

Establishment of an *oriP* Replicon Is Dependent upon an Infrequent, Epigenetic Event

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Plasmids containing *oriP*, the latent origin of replication for Epstein-Barr virus, support efficient replication in selected cell clones when the viral protein EBNA-1 is provided, being lost at a rate of 2 to 4% per cell generation after removal of selection (A. L. Kirchmaier and B. Sugden, *J. Virol.* 69:1280–1283, 1995; B. Sugden and N. Warren, *Mol. Biol. Med.* 5:85–94, 1988). We refer to these plasmids as established replicons in that they support efficient DNA synthesis and partitioning each cell cycle. Unexpectedly, we have found that upon introduction of *oriP* plasmids into a population of EBNA-1-positive cells, *oriP* plasmids replicate but are lost precipitously from cells during 2 weeks posttransfection (>25% rate of loss per cell generation). Upon investigation of these disparate observations, we have found that only 1 to 10% of cells transfected with an *oriP* plasmid expressing EBNA-1 and hygromycin phosphotransferase give rise to drug-resistant clones in which the *oriP* replicon is established. A heritable alteration in these drug-resistant cell clones, manifested at the genetic or epigenetic level, does not underlie the establishment of *oriP*, as newly introduced *oriP* plasmids replicate but are also lost rapidly from these cells. In addition, a genetic alteration in the *oriP* plasmid is not responsible for establishment, as *oriP* plasmids isolated from an established cell clone, propagated in *Escherichia coli*, and reintroduced into EBNA-1-positive cells are likewise established inefficiently. Our findings demonstrate that *oriP* replicons are not intrinsically stable in EBNA-1-positive cell lines. Rather, the establishment of an *oriP* replicon is conferred upon the replicon by a stochastic, epigenetic event that occurs infrequently and, therefore, is detected in only a minority of cells.

It is essential for cells and latently infecting viruses to duplicate their genomes and transmit their genetic information to daughter cells during each cell cycle, processes that we condense into the term replication. The plasmid replicon of Epstein-Barr virus (EBV) serves as a model for studying replication in that, akin to the genome of its host cell, this replicon is synthesized only once per cell cycle during S phase (1, 51) and is efficiently partitioned to daughter cells (20, 25, 45, 47). Only two viral components are required for the replication of EBV's genome, the latent origin *oriP* and its binding protein Epstein-Barr-associated nuclear antigen 1 (EBNA-1); all else is contributed by the cell (26, 49, 52). Plasmids containing *oriP* support efficient replication in EBNA-1-positive cells selected to maintain them, being lost at a rate of 2 to 4% per cell generation after removal of selection (20, 47), a rate of loss which resembles that of *ARS/CEN* plasmids in *Saccharomyces cerevisiae* (23). We define these plasmids as established replicons in that they support efficient DNA synthesis and partitioning each cell cycle.

What events ensure efficient replication of *oriP* plasmids? The answer to this question largely remains enigmatic but should provide insights into the replication of EBV and perhaps into the replication of mammalian genomes. Genetic and biochemical studies of the eukaryotes *S. cerevisiae* and *Schizosaccharomyces pombe* have unveiled three regulatory steps required for high-fidelity replication which occur at distinct time points: during the initiation of DNA synthesis, during the com-

pletion of DNA synthesis, and during the partitioning of daughter molecules at mitosis. The mechanisms underlying these checkpoints may provide a framework for understanding the efficient replication of EBV's plasmid replicon.

Efficient initiation of DNA synthesis in eukaryotes depends upon genetic and epigenetic parameters. *cis*-acting elements known as origins must effectively recruit components of the prereplication complex (pre-RC), including ORC and MCM proteins (36). These specific initiation sites are defined by nuclear structure as demonstrated by studies of the dihydrofolate reductase origin in *Xenopus* egg extracts (10, 13), and chromatin, a component of nuclear structure, affects the timing of DNA synthesis at these origins as well (43). Epigenetic factors not only can define origin usage and timing but also can modulate origin activity as illustrated by experiments in which the activity of *ARS1*, an origin of DNA synthesis in *S. cerevisiae*, is compromised when located within a nucleosome but restored upon the positioning of the nucleosome outside the origin (41).

