JOURNAL OF VIROLOGY, Jan. 1991, p. 483–488
0022-538X/91/010483-06$02.00/0

Vol. 65, No. 1

Epstein-Barr Virus-Derived Plasmids Replicate Only Once per Cell Cycle and Are Not Amplified after Entry into Cells

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Received 3 July 1990/Accepted 18 October 1990

Some possible ways in which replication of plasmids containing the Epstein-Barr virus (EBV) plasmid maintenance origin, *oriP*, might be controlled were investigated. Virtually all plasmid molecules were found to replicate no more than once per cell cycle, whether replication was observed after stable introduction of the plasmids into cells by drug selection or during the first few cell divisions after introducing the DNA into cells. The presence in the cells of excess amounts of EBNA1, the only viral protein needed for *oriP* function, did not increase the number of *oriP*-replicated plasmids maintained by cells under selection. In the cell lines studied, EBNA1 and *oriP* seem to lack the capacity to override the cellular controls that limit DNA replication to one initiation event per DNA molecule per S phase. The multicopy status of EBV-derived, selectable plasmids appears to result from the initial uptake by cells of large numbers of plasmid molecules, the efficient maintenance of these plasmids, and the pressure of genetic selection against plasmid loss. Other unknown controls must be responsible for the amplification of EBV genomes soon after latent infection of cells.

Epstein-Barr virus (EBV) is a human herpesvirus that immortalizes human B cells in culture and is associated with several human cancers. The ~170-kbp EBV genome is nearly always maintained in these infected cells as a multicopy, circular plasmid (13, 16). In B-cell clones that have been immortalized by infection at much less than one viral particle per cell, the viral plasmid genomes are amplified soon after infection, resulting in cell lines that carry anywhere from fewer than 10 to as many as several hundred copies per cell (26). The multiple EBV genomes carried by EBV-associated Burkitt's lymphomas and nasopharyngeal carcinomas also appear to have descended from a single infecting viral genome (20). The number of copies of EBV plasmids carried by established cell lines is usually rather stable for long periods in culture. Little is known about how the number of EBV plasmid genomes is governed, although the principal *cis*- and *trans*-acting functions required for EBV plasmid maintenance have been identified and studied (reviewed in reference 18).

A 1,700-bp *cis*-acting region of the EBV genome, termed the plasmid maintenance origin, or *oriP*, can support the stable maintenance of recombinant plasmids carrying selective markers in many human cell lines, requiring the presence of only a single EBV-encoded protein, EBNA1 (17, 30, 31). Plasmids containing *oriP* are maintained under selection in different cell lines at anywhere from a few copies to approximately 100 copies per cell and are lost from the cell populations at rates of close to 5% per generation in the absence of selection (25, 30, 31). A distinct element of the EBV genome, *ori-Lyt*, supports the amplification of recombinant plasmids in cells that are supporting lytic replication of EBV and requires the EBV-encoded DNA polymerase (8). These two functionally distinct origins are believed to be responsible for the two distinct modes of EBV DNA replication.

As an initial step towards understanding the regulation of EBV plasmid replication, we sought to investigate what might govern the replication of recombinant plasmids carrying *oriP*. With the Burkitt's lymphoma cell line Raji, which carries approximately 50 plasmid copies of the EBV genome, studies involving labeling of replicated DNA with the dense thymidine analog bromodeoxyuridine (BrdU) revealed that most, if not all, of the EBV plasmids replicate once and only once per cell division cycle (1). The limitation of each segment of chromosomal DNA to one round of replication per S phase is indeed a hallmark of eucaryotic DNA replication (reviewed in reference 14). Because EBV plasmid genomes must escape this form of regulation at some time after infection, we sought to determine whether plasmids carrying *oriP*, which are also maintained at multiple copies in cells, are subject to this type of regulation either after stable introduction into cells or when introduced transiently.

Replication of *oriP*-containing plasmids introduced stably into cells by drug selection. Raji cells carrying the hygromycin B-selectable plasmid pHEBo (25) at 10 to 20 copies per cell were studied initially. Cells were grown in the presence of 0.1 mM BrdU plus 0.2 mM deoxycytidine, which has been reported to reduce the toxicity of BrdU to human cells (19). After 13 or 27 h of labeling, total DNA was isolated from 2.5 $\times 10^6$ cells, mixed with cesium chloride to a density of 1.740 g/ml in 5 ml, centrifuged at 48,000 rpm in a Beckman VTi65.2 rotor for 20 h, and separated into 24 or 26 fractions. For each fraction, duplicate portions containing 25, 1, and 0.2% of the DNA were dot-blotted onto nylon membranes which were then hybridized to random-prime-labeled DNAs: pHyg (25) to detect pHEBo, the 3.1-kb IR1 repeat of EBV (*Bam*HI W fragment [2]) to detect EBV DNA, or the 300-bp *Alu* repeat isolated from pBlur-8 (22) to detect human DNA, respectively. Hybridization was quantified by liquid scintillation counting.

The density gradient profiles are shown in Fig. 1. The data are summarized in Table 1 as the percentages of pHEBo DNA, EBV DNA, and human DNA that had incorporated BrdU into neither strand (light-light), into one strand (heavy-light), or into both strands (heavy-heavy). Plasmid pHEBo, EBV DNA, and human DNA showed very similar gradient profiles after 13 h of labeling, at which time approximately

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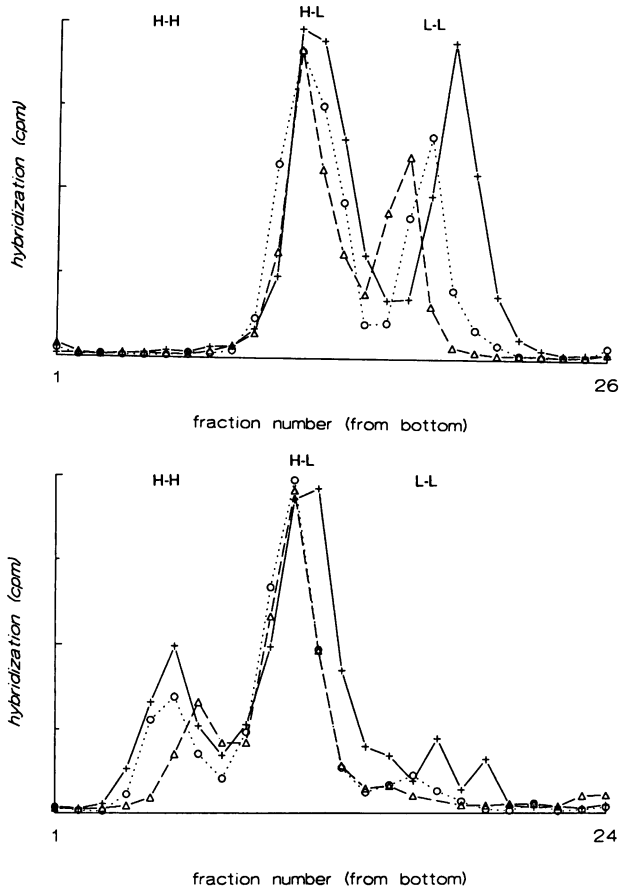

FIG. 1. Cesium chloride density gradient analysis of replication of pHEBo and EBV genomes carried by Raji cells exposed to BrdU for 13 (upper graph) or 27 (lower graph) h. H-H, H-L, and L-L mark the regions of the gradients occupied by heavy-heavy, heavy-light, and light-light DNAs, respectively. Maximum ordinate values for detection of the DNAs ranged from 16,000 cpm for human DNA to 1,300 cpm for EBV DNA. The relative positions of the peaks of BrdU-substituted and unsubstituted DNAs are in accordance with their A+T contents: 60% for human DNA, 47% for pHEBo, and 40% for EBV DNA (2). Symbols: +, cell DNA; Δ, EBV DNA; O, pHEBo.

