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Plasmid Maintenance of Derivatives of *oriP* of Epstein-Barr Virus

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***oriP* is the origin of plasmid replication of Epstein-Barr virus. Replication from *oriP* requires both the *cis*-acting elements (the family of repeats and the dyad symmetry element) and the viral origin-binding protein, EBNA-1. The ability of plasmids containing *oriP* to be maintained stably in EBNA-1-positive cells reflects the efficiency both of their replication and of their segregation each cell cycle. The efficiency of plasmid maintenance was determined for plasmids containing derivatives of *oriP* with one copy of the dyad symmetry element and two copies of the family of repeats by measuring the rate at which they were lost from cells in the absence of selection. These measurements demonstrated that plasmids with derivatives of *oriP* with two copies of the family of repeats in one orientation are maintained only slightly less efficiently than is wild-type *oriP*. To determine whether plasmid maintenance could be affected by reinitiation at the dyad symmetry element (T. A. Gahn and C. L. Schildkraut, Cell 58:527–535, 1989), plasmids containing derivatives of *oriP* with two copies of the dyad symmetry element and one copy of the family of repeats were compared with plasmids containing wild-type *oriP* in EBNA-1-positive cells. These measurements showed that plasmids containing a derivative of *oriP* with two copies of the dyad symmetry element are maintained as efficiently as is wild-type *oriP* and are not amplified relative to wild-type *oriP*. These observations indicate that the *trans*-acting factors that regulate DNA to replicate once per S phase are insensitive to multiple *cis*-acting regulatory sites within a replicon.**

Two essential *cis*-acting elements comprise Epstein-Barr virus's plasmid origin of DNA synthesis, *oriP*: a dyad symmetry element (DS) and a family of repeats (FR) (22). Each of these elements is composed of multiple binding sites, 4 for the DS and 21 for the FR, for Epstein-Barr virus nuclear antigen 1 (EBNA-1) (1, 17, 18), the only viral gene required in *trans* for *oriP* to function (26). The DS is the site or close to the site at which DNA synthesis initiates (6); the FR is a site at which replication forks accumulate (4, 6, 21) and has been interpreted to be the site at which they terminate. Were replication forks to terminate efficiently at the FR, we hypothesized that a replicon formed with one DS flanked by two copies of the FR would not replicate to completion. This hypothesis is readily consistent with our observation that a replicon with two intact copies of *oriP* replicates and is maintained in proliferating cells as efficiently as is a replicon with one copy of *oriP* (25) if there is a directionality to the proposed termination within the FR. (Termination sites on the *Escherichia coli* chromosome and plasmid R6K do act in an orientation-specific manner [3, 9, 11, 12, 15].) We have tested this possibility by measuring maintenance in proliferating cells of plasmids which have one copy of the DS flanked by two copies of the FR with one copy in either orientation. We have also measured maintenance of plasmids with one copy of the FR flanked by two copies of the DS to search for their possible amplification. We find that all of these modified versions of *oriP* replicate in and segregate to daughter cells with composite efficiencies similar to that of wild-type *oriP*. One modified version of *oriP* with two copies of the FR in a tail-to-tail orientation flanking the DS (Fig. 1) is maintained in proliferating cells, albeit detectably less efficiently than is wild-type *oriP*, but the difference is small.

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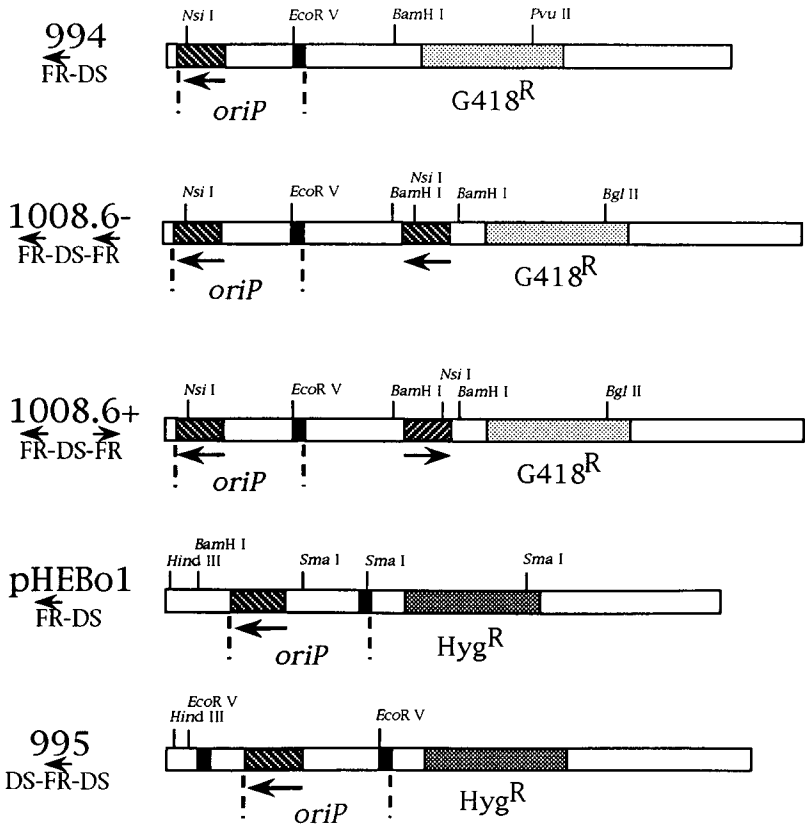
The five vectors with wild-type *oriP* or its modifications were constructed by using standard methods and are diagrammed in Fig. 1. These vectors were introduced into two cell recipients, 143/630 (20) and D98AH2/Raji (7), which express EBNA-1 from either an integrated or a viral genomic template. The treated cells were selected for the drug-resistant marker expressed by the vectors, and clones arose at similar frequencies independent of the structure of the introduced vector. Six to eight drug-resistant clones of each derivative of *oriP* in each cell type were isolated and characterized by Southern analysis (23) to determine the amount of the vector DNAs present as plasmids and whether the clones contained integrated or rearranged copies of the input plasmids. Only those clones with unrearranged plasmids were studied further. Extrachromosomal DNAs isolated from Hirt extracts or total cellular DNAs (13, 19, 25) were digested with endonuclease *Eco*RV, *Nsi*I, *Bam*HI, *Sma*I, *Bgl*II, *Pvu*II, or *Hin*dIII. The plasmid DNAs in the 39 cell clones studied, tabulated in Fig. 1, were intact and present in average numbers that did not depend on the structure of the introduced vector. Clones of 143/630 cells contained approximately 1 to 3 copies per cell, and clones of D98AH2/Raji cells contained 1 to 35 copies per cell (Fig. 1 and 2 and data not shown). Five clones also contained integrated vector DNA, which did not affect these studies. This finding indicates grossly that plasmid replicons with two copies of the FR flanking the DS do replicate and segregate successfully and that plasmid replicons with two copies of the DS flanking one copy of the FR do not amplify disproportionately relative to wild-type *oriP*.

To search for subtle effects that these modifications might have on *oriP*'s functions, we measured the rate at which the plasmids were lost from their host cells in the absence of selective drugs (25). This measurement reflects the efficiency of both replication and segregation and provides a sensitive assay for small differences in either function because these differences would be compounded each cell division. Four cul-



	Avg. plasmid copy number/cell in the clones of cells studied	
	D98AH2/Raji	143/630
994	6, 15, 17, 35	1, 1, 1, 3
1008.6−	3, 3, 5, 6, 7, 10, 13, 15	1, 1, 1, 1
1008.6+	2, 2, 3, 4, 4, 6, 10, 16	1, 1, 1
pHEBo1	3, 4, 4, 5	ND
995	1, 3, 4, 13	ND

FIG. 1. Derivatives of *oriP*. Plasmid 994 (7,466 bp), derived from pKan2 (27), contains wild-type *oriP* (FR [▨] and DS [■]) and neomycin resistance ($G418^R$ [▩]) (2). Plasmid 1008.6− (8,352 bp) contains two copies of the FR flanking the DS, and plasmid 1008.6+ (8,352 bp) encodes two copies of the FR flanking the DS with one copy of the FR in the opposite orientation to that found in 1008.6−. Plasmid pHEBo1 (24) (7,068 bp) contains wild-type *oriP* and hygromycin resistance (Hyg^R [▩]) (8). Plasmid 995 (7,220 bp) encodes two copies of the DS flanking the FR. The orientation of the second copy of the DS has not been determined. The arrow below the FR represents its orientation relative to the DS and designates the direction in which replication fork movement through the FR has been shown to stall (6). Restriction sites used during Southern analysis are noted. (Plasmid maps are not to scale.) The average copy number of plasmids per cell for each clone of each derivative of D98AH2/Raji and 143/630 cells used in this study is listed on the right. Clones of D98AH2/Raji and 143/630 cells were analyzed as described for Fig. 2 except that extrachromosomal DNA from each clone of 143/630 cells was isolated by use of Hirt extracts (13) and densitometry was used to quantify the plasmid DNA present. ND, not done.

tures of each of the 39 clones were propagated for approximately 20 and 30 cell doublings in the absence of selection and counted during each passage to calculate the number of accumulated generations. After both the fourth and sixth passages, 100, 300, and 1,000 viable cells from two cultures of each of the clones were plated in selective media, and 100 and 300 viable cells from two cultures of each of the clones were plated in nonselective media to determine their plating efficiency. The number of surviving colonies was measured after 2 weeks, and the percentage of colonies resistant to selection was calculated. These numbers were used to determine the rate of loss of the plasmids, which follows simple exponential decay (25). In this study, we have assumed that plasmid maintenance is independent of cell type because no cell-type-dependent effect on replication of *oriP* in human cells has been previously observed. Therefore, the results of independent experiments were jointly analyzed by using the Wilcoxon rank sum test for multiple experiments. (When the data from the 1008.6− clones of 143/630 and D98AH2/Raji cells are analyzed separately by using the Wilcoxon rank sum test, the rate of loss of 1008.6− does not differ from that of 994 in either cell type, $P = 0.058$ and 0.93, respectively [14].) These experiments indicate that derivatives of *oriP* with two copies of the FR flanking the DS are maintained in proliferating cells similarly to wild-type *oriP*, although one orientation, 1008.6+, is lost from cells detectably more rapidly than is the wild type (Table 1). They also indicate that derivatives of *oriP* with two copies of the DS flanking the FR are maintained in proliferating cells similarly to wild-type *oriP* (Table 2) (14).

In an independent experiment, the average copy number of plasmid DNAs per cell in three clones of cells with 995 and three clones of wild-type *oriP* were measured after reselection.

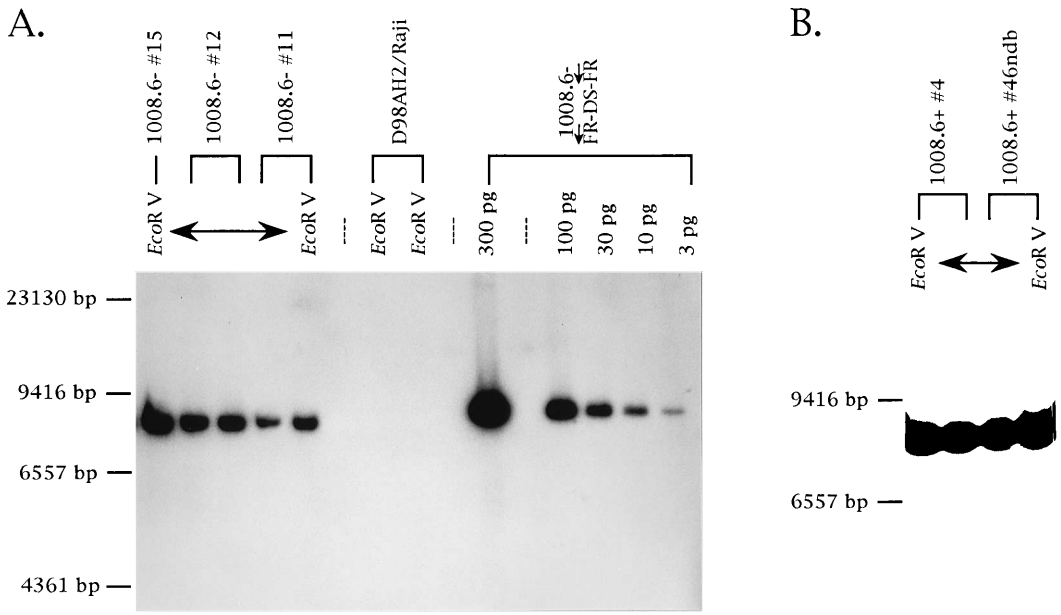


FIG. 2. Average copy number of plasmids per cell in D98AH2/Raji clones as determined by Southern analysis. (A) Ten micrograms of total cellular DNA of D98AH2/Raji clones 1008.6− 15, 1008.6− 12, and 1008.6− 11, 10 μg of total cellular DNA of the nontransfected D98AH2/Raji cell line, and 300, 100, 30, 10, and 3 pg of linearized input plasmid 1008.6−. No sample was loaded in lanes labeled with dashes. (B) Ten micrograms of total cellular DNA of D98AH2/Raji parental clone 1008.6+ 4 and subclone 1008.6+ 46ndb, which was generated after propagating the parental cells in the absence of selection for approximately 30 generations before reintroducing the selective drug. DNA samples in each blot were digested with *Eco*RV, separated by agarose gel electrophoresis, transferred to nylon membranes (Hybond N; Amersham), and probed with pKan2 labeled with [α-^{32}P]dCTP, using the Amersham Multiprime DNA labeling system. Betagen scanning of the blots was used to quantify the plasmid DNA present in each clone of D98AH2/Raji cells by interpolating values for sample concentrations from an internal standard curve of known concentrations of plasmid DNA loaded on the same gel. DNA molecular weight standards are noted to the left of the blots.

This average copy number per cell was compared with the original copy number, and their distribution was found to be similar (data not shown); that is, the average copy number of 995, a replicon containing two copies of DS, had not amplified relative to that of wild-type *oriP* in cells treated similarly. These results distinguish *oriP* from *oriS* and *oriL* of herpes simplex virus, in which additional copies of origins of replication provide a selective advantage (5).

We have found that plasmids with modifications of *oriP* are lost from cells at rates similar to that of wild-type *oriP*; conversely, they are maintained in proliferating cells at rates similar to that of *oriP*. Plasmid maintenance reflects the sum of plasmid replication and segregation. Our findings therefore indicate that plasmids with two copies of the FR flanking one copy of the DS and plasmids with two copies of the DS flanking one of the FR replicate with efficiencies similar to that of wild-type *oriP*. These observations mean that for each cell cycle, replication usually neither initiates nor terminates more than once per replicon even for those replicons which have two copies of the DS or of the FR. Initiation of replication in derivatives of *oriP* is presumably controlled as is that of chromosomal DNA in which replicated DNA is not found to support reinitiation of DNA synthesis during one S phase. There is no such model to explain a possible unique termination in

TABLE 1. Multiple copies of the FR affect the efficiency of plasmid maintenance

Derivative of *oriP*	Cell line[a]	No. of clones	% of cells that lost plasmids/ generation ± SD	*P*[b]
994	D98AH2/Raji	4	1.8 ± 1.2	
←FR-DS	143/630	4	2.0 ± 0.42	
1008.6−	D98AH2/Raji	8	2.4 ± 1.7	0.15
←FR-DS-FR	143/630	4	4.2 ± 1.6	
1008.6+	D98AH2/Raji	8	4.4 ± 3.4	0.034
←FR-DS-FR→	143/630	3	4.0 ± 1.7	

[a] Selection used: for 143/630 clones, 600 μg of G418 per ml; for D98AH2/Raji clones, 1,000 μg of G418 per ml.

[b] The rate of loss of each derivative of *oriP* was compared with the rate of loss of wild-type *oriP* for each cell line. Results from the two independent experiments were jointly analyzed by using the Wilcoxon rank sum test. *P* is shown for the hypothesis that the measured rate of loss of the *oriP* derivative does not differ from that of *oriP*. $P < 0.05$ is considered significant, which indicates that in this analysis the rate of loss of 1008.6+ differs from that of 994.

TABLE 2. Derivatives of *oriP* containing an additional copy of the DS are maintained as efficiently as are plasmids containing wild-type *oriP*[a]

Derivative of *oriP*	% of cells that lost plasmids/ generation ± SD	*P*[b]
pHEBo1	3.8 ± 1.4	
←FR-DS		0.56
995	3.9 ± 2.5	
DS-←FR-DS		

[a] Assay were done with D98AH2/Raji clones, using 150 μg of hygromycin per ml for selection. Four clones of each *oriP* derivative were analyzed.

[b] The rate of loss of the derivative of *oriP* was compared with the rate of loss of wild-type *oriP* by using the Wilcoxon rank sum test. *P* is shown for the hypothesis that the measured rate of loss of the *oriP* derivative is the same as that of *oriP*. $P < 0.05$ is considered significant, which indicates that in this analysis, 995 is lost from cells at the same rate as pHEBo1.

derivatives of *oriP*. It is possible that termination occurs at only one copy of the FR because it is in a complex such as a matrix attachment site (16) which precludes a second, similar, nearby complex. However, termination of replication at two juxtaposed sites in plasmid R6K occurs efficiently at each site (10, 15). It is also possible that the accumulation of replication forks observed at the FR reflects inhibition of their movement, not termination, which might result from avid binding of 21 dimers of EBNA-1 to the FR.

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