After initiation of DNA synthesis within the eukaryotic cell, several checkpoints are imposed to ensure completion of genome duplication prior to entry into mitosis and to prevent additional rounds of DNA synthesis in the absence of intervening mitoses. The former of these checkpoints involves components of the replication fork, such as DNA polymerase epsilon in *S. cerevisiae*, which is thought to sense single-stranded DNA accumulated during an S-phase block and transduce a Rad-dependent signaling cascade which halts mitosis (6, 31). When the genome is completely duplicated, additional rounds of DNA synthesis must be inhibited. The M-phase kinase Cdk1/cyclin B regulates this process by inhibiting pre-RC formation until mitosis is complete (8, 32). Deletion of the mitotic

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B cyclin p56^{cdc13} in *S. pombe* has disastrous results, with cells accumulating up to 32 copies of their genome due to the lack of intervening mitoses (16).

Upon completion of a single round of DNA synthesis within the eukaryotic cell, a single sister chromatid must be partitioned to each daughter cell. The fidelity of this process is ensured in part by the assembly of functional centromeres. In *S. pombe*, an epigenetic event involving maintenance of underacetylated centromeric chromatin is essential for centromere function (11, 14, 42). Upon association of the centromere-bound kinetochore with the mitotic spindle, a MAD2 checkpoint is imposed in which MAD1, MAD2, MAD3, and BUB1 inhibit the activity of the anaphase-promoting complex activator Cdc20 (APC^{cdc20}), thereby blocking sister chromatid separation (4, 7). Anaphase can ensue upon attachment of these chromatids to spindle fibers emanating from opposing poles.

Eukaryotes have orchestrated multiple regulatory events to ensure high-fidelity replication of their genomes, which EBV is likely to use. EBV, in contrast to latently infecting viruses such as bovine papilloma virus, does not override the cellular synthetic machinery to maintain its genome extrachromosomally but rather appears to insinuate itself into this machinery. For example, initiation of DNA synthesis within EBV and *oriP* plasmids occurs at or near the dyad symmetry element (DS) of *oriP* (12), an origin that is licensed analogously to origins in eukaryotic cells (39). Likewise, partitioning of *oriP* plasmids is nonrandom, akin to eukaryotic genomes, in that cells containing, on average, two *oriP* plasmids lose these plasmids at the same rate as do cells having 10 or more *oriP* plasmids (20). The events that underlie the establishment of *oriP* plasmids therefore likely mirror those of eukaryotic cells.

Our studies on the replication of *oriP* plasmids have provided insights into an event required for the establishment of a replicon. While *oriP* plasmids support efficient replication in selected cell clones, we have found that upon introduction of *oriP* plasmids into a population of EBNA-1-positive cells, the *oriP* plasmids support replication but are lost precipitously from these cells during 2 weeks posttransfection. Investigation of these disparate findings has revealed that only 1 to 10% of those cells transfected with an *oriP* plasmid expressing EBNA-1 and hygromycin phosphotransferase gave rise to a drug-resistant clone in which an *oriP* replicon was established. A heritable alteration in these cell clones was not responsible for the establishment of *oriP*, as newly introduced *oriP* plasmids supported replication but were also lost precipitously in these cells. In addition, establishment is not dependent upon genetic alteration of the *oriP* plasmid, as *oriP* plasmids which were rescued from an established cell clone, propagated in *Escherichia coli*, and reintroduced into EBNA-1-positive cells were likewise established inefficiently. Our experiments demonstrate that the stable replication of an *oriP* plasmid is not intrinsic to the plasmid but is conferred upon the replicon by a stochastic, epigenetic event that occurs infrequently and therefore is detected in a minority of cells. This epigenetic event must allow efficient completion of one or more of the regulatory steps described above. Defining this epigenetic event will provide insights into the cellular and viral contributors to the establishment of *oriP* and perhaps to that of other cellular and viral replicons.