60% of each DNA had undergone one round of replication, in accordance with the 21- to 22-h doubling time of the cell population. For this labeling period, there were no discernible peaks of hybridization in the region of the gradient in which heavy-heavy DNA would have banded. The sum of the hybridization signals of all fractions over the most dense one-third of the gradient amounted to approximately 2% of the amount of each DNA that was detected over the entire gradient. This represents an upper limit for the amount of EBV or pHEBo DNA that could have replicated more than once during the 13-h period. After 27 h of labeling, the three DNAs were substituted on one or both strands to indistinguishable extents. Thus, in an asynchronous population of Raji cells, the replication of the *oriP*-carrying plasmid pHEBo, as revealed by BrdU incorporation, is indistinguishable from the replication of the EBV plasmid genome or from that of the human genome.

The 21- to 22-h doubling time of the cell population was not affected by the BrdU until after 30 to 40 h of labeling, at which time growth began to slow. Because the amounts of once- and twice-replicated human DNA observed after 13 and 27 h of labeling are in close accordance with the doubling time for the cells, the delayed toxicity of the BrdU is not expected to have influenced the results.

TABLE 1. Percentages of Raji cell DNA, EBV DNA, and pHEBo with BrdU incorporated into one, both, or neither strand[a]

Time in BrdU (h)	Density region	% of DNA with BrdU incorporated		
		Cell DNA	EBV DNA	pHEBo
13	Light-light	41	37	35
	Heavy-light	57	61	64
	Heavy-heavy	<2.0[b]	<2.3[b]	<1.4[b]
27	Light-light	12	11	10
	Heavy-light	64	66	66
	Heavy-heavy	25	23	24

[a] The data are from the density-labeling experiment of Fig. 1. Gradient fractions were apportioned into the three density regions, which differed slightly for each DNA owing to their different base compositions. The sum of hybridization signals over each density region are expressed as a percentage of the total hybridization across the entire gradient.

[b] Because these values were obtained by summing hybridization signals over the entire lower one-third of the gradients where no peaks could be discerned, they represent upper limits.

Similar density-labeling experiments were performed with human 293 cells carrying the *oriP*-containing EBNA1-expressing plasmid p220 (similar to p201 [31]) at approximately 50 copies per cell. As with Raji cells carrying pHEBo, no plasmids were detected that had replicated twice when the labeling period was short enough so that no detectable cell DNA had replicated twice (data not shown).

Replication of *oriP*-carrying plasmids during the initial cell doublings after their entry into cells. If *oriP*-replicated plasmids only replicate once per cell cycle after being stably introduced into cells by drug selection, how do the cells obtain multiple copies of these plasmids? One possibility was that the plasmids reach multicopy levels by replicating more than once per cell cycle during the first few cell cycles after being transfected into cells, as is believed to occur with bovine papillomavirus (BPV) DNA or with BPV-derived plasmids (3). To test this possibility, the replication of *oriP*-containing plasmids was monitored after transiently transfecting them into cells.

Plasmid DNAs isolated from *Escherichia coli* can be monitored for replication in mammalian cells by determining the presence or absence of *dam*-specific methylation on one or both strands of the plasmids after recovery from transfected cells. *Dpn*I cleaves the 4-bp *dam* methylation sequence GA^{7m}TC if and only if the adenines are methylated on both strands. Initially, transfected DNAs are sensitive to *Dpn*I, but after one round of replication in mammalian cells, they become resistant to digestion. The enzyme *Mbo*I also cuts at GATC, but only if the adenines on both strands are not methylated. Thus, after one round of replication in human cells, plasmids will be resistant to digestion by both of these endonucleases; after two rounds of replication, half the molecules will become sensitive to cleavage by *Mbo*I.

Initial experiments were performed by using the cell line 293 because it carries EBV-replicated plasmids at unusually high numbers of copies per cell under selection (for p201 or p205 [31], 50 to 100 copies per cell [unpublished data]). It was found that if cells were harvested before completing more than one cell cycle after transfection, then the *Dpn*I-

resistant (replicated) DNA was resistant to *Mbo*I, indicating that most or all of the plasmid molecules replicated no more than once. In other transient replication studies in which the cells were grown for 3 to 4 days after transfection (such as in reference 29), 70 to 80% of the *Dpn*I-resistant plasmid DNA was found to lack adenine methylation on both strands (*Mbo*I sensitive), indicating that the replicated plasmids had been replicated close to three times on average, consistent with the rate of one round of plasmid replication per round of cell division cycle (data not shown).

For a more sensitive test to determine whether even a small fraction of the plasmids overreplicate after transfection, the *oriP*-carrying plasmid p119 (called pCΔJ in reference 30) was used because it carries a *Bcl*I site. The *Bcl*I recognition site contains the *dam* methylation site GATC, and like *Mbo*I, it cleaves only if the site lacks adenine methylation on both strands. 293 cells were transfected with p119 mixed with an equimolar amount of a second *oriP*-containing plasmid, p367 (29), which provided a source of EBNA1. Sixteen or 37 h after transfection, small-molecular-mass DNA was isolated from the cells by the method of Hirt (12), digested with *Dpn*I, *Bcl*I, and *Bam*HI (to linearize both plasmids), electrophoresed through a 0.7% agarose gel, transferred to a membrane, and hybridized to a radiolabeled plasmid probe. The autoradiograph is shown in Fig. 2A. The band representing full-length, *Dpn*I-resistant p119 (10.4 kb) is apparent, migrating just above the band of replicated p367 (9.9 kb). An 8.4-kb DNA that would be produced by cutting with *Bcl*I could not be detected even in a long exposure of the autoradiograph. On the basis of densitometric scans of autoradiographs exposed for appropriate lengths of time, it was concluded that less than 5% of the replicated p119 had been cut by *Bcl*I.

When DNA was isolated from the cells 37 h after transfection in the same experiment, more of the transfected plasmids were found to have replicated, and a readily detectable portion of the p119 was sensitive to cutting by *Bcl*I (Fig. 2A).

Additional experiments that were performed with Raji cells also failed to reveal any overreplication of the *oriP*-containing plasmid. Because Raji cells are grown in suspension, exponential growth of the transfected cells could be easily sustained and concurrently monitored with measurements of plasmid replication. Duplicate populations of 8×10^7 cells were electroporated with p119 DNA, cultured at 37°C in growth medium, and harvested in portions of 2.5×10^7 to 4×10^7 cells after incubation for analysis. For electroporation, a Bethesda Research Laboratories Cell-Porator was set at 300 V and 330 μF to electroporate 10^7 cells at a time in 0.6 ml of complete RPMI 1640 medium with 2 μg of DNA at room temperature. *Dpn*I-resistant, replicated p119 was detected after 14.5 and after 22 h, but very little, if any, of the DNA could be cut with *Bcl*I (Fig. 2B). In a long exposure of the autoradiograph, faint bands were seen at the position expected for the *Bcl*I-cut DNA, but their intensities were less than 3% of the intensities of the bands representing the full-length DNA and no greater than the intensities of other faint bands of unknown origin that were seen at other positions in these lanes. To check for complete cutting by *Dpn*I, 200 pg of p119 was mixed with DNA prepared from Raji cells that had not been electroporated and analyzed in parallel (Fig. 2B, lane bl.). Complete digestion by *Bcl*I was apparent from the pattern of mitochondrial DNA fragments visible on the ethidium bromide-stained gel.

The doubling time of the cultures (grown in dishes filled more deeply with medium in this experiment) was approximately 25 h. Some of the plasmids harvested from the asynchronous cultures after 22 h could have been present in cells for parts of two S phases, so some twice-replicated plasmid DNA might have been expected. It is not known, however, how much time after electroporation is needed for the cells to become competent to replicate DNA or for the introduced DNA to become accessible for replication.

It is apparent from inspection of Fig. 2B and 3 that the amount of replicated plasmid DNA per Raji cell increased until some time between 22 and 42 h after electroporation. This increase was almost entirely due to unreplicated, transfected DNA being replicated for the first time. Thereafter,

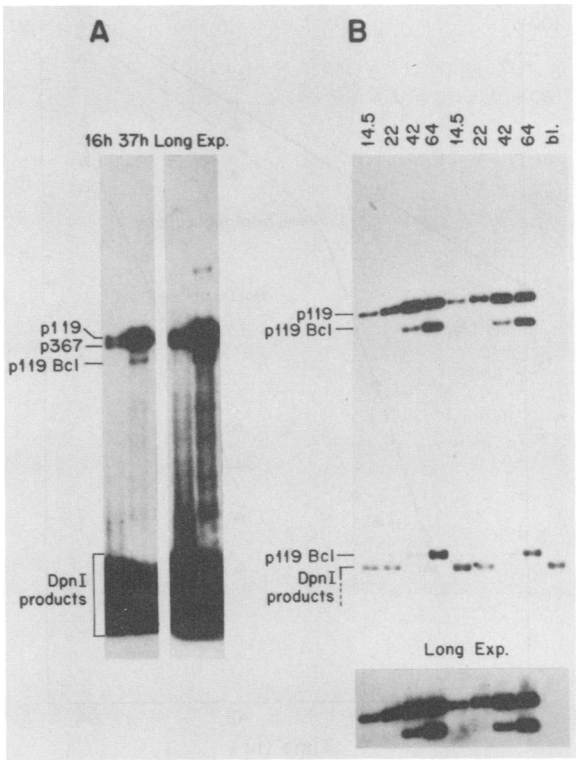

FIG. 2. The initial rounds of *oriP*-dependent replication in transfected cells, monitored by disappearance of *dam* methylation on one or both DNA strands. (A) Plasmid replication in 293 cells. Cells were cotransfected with p119 and p367 (5 μg of each per 10-cm dish) by using the calcium phosphate method (7) with a 2-min, 20% glycerol shock 5 h later. Hirt supernatant DNAs (12) were isolated 16 or 37 h after the glycerol shock, and portions corresponding to half of a dish of transfected cells were digested with *Bam*HI, *Bcl*I, and *Dpn*I to monitor replication (see the text) and subjected to Southern analysis. A four-times-longer autoradiographic exposure of the same blot is shown at the right (Long Exp.). For details of the methods, see reference 28. (B) Replication of p119 in Raji cells. For duplicate populations of Raji cells electroporated with p119, replication of the plasmid was determined after 14.5, 22, 42, and 64 h as described for panel A. DNA from 7.5×10^6 cells for the 14.5-h samples and from 10^7 cells for the other samples was analyzed. In lane bl., 200 pg of p119 was mixed with an equivalent amount of DNA from Raji cells that had not been electroporated and analyzed in parallel. Indicated at the left are the positions of full-length p119 linearized with *Bam*HI (10.4 kb), of the 8.4- and 2.0-kb fragments resulting from additional cleavage with *Bcl*I, and of the largest hybridizing *Dpn*I fragment. Several smaller *Dpn*I fragments were run off the gel. Below is shown a three-times-longer exposure of the upper relevant portion of the blot.

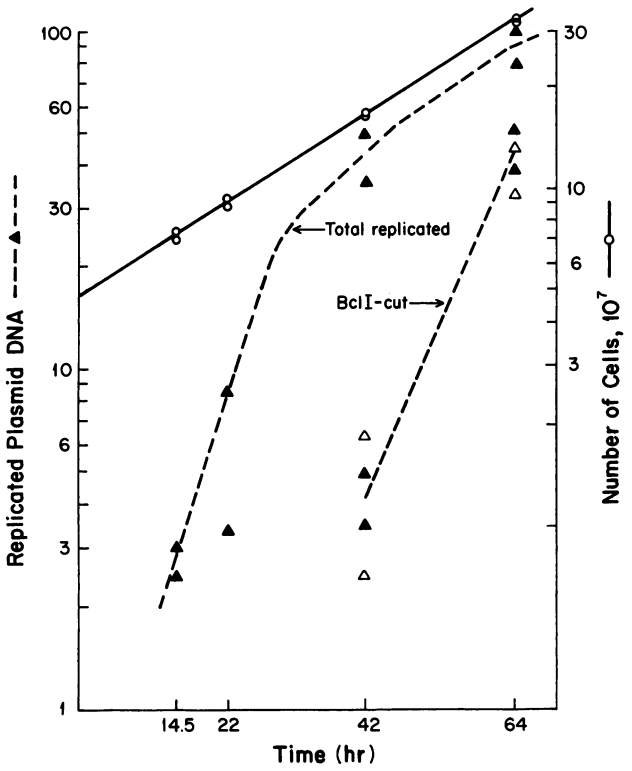

FIG. 3. Replication of *oriP*-containing plasmid, p119, transiently introduced into Raji cells. Presented graphically from the experiment of Fig. 2B are growth curves for the duplicate cultures (○), for the amounts of replicated DNA (▲), both as total replicated (*Bcl*I-sensitive and *Bcl*I-resistant) and as *Bcl*I-sensitive DNA, and for the amounts of *Bcl*I-sensitive plasmid predicted by calculation from the amounts of replicated plasmid DNA observed at the previous times (△). Note that the increase in the amount of replicated plasmid per cell occurred before very much of it could be cut by *Bcl*I; that is, most of the increase resulted from unreplicated plasmids being replicated for the first time. Growth curves were corrected for the numbers of cells removed for analysis. The amounts of replicated plasmid DNA, determined by densitometric analysis of the autoradiographs, have been corrected to represent the total amounts present in the cultures. The lower value for 22 h is considered to be in error. To calculate the expected amounts of plasmid resulting from two or more rounds of replication and lacking *dam* methylation on both strands (*Bcl*I-sensitive DNA), it was assumed that the plasmids would be replicated asynchronously in the population and that all plasmids would replicate once per cell generation of 25 h. *Bcl*I-cleavable DNA at time 2 ($t2$) was estimated from the amounts of replicated DNA at time 1 ($t1$) to equal: (*Bcl*I-cut DNA)$_{(\text{at } t1)}$ + (total replicated DNA)$_{(\text{at } t1)} \times (2^{(t2 - t1)/25\text{ h}} - 1)$. The final factor equals the fraction of molecules present at $t1$ that will have replicated by $t2$. Replication of each hemi-*dam*-methylated molecule yields one unmethylated molecule; replication of each unmethylated molecule yields two unmethylated molecules, but one of them has already been counted in the first term of the equation.

while plasmids that had been replicated more than once accumulated, the number of replicated plasmids per cell appeared to have reached a plateau. These observations indicate that *oriP*-replicated plasmids are not amplified after transfection into cells.

It might be argued that, perhaps due to cell injury during electroporation, the transiently introduced plasmids replicate with a very low efficiency, much less than once per molecule per cell cycle, so that very few molecules would have been observed to replicate more than once per cell cycle even if *oriP*-initiated replication normally involves such events. However, the quantitative data (Fig. 3) are consistent with rather efficient replication of the transiently introduced plasmids. First, it appears that the cells recovered quickly after electroporation, generating exponential growth curves from the first time point at 14.5 h onward. Second, between 42 and 64 h after transfection, the increases of replicated DNA in the two populations matched the cell growth rates. Finally, the accumulation of plasmid molecules lacking *dam* methylation on both strands followed the accumulation of once-replicated plasmids at a rate consistent with that of each molecule replicating once per cell cycle. The amounts of *Bcl*I-cut DNA observed at 42 and 64 h were close to the amounts that could be predicted by assuming a once-per-cell-cycle replication of previously replicated (mostly once-replicated) plasmids observed at the previous times (Δ in Fig. 3; see legend).

The data presented here do not rule out the possibilities that amplification of *oriP* plasmids occurs in an undetectably small subset of the cells and that it is these cells that are the progenitors of the stably transfected, drug-resistant clones. However, if amplification of plasmids in a small subset of the cells had been responsible for the accumulation of plasmids that had replicated more than once, then the appearance of the *Bcl*I-digestable plasmid DNA in Fig. 3 need not have borne any relationship to the once-replicated plasmids in the bulk population. In addition, previous studies have shown that Raji cells electroporated with pHEBo establish drug-resistant clones almost as efficiently as they take up DNA (25). Therefore, the present studies, in which DNA replication is observed following transient transfection, are likely to reveal the events that lead to stable maintenance of the plasmids in cell populations.

Inability of excess EBNA1 to increase the copy of *oriP*-containing plasmids. The possibility that limiting levels of EBNA1 protein might limit *oriP*-specific replication, especially under conditions of the transfections, was considered. The plasmid p220, derived from pHEBo, contains *oriP* and the hygromycin B-resistance gene, *hph*, but also encodes ENBA1, allowing it to be maintained efficiently at multiple copies in a variety of human cell lines. (p220 is identical to p201 [31] except for the presence of a polylinker). It was found that Raji cells carrying p220 under selection express EBNA1 at elevated levels for a few weeks after introduction of the plasmid. The additional EBNA1 in the cells had no apparent effect on the number of copies per cell at which this *oriP*-replicated plasmid was maintained.

Populations of Raji cells carrying pHEBo or p220 were established by growing 2×10^5 electroporated cells in the presence of 250 μg of hygromycin B per ml for 3 weeks before analysis. Because approximately 1% of the cells was shown capable of founding drug-resistant clones by limiting dilution, the drug-resistant populations used were mixed populations of up to 1,000 initial founding clones. Figure 4 shows the analysis of EBNA1 protein and plasmid DNA present in these populations. The EBNA1 protein encoded by p220 from strain B95-8 is larger than the protein encoded by the Raji EBV strain because of a longer IR3 repeat in the EBNA1 open reading frame (11). EBNA1 protein expression due to p220 was more than twice the level of that expressed from the EBV genomes carried by Raji cells (Fig. 4A). Despite this greater than threefold elevation in the total amount of EBNA1 in the cells carrying p220, p220 and pHEBo were maintained at similar levels in cells (Fig. 4B). Quantitation of the hybridization signals obtained for these

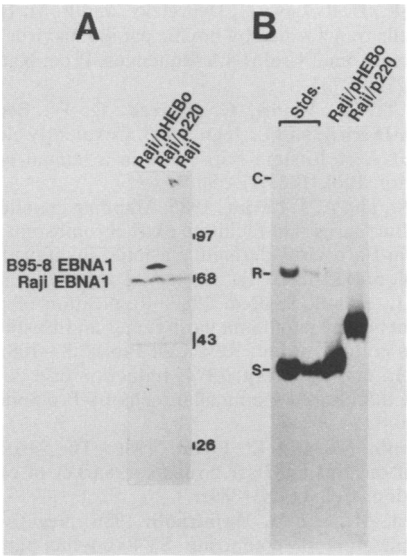

FIG. 4. Insensitivity of copy number of *oriP*-replicated plasmids to excess EBNA1 protein. Raji cells carrying pHEBo or the EBNA1-expressing plasmid p220 were analyzed for levels of EBNA1 protein (A) and levels of plasmid DNA (B). (A) Whole-cell extracts of 5×10^5 Raji cells alone or Raji cells carrying the indicated plasmid were electrophoresed in a sodium dodecyl sulfate-12% polyacrylamide gel and analyzed by immunoblot by using a rabbit antiserum specific to EBNA1 and an alkaline phosphatase-conjugated secondary antibody (29). The positions of prestained protein size markers (Bethesda Research Laboratories) are indicated by vertical marks at the right, alongside the M_r (in thousands) of each. (B) Hirt supernatant DNAs obtained from 1.6×10^6 cells were analyzed without restriction enzyme digestion in agarose gels followed by Southern analysis with pHyg (25) as a probe. Standards (Stds.) were 100 and 20 pg of pHEBo, corresponding to 7.9 and 1.6 molecules recovered per cell, respectively. The positions of supercoiled (S) and relaxed circular (R) pHEBo and of linear human chromosomal DNA fragments (C) are indicated at the left.

plasmids by using an Ambis β-scanner revealed that slightly fewer p220 molecules (7 per cell) than pHEBo molecules (11 per cell) were actually present in the extracted DNA. It is not known whether or not this difference is significant, but these values are within the ranges previously observed for the levels of maintenance of pHEBo and its derivatives in Raji cells by using these methods of measurement (28).

For unknown reasons, EBNA1 expression from p220 was observed to drop reproducibly to very low levels during propagation of Raji cells carrying p220 for 2 to 3 subsequent weeks. Nevertheless, the above experiment indicates that *oriP*-dependent replication is unlikely to be limited by the level of EBNA1 protein, either during transient transfections or during the establishment of stably maintained plasmids in Raji cells.

A cellular control mechanism for *oriP*-initiated replication. The replication of *oriP*-containing plasmids in a once-per-cell-cycle fashion suggests that initiation of replication at *oriP* may be limited by the same mechanism that limits chromosomal DNA to one replication per S phase. The failure of excess amounts of EBNA1 protein to elevate the copy level of an *oriP*-replicated plasmid is consistent with this conclusion. It has recently been shown that EBV immortalized B-cell clones that differ 40-fold in the number of EBV genomes carried have very similar levels of EBNA1 protein (24). A previous finding also consistent with this conclusion is that addition of a second copy of *oriP* to an *oriP*-containing plasmid had no effect either on the number of plasmids maintained per cell under selection or on the rate at which plasmids were lost from cells in the absence of selection (27). In these studies it was also shown that Raji cells acquire multiple plasmid molecules by electroporation; cells simultaneously electroporated with two *oriP*-containing plasmids carrying different selective markers were found to acquire and maintain both plasmids efficiently when selected for either one alone (27). Although the possibility remains that plasmid amplification could occur later than the times observed in the transient studies but before drug-selected cell populations are established, the current data suggest that the multiple copies of plasmids carrying *oriP* result from the initial uptake by cells of many plasmid molecules and the subsequent pressure of selection against plasmid loss.

In these studies of plasmids containing *oriP*, there is no indication of the amplification that must occur with the EBV plasmid genome in infected cells. It is suggested here that the mechanisms responsible for this amplification do not operate on *oriP*-containing plasmids in Raji cells and are not provided by EBNA1 and *oriP* when introduced into EBV-negative cells. It is likely either that an additional *trans*-acting factor is required to allow more than a single initiation at *oriP* per S phase at some time after infection or that a different replication origin is used. The EBV lytic replication system is unlikely to be important for the amplification of latent viral genomes for two reasons. The viral replication inhibitor acyclovir was found to have no effect either on the frequency of immortalization of B cells or on the average number of genomes carried by the established B-cell lines (23). In addition, the EBV genomes carried by Raji cells have a deletion in a gene essential to herpesvirus lytic replication, yet the multiple (~50)-plasmid copies per Raji cell must have been amplified from a single mutant molecule (10).

Bearing some biological resemblance to EBV, BPV can latently infect cells via a single infecting particle, leading to stable maintenance of approximately 40 to over 100 plasmid copies of the viral genome in the resulting growth-transformed cells (5, 15). Two studies led to diametrically opposed views as to whether BPV plasmid genomes are each replicated once per cell cycle or whether plasmids are replicated randomly from among the pool of unreplicated and previously replicated molecules within an S phase (4, 6). It has been proposed that the E1 open reading frame, essential for BPV plasmid maintenance, also encodes a function that specifically limits BPV plasmid replication (3, 21), although the observations leading to this hypothesis have not been extended.

Might EBV provide such specific negative control over its own plasmid replication? Plasmids containing *oriP* replicate once per cell cycle in 293 cells when EBNA1 is the only EBV-derived protein present, and EBNA1 is unlikely to possess a negative control function that is separable from its positive replication function. This is the conclusion of an extensive deletion analysis of the EBNA1 coding region in which only small regions of the DNA-binding domain were found to be uniquely required for replication and no deletion led to overreplication (29, 29a).

It seems more likely that the cellular mechanisms that ensure that each segment of chromosomal DNA is replicated no more that once per S phase apply to initiation of replication at *oriP*. Indeed, such regulation of replication in eucaryotes may not depend on any site-specific signals. Any

circular DNA of bacterial origin injected into *Xenopus laevis* eggs is replicated only once per S phase, suggesting that a mechanism exists to distinguish replicated from unreplicated DNA (9). EBNA1 and *oriP* of EBV appear to be subservient to this control.

The initial studies that indicated that EBV-derived plasmids replicate only once per cell cycle following transient transfections were performed by Denise Liu and Laurel Sherwood, high school and undergraduate students, during the summer of 1987. We thank Bill Sugden and Lynne Maquat for comments on the manuscript.

This work was supported by grants CA43122 and RR056482 from the National Institutes of Health.

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