *oriP* rendered the plasmid inactive in these assays. Since we cannot exclude the possibility that DNA sequences within the 4.6-kilobase-pair lambda insert may encode products that have deleterious effects on the cell, we are in the process of testing *oriP* derivatives containing other large insertions.

To test whether *oriP* activity requires a specific orientation of one *cis*-acting component relative to the second, the 876-bp *Eco*RI-to-*Sma*I restriction fragment encompassing the family of repeats was inserted in both orientations at the *Cla*I restriction site of the *oriP* deletion plasmid pΔBal 17.1 (Fig. 1 [p132.7] and 3B. Introduction of the restriction fragment containing the family of repeats into pΔBal 17.1 at the *Cla*I site resulted in the insertion of 352 bp of pBR322 DNA between the deletion endpoint of pΔBal 17.1 and the introduced *Eco*RI-to-*Sma*I fragment. Such plasmids containing the family of repeats in either the native or the inverted orientation yielded a high number of stable G418-resistant transfectants after introduction into D98/Raji and were maintained as plasmids in these cells (Fig. 3B).

From these results, we conclude that neither the precise distance between the two components of *oriP* nor their orientation relative to one another is critical for *oriP* activity.

Neither component of *oriP* alone permits plasmid replication, but together they are necessary and sufficient for plasmid replication. The assays used above to define *oriP* activity (number of stable drug-resistant colonies and plasmid maintenance) are based on the selection of drug-resistant cells and therefore require that the plasmids both replicate and partition efficiently to daughter cells to be scored as functional. Thus, recombinant plasmids scored as not having *oriP* activity could be defective in replication or in some other property necessary to partition replicated molecules during cell division. To eliminate the need to select for drug-resistant cells, the functional activity of *oriP* and each of its *cis*-acting components was assessed in a plasmid replication assay (described above). This assay takes advantage of the observation that plasmid DNA grown in DNA adenosine methylase-positive *E. coli* (*dam*$^+$) is cut by restriction endonuclease *Dpn*I and not by endonuclease *Mbo*I, whereas DNA that has replicated in mammalian cells is not cut by *Dpn*I but is by *Mbo*I due to the loss of methylated adenines.

Plasmids tested for replication were derived from pHyg, a hygromycin B resistance plasmid (see below; Table 2). pHyg or derivatives of pHyg carrying the family of repeats on an 876-bp *Eco*RI-to-*Sma*I restriction fragment (p2.8), the region

TABLE 2. The 20-member family of repeats confer transient drug resistance

Plasmid[a]	No. of hygromycin B-resistant colonies[b] of:			
	143 at:		143/EBNA at:	
	14 days[c]	21 days[c]	14 days[c]	21 days[c]
pHyg	13	13	0	0
pHEBo (*oriP*)	0	0	4,500	4,500[d]
p2.8 (30-bp repeats)	3	3	5,500	10[e]
p3.2 (dyad symmetry)	0	0	0	0
p7.10 (repeats plus dyad symmetry)	NT	NT	1,800	1,800[d]

[a] The family of repeats was isolated on the 876-bp *Eco*RI-to-*Sma*I restriction fragment, ligated to *Bam*HI linkers, and cloned into the *Bam*HI site of pHyg (35) to yield p2.8. The region of dyad symmetry was isolated on the 140-bp *Eco*RV-to *Hpa*I fragment and cloned into the *Bam*HI site of pHyg to yield p3.2. p7.10 was constructed by cloning the region of dyad symmetry onto p2.8, 275 bp away at an *Sal*I site that had been converted to a *Bgl*II site by the addition of synthetic linkers.

[b] Approximately 10^6 cells were transfected with 2.5 μg of plasmid DNA and grown in the presence of 250 μg of hygromycin B per ml. Values represent the average of two experiments. NT, Not tested.

[c] Number of days after transfection.

[d] Hygromycin B-resistant colonies were pooled, expanded, and assayed for plasmid DNA. On the average, approximately five plasmid molecules per cell of pHEBo and one plasmid molecule per cell of p7.10 were recovered after 30 generations in culture in the presence of hygromycin B.

[e] At 21 days posttransfection there were 10 colonies present in the culture. They were pooled, expanded, and assayed for plasmid DNA as described in the text. No free plasmid could be detected.

of dyad symmetry on a 140-bp *Eco*RV-to-*Hpa*I fragment (p3.2), or *oriP* intact (pHEBo) were introduced into Raji cells. After approximately two population doublings in the absence of selection, plasmid DNA was isolated by the Hirt lysis procedure. The DNA was then divided into two portions and digested with either *Dpn*I or *Dpn*I plus *Mbo*I. The *Mbo*I digestion was included to ensure that any *Dpn*I-resistant products were due to demethylation and not to incomplete digestion by *Dpn*I. The digestion products were analyzed by Southern transfer analysis. The subsequent autoradiogram is shown in Fig. 4. *Dpn*I-resistant, *Mbo*I-sensitive plasmid DNA (i.e., replicated DNA) can be seen as a major species only in Raji cells that had been transfected with plasmids containing an intact *oriP*, pHEBo, demonstrating that this plasmid had undergone replication. A comparison of plasmid-specific bands on the autoradiograms by scanning densitometry indicated that we could detect a signal that had approximately 100 times lower intensity than the signal obtained with replicated pHEBo. Since approximately equivalent amounts of each plasmid DNA were present in the cells,and since, except for pHEBo, little if any recovered plasmid DNA is both *Dpn*I resistant and *Mbo*I sensitive, we conclude that neither the vector nor either of the two *cis*-acting components of *oriP* alone supports detectable plasmid replication after two cell population doublings. This result indicates that neither component of *oriP* alone is sufficient for replication, but together with the DNA between them they do permit replication of plasmid DNAs. The DNA between the two components of *oriP* is not required for replication because a hygromycin B resistance plasmid carrying only the family of 30-bp repeats and the dyad symmetry region of *oriP*, p7.10, had the capacity to be stably maintained as a plasmid (Table 2, see below) and, therefore, to be replicated. As expected, p7.10 was observed to replicate when introduced into Raji cells in a similar experiment (data not shown) to that shown in Fig. 4. Either component of *oriP* plus the DNA between them is insufficient for plasmid replication. Plasmids carrying deletion derivatives of *oriP* that lack only the family of 30-bp repeats or only the region of dyad symmetry (pΔBal 14.2 and pΔBal 2, respectively; Fig. 2) did not replicate detectably in a short-term assay (limit of detection, <0.5 replicated molecules per cell [data not shown]). These results indicate that the two identified components of *oriP* are necessary and sufficient for plasmid replication.

Selectable plasmids that carry only the 20-member family of repeats of *oriP* confer transient drug resistance. We consistently observed that transfection of EBV-positive, but not EBV-negative cells, with plasmids that contain the 20-member family of repeats in the absence of the region of dyad symmetry leads to a significant number of colonies that are transiently resistant to the selective drug for approximately 8 to 11 generations. These colonies occurred at approximately the same frequency as did stable colonies when compared to a plasmid carrying all of *oriP*, such as pΔSstII3 (100-fold over pKan2 [Fig. 2A]). However, the transiently resistant colonies stopped growing after reaching 300 to 3,000 cells and eventually died and detached from the culture dish. These colonies could not be propagated under selection after trypsinizing them and transferring them to new culture dishes. However, when the selective drug was removed, these colonies continued to proliferate. This transient drug resistance was also observed after transfection of EBV-positive cells with a deletion derivative of *oriP* that carries only 11 to 12 copies of the 30-bp sequence (pΔBal 15 [Fig. 2A]).

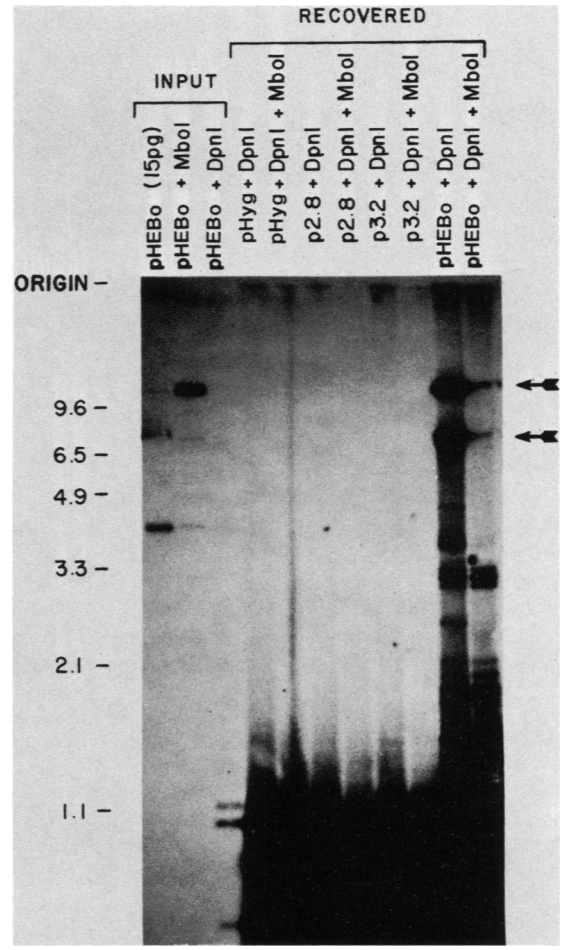


FIG. 4. Replication of *oriP* plasmids. Raji cells were transfected with each of the following four plasmids: pHEBo (all of *oriP*), p2.8 (30-bp repeats), p3.2 (dyad symmetry), and pHyg (vector), as described in the text. After two population doublings, replication was assayed as described in the text. After electrophoresis through 1% agarose, transfer to nitrocellulose, and hybridization to ^{32}P-pHyg DNA, replicated plasmid can be visualized as the *Dpn*I-resistant, *Mbo*I-sensitive bands that comigrate with the pHEBo-nicked circular and linear DNA markers (arrows). The hybridizing material at the bottom of the autoradiogram represents the *Dpn*I-sensitive plasmid DNA that has not replicated. The incomplete digestion products in the lanes containing pHEBo DNA are probably due to plasmid molecules that have not completed replication and are therefore partially methylated. The molecular weights (kilobase pairs) were taken from bacteriophage lambda DNA digested with *Hin*dIII and electrophoresed in the same gel. Assuming that 4 to 5% of the viable Raji cells took up and expressed DNA (35), the bands of demethylated (replicated) pHEBo represent approximately 10 to 15 replicated molecules per cell.

Since expression of the EBV-encoded nuclear antigen EBNA-1 is required for replication of *oriP*-containing plasmids (42), we asked whether EBNA-1 is also responsible for the transient drug-resistant phenotype conferred by plasmids carrying the family of repeats. We transfected human 143 cells and 143/EBNA cells, which carry and express an integrated EBNA-1 gene (42), with hygromycin B resistance plasmids that carry *oriP* (pHEBo), the region of dyad symmetry (p3.2), the family of repeats (p2.8), or both the repeats and the dyad symmetry (p7.10). The *oriP*-containing plasmids pHEBo and p7.10 yielded a high number of stable

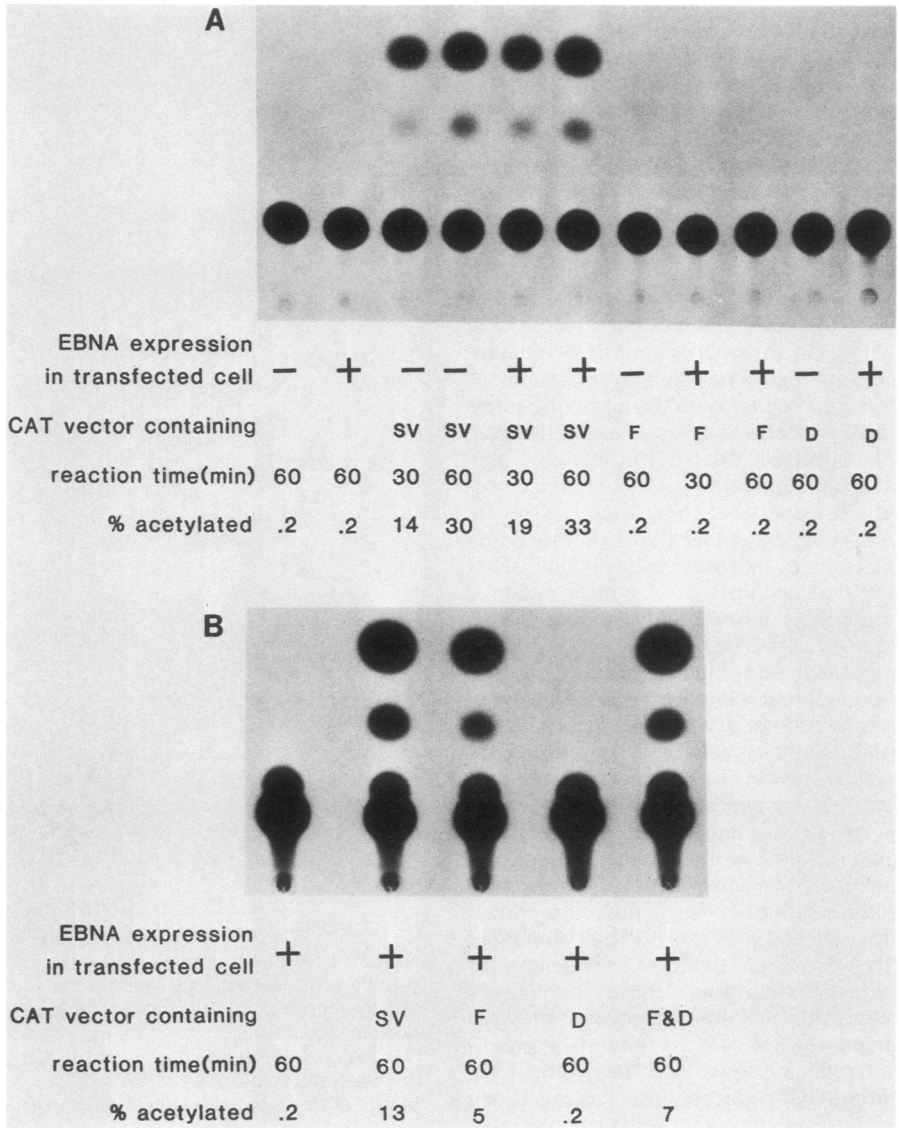


FIG. 5. (A) Assay of CAT activity in 143 (negative for EBNA expression) or 143/EBNA (positive for EBNA expression) cells transfected with CAT vectors containing the two *cis*-acting components from *oriP* (F, 30-bp family of repeats; D, region of dyad symmetry) or the SV40 enhancer element (SV). At 72 h after transfection, cell extracts were prepared from 10^7 cells (approximately 1% of the cells expressed the introduced DNA), and 50% was assayed for CAT activity (9). After 30- or 60-min incubations, or both, the reaction products were spotted on thin-layer silica gels and chromatographed with chloroform-methanol (95:5). After autoradiography, the acetylated and unacetylated forms of [^{14}C]chloramphenicol were eluted with ethyl acetate and quantified by scintillation counting. Approximately 1.5×10^5 cpm of [^{14}C]chloramphenicol was included for each time point. (B) Assay of CAT activity in Raji cells. At 72 h after introduction of the plasmids into the cells by electroporation, where approximately 1% of the cells expressed the introduced DNA, cell extracts were prepared from 10^7 cells, and 50% was tested for CAT activity. The products were assayed as described above.

hygromycin B-resistant colonies in 143/EBNA but not in human 143 cells (Table 2). The plasmid containing the region of dyad symmetry, p3.2, yielded a low number of hygromycin B-resistant colonies. At 2 weeks, the plasmid that contains the 20-member family of repeats, p2.8, yielded a similar number of hygromycin B-resistant colonies in 143/EBNA cells as did pHEBo. These colonies were not stably resistant to hygromycin B and died over the course of an additional week of selection. Such abortive colonies were not observed in human 143 cells. This result suggests that the EBNA-1 protein interacts either directly or indirectly with the family of direct repeats of *oriP* and that this interaction leads to an elevated level of transient drug resistance.

The family of 30-bp repeats enhances transcription from the SV40 early promoter detectably in some but not all cells that support replication of *oriP*. In the papovaviruses, some *cis*-acting elements, such as the polyoma *Pvu*II-4 fragment and the SV40 72-bp repeat, enhance the efficiency of DNA replication and also can act as transcriptional enhancers (5, 7, 21, 25; G. Hertz and J. Mertz, personal communication). In view of these observations it seemed possible that the family of 30-bp repeats of *oriP* might act to enhance transcription in the presence of EBNA-1 and that this activity might be responsible for the increased level of transient drug resistance from plasmids that carry the family of 30-bp repeats.

To test this possibility, the two *oriP cis*-acting components were cloned into the CAT expression vector $pA_{10}CAT_2$ at the unique *Bgl*II restriction site 200 bp upstream of the CAT coding sequences. $pA_{10}CAT_2$ contains the *E. coli* CAT gene downstream from the SV40 early promoter and lacks the SV40 72-bp repeat enhancer element (18). One measure of whether a DNA sequence can serve as a transcriptional enhancer is to insert it into $pA_{10}CAT_2$, introduce the recombinant DNA into cells, and measure the resulting level of CAT activity in cell extracts. Constructions containing either of the two *oriP cis*-acting components were introduced into human 143 cells (EBNA-1 negative), 143/EBNA cells (EBNA-1-positive cells), and Raji cells (EBV-positive Burkitt's lymphoma cell line). After 72 h the cells were harvested, and cell lysates were assayed for CAT activity with [^{14}C]chloramphenicol and acetyl-coenzyme A as substrates. The values obtained were compared to values obtained after transfection of the vector alone as a negative control and a vector containing the SV40 72-bp enhancer element pSV2CAT as a positive control for transcriptional enhancement.

Extracts from human 143 cells or 143/EBNA cells transfected with CAT constructions carrying either of the two *cis*-acting components of *oriP* did not detectably express the CAT enzyme, whereas extracts from cells transfected with the CAT construction carrying the SV40 enhancer expressed the CAT activity at least 150-fold above background (Fig. 5A). In contrast, when the plasmids were tested in Raji cells, the family of 30-bp repeats allowed levels of CAT expression that were comparable to levels obtained with the SV40 enhancer (about twofold lower in the experiment shown (Fig. 5B) and about twofold higher in a second experiment not shown). Thus, the family of 30-bp repeats can enhance transcription from the SV40 early promoter in Raji cells but does not do so detectably in another cell line, 143/EBNA, that also supports replication of *oriP*-bearing plasmids.

We conclude from this experiment that at least under some conditions, the 30-bp repeats of *oriP* can enhance transcription. Therefore, the increased expression of drug resistance from the HSV-1 thymidine kinase (TK) promoter on plasmids that carry the 30-bp repeats in D98/Raji cells and in 143/EBNA cells could be due to an enhancement of the TK promoter by the 30-bp repeats that are dependent on the presence of EBNA-1. This possibility remains to be tested directly. There are several possible explanations for the observed enhancement of the SV40 early promoter by the 30-bp repeats in Raji cells and the lack of detectable enhancement by this assay in 143/EBNA cells. First, because we could not detect a basal level of CAT expression from $pA_{10}CAT_2$ in 143/EBNA cells, a several-fold increase of this level could have gone undetected. Second, the enhancer activity of the 30-bp repeats may depend on the presence of EBNA-1 as does the increased expression of transient drug resistance in 143 cells, and Raji cells may express EBNA-1 at higher levels than do 143/EBNA cells. Finally, the 30-bp repeats or the 30-bp repeats in conjunction with EBNA-1 could require a B-cell-specific factor for efficient enhancer activity. Experiments to test these possibilities are in progress.

DISCUSSION

oriP of EBV serves a different biological function than do most of the origins of DNA replication from animal viruses that have been characterized previously. The replicative origins of SV40, polyomavirus, the adenoviruses, and HSV-1 permit an exponential rate of DNA replication during the cell cycle that eventually leads to cell death. *oriP*, in contrast, permits a controlled replication of plasmid DNA which is consistent with cell survival and proliferation characteristic of EBV-transformed B-cells. This functional difference of *oriP* presumably is a reflection, at least in part, of its structure. The analysis reported here reveals that although some structural features of *oriP* are common to replicative origins, some of its features are without precedent.

oriP is approximately 1,700 bp in length and contains two identified *cis*-acting components, both of which are required for function: a family of 30-bp repeats and a region of dyad symmetry, separated by about 1,000 bp (Fig. 6). At most, 12 copies of the 30-bp repeated sequence and, at most, 114-bp containing the region of dyad symmetry are required for the replication and maintenance of recombinant plasmids in EBNA-1-positive cells (Fig. 2). Deletions, insertions, and inversions made within *oriP* indicate that neither a precise distance nor a particular orientation of the two components relative to one another is required for the activity of *oriP* (Fig. 2 and 3). It is possible that additional sequences within the 1,000 bp separating these two components or within the 400 bp to the right of the region of dyad symmetry (between the *Hpa*I and *Sst*II sites) may improve plasmid replication or stability. Plasmids lacking these sequences are maintained at fewer molecules per cell (Fig. 2). However, if the DNA between the two components affects plasmid segregation, its effect is not dramatic (Table 1).

The roles of the two components of *oriP* in replication have not been determined. However, we hypothesize that the region of dyad symmetry serves as an origin for DNA synthesis. Regions of dyad symmetry have been observed at replicative origins from bacteria (24) and mitochondria (1), as well as from papovaviruses (30, 34) and HSV-1 (33; S. Weller, personal communication). The dyad symmetry of the SV40 origin includes symmetrically positioned binding sites for large T antigen (39). Genetic and biochemical evidence indicates that binding of large T antigen to these sites is required to initiate DNA replication (29, 38). Located on both sides of the center of dyad symmetry of *oriP* and at two positions immediately to the right are four copies of a 15- to 18-bp sequence that is also found in the 30-bp tandemly repeated sequence (Fig. 6). By analogy to SV40 and polyomavirus and for reasons discussed below, we hypothesize that the EBNA-1 protein binds to this repeated sequence at the dyad symmetry to activate *oriP*.

Plasmids that carry only the family of 30-bp repeats from *oriP* and a selective marker yield a high number of transient drug-resistant colonies in EBNA-1-positive cells (Fig. 2A and Table 2). Although the mechanism responsible for this property is not known, it is unlikely to involve limited replication of the plasmids, since plasmid molecules isolated from abortive drug-resistant colonies are unreplicated (data not shown) and since the family of repeats is insufficient to allow plasmid replication (Fig. 4). Regardless of the mechanism, the observed transient drug resistance requires the expression of the EBNA-1 protein and thus suggests binding of EBNA-1 to the 30-bp repeated sequence, presumably to the protion of it that is also present at the region of dyad symmetry (open boxes in Fig. 6). A tandem array of binding sites might serve to increase the probability of EBNA-1 occupying, at any one time, one or more sites in this region of *oriP* under conditions in which EBNA-1 protein is at a low concentration in the cell (19). This in turn might increase the local concentration of EBNA-1 that is available to bind to

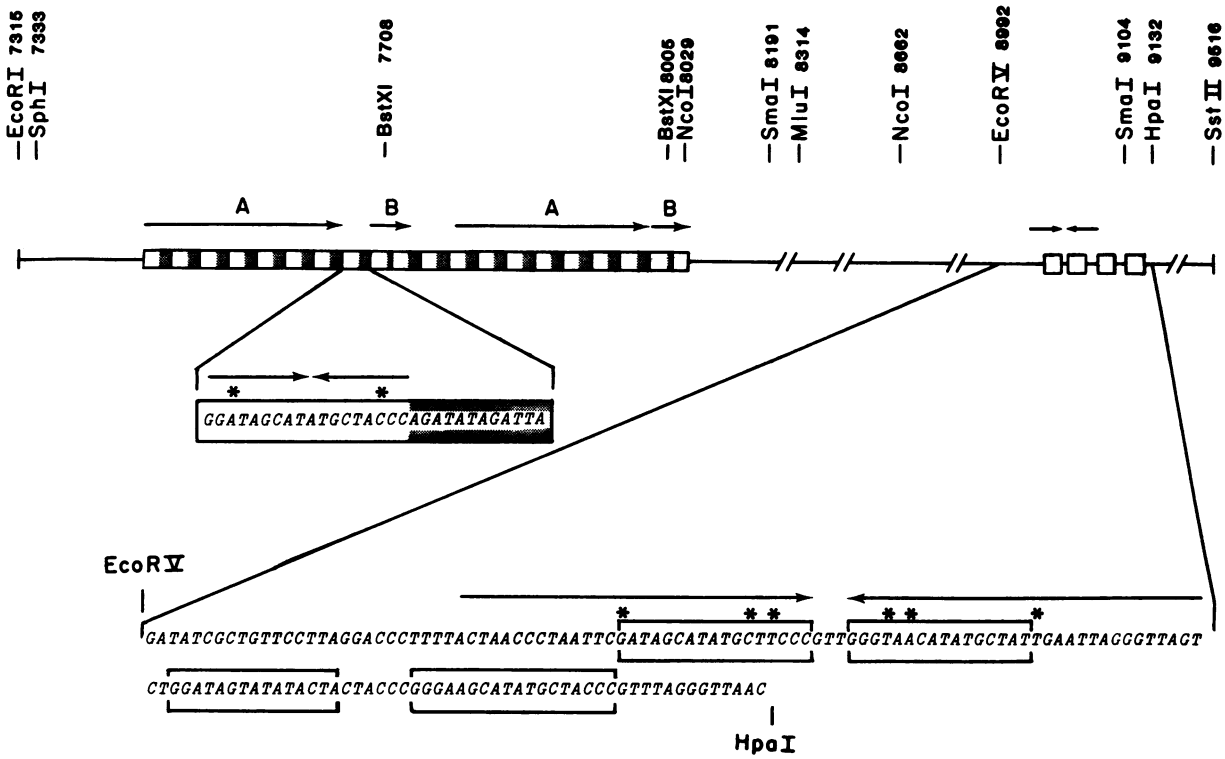


FIG. 6. Structure of *oriP*. At the top are the restriction endonuclease sites used to generate and map deletions. The number above each restriction site represents the position of the site and is expressed as nucleotide pairs on the B95-8 EBV genome (2; Hudson et al., submitted for publication). At the left of this region of DNA, there is a 20-member family of tandemly repeated sequences. This family, represented by the series of 20 boxes, consists of 17 imperfect copies of a 30-bp sequence and three truncated copies. A consensus sequence, shown below, is 30 bp in length and contains a 9-bp inverted repeat (thin arrows) with a 1-bp mismatch (asterisk), followed by 12 bp containing a 4-bp direct repeat that is separated by 2 bp (shaded area). Superimposed upon this block of tandem repeats is a 210-bp direct repeat (arrow labeled A) and a 45-bp direct repeat (arrow labeled B). The B sequences contain the truncated copies of the consensus sequence. At the right of *oriP* and within the 140 bp between the *Eco*RV restriction site and the *Hpa*I restriction site are four truncated copies of the 30-bp sequence described above. The sequence of this region is shown below. Each truncated copy of the 30-bp sequence is within a box. Two of these blocks of repeats plus additional flanking sequences make up a 65-bp dyad symmetry containing three mismatches (asterisk). This inverted repeat is indicated by the inverted arrows above the sequence. Between the tandem family of repeats at the left of *oriP* and the region at the right that contains the 65-bp dyad symmetry are 960 bp of unique DNA. These unique sequences can be deleted without eliminating *oriP* activity.

the region of dyad symmetry. Although the amount of EBNA-1 protein present in latently infected cells is not known, the amount of its mRNA has been determined to be only a few copies per cell (13; unpublished data). The hypothesis that EBNA-1 binds to the sequence within the 30-bp repeats and the region of dyad symmetry is supported by recent nuclease protection experiments that show binding of EBNA-1 to these sequences in vitro (D. Rawlins, G. Milman, S. D. Hayward, and G. S. Hayward, personal communication).

When cloned onto $pA_{10}CAT_2$ of Laimins et al. (18), the family of repeats was found to enhance expression from the SV40 early promoter in Raji cells but not detectably in 143/EBNA cells (Fig. 5). In 143/EBNA cells, the 30-bp repeats may not enhance expression from the SV40 early promoter sufficiently to be detected in our CAT assay, but they may enhance expression of drug resistance from the HSV TK promoter sufficiently to yield the colony formation that is eventually abortive (Fig. 2A and Table 2). If the observed transient drug resistance is due to an enhancer activity of the 30-bp repeats, then this enhancement requires the presence of EBNA-1. A contrasting view is that the enhancer activity observed in Raji cells is specific to lymphoid cells and for this reason is not observed in 143/EBNA cells. If the 30-bp repeats do not act as an enhancer in 143/EBNA cells, then the enhancer activity is not required for plasmid replication, since 143/EBNA cells support *oriP* function. By this view, the transient drug resistance would result from an additional EBNA-dependent function of the 30-bp repeats. Such an additional function could influence the partitioning of plasmids bound by EBNA-1 to the nucleus after mitosis or to a compartment of the nucleus that would permit prolonged expression of the selective marker in the absence of replication.

The observation that the family of 30-bp repeats of *oriP* can serve, under at least one set of conditions, as a transcriptional enhancer is intriguing, considering what has been found with polyomavirus and SV40. The polyomavirus origin apparently requires a transcriptional enhancer for activity (5, 21, 25), and although the 72-bp enhancer of SV40 is not required for replication, it can obviate the need for the 21-bp repeats for SV40 replication (6; Hertz and Mertz, personal communication). Enhancement of transcription and activation of replication for these viral plasmids could thus involve a common mechanism. In contrast to the papovaviruses, however, polyadenylated transcripts have not been found within several kilobase pairs of *oriP* in cells latently infected by EBV (Hudson et al., submitted for publication).

Two structural features of *oriP*, its length and the presence

of the family of repeats, are without precedent among eucaryotic origins of replication. For HSV-1, the only other herpesvirus for which replicative origins have been well characterized, both ori_S and ori_L differ functionally from *oriP* of EBV in their dependence on virally encoded DNA polymerase during lytic infection and in the generation of concatenated replication intermediates, presumably by a rolling circle mechanism (for a review, see reference 28). Functional ori_S consists of less than 100 bp which contains a 45-bp dyad symmetry (33), and ori_L contains a 114-bp dyad symmetry (S. Weller, A. Sparado, J. E. Schaffer, A. W. Murray, A. M. Maxam, and P. A. Schaffer, submitted for publication). We identified no sequence similarities between these origins and *oriP* other than the presence of dyad symmetry.

oriP shares some structural, and perhaps functional, similarities with the γ-origin of the *E. coli* plasmid R6K. This origin of replication consists of seven 22-bp tandem direct repeats plus a segment to the left of the repeats that is 80% A+T. At least some of these repeats have been shown to be required for plasmid stability and plasmid replication (15). In addition, the R6K-encoded protein π apparently binds to the repeats and is required for the initiation of R6K replication (16). Perhaps the bacterial plasmid R6K and the human herpesvirus EBV have in their evolution independently arrived at a common mechanism for the control of plasmid replication or maintenance, or both, in dividing cells.

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