MATERIALS AND METHODS

Plasmids. 2278, the *oriP* test plasmid, and 2276, the prokaryotic backbone plasmid, are derived from 2275, a plasmid containing a ColE1 origin and *supF* marker for propagation in *E. coli* and a neomycin phosphotransferase gene driven by the thymidine kinase promoter of herpes simplex virus. 2278 was constructed by inserting *oriP* between the *HpaI* and *NsiI* sites of 2275. 2276 was constructed by replacing the neomycin phosphotransferase gene with the firefly luciferase gene, thereby generating a length polymorphism upon amplification with primers 1588 and 86, as described below. 1728 contains *oriP* and encodes hygromycin B phosphotransferase and a derivative of EBNA-1 containing only five copies of the Gly-Gly-Ala repeat (2). 2048 is pCDNA3 (Invitrogen) in which the neomycin phosphotransferase gene was deleted by digestion with *EcoRV* and *Bst1107I* and religated. 2145 encodes enhanced green fluorescent protein (EGFP), whose expression is driven by the cytomegalovirus promoter, and lacks the neomycin phosphotransferase cassette. 2264, the competitor DNA, contains a fragment of DNA composed of pBR322 and *LEU2* flanked by primer 1588 and primer 86 binding sites, such that amplification by PCR yields a 656-bp product. 2446 contains the luciferase gene from pGL2 Basic (Stratagene) cloned in place of EGFP in a plasmid construction (1783) containing *oriP*, and a cytomegalovirus promoter-driven IRES-EGFP cassette.

p220 was constructed by addition of the *SmaI/HaeIII* polylinker from pUC12 into the *NarI* site of p201 (52). This plasmid contains *oriP*, the beta-lactamase gene and origin of replication from pBR322, and encodes hygromycin B phosphotransferase and EBNA-1. The DS of *oriP* was removed from p220 by digestion with *EcoRV* and *HpaI* and religated, resulting in p220ΔDS (plasmid 2368, a kind gift from Jun Komano). pHEBo is the parental plasmid of p220 and lacks EBNA-1-encoding sequences (46). Low-molecular weight DNA from an established 293 cell clone harboring p220 (293/p220#2) was isolated (a kind gift from Jun Komano) and introduced into STBL2 *E. coli* (Gibco BRL). Two pools of approximately 1,000 colonies each were propagated on a large scale and plasmid DNA was purified (37), resulting in plasmids 2798 and 2799. The establishment efficiency of these plasmids (collectively referred to as "rescued p220") in 293 cells was monitored as described for plasmid 1728 (see below).

Plasmid constructions were confirmed by sequencing and/or restriction enzyme digestions. Our plasmid database is accessible at: <http://mcardle.oncology.wisc.edu/sugden/>.

Cell lines and transfections. The cell lines used for the replication assays include (i) H1299, a p53-null human lung carcinoma cell line (ATCC CRL-5803); (ii) 293, a human embryonic kidney cell line (ATCC CRL-1573); (iii) 143B, a human osteosarcoma cell line (ATCC CRL-8303); (iv) 293/EBNA-1, a 293 cell line which stably expresses EBNA-1 and neomycin phosphotransferase (ATCC CRL-10852); and (v) C33A/EBNA-1, a human cervical carcinoma cell line (ATCC HTB-31) into which the plasmid 1553, which expresses EBNA-1 and hygromycin B phosphotransferase, was integrated (2). Cell lines were grown in Dulbecco's modified Eagle's medium-high glucose supplemented with 10% fetal bovine serum, 200 U of penicillin per ml and 200 μg of streptomycin sulfate per ml. 143B cells were grown in medium containing calf serum instead of fetal bovine serum. 293/EBNA-1 and C33A/EBNA-1 cells were also grown in the presence of 200 μg of G418 sulfate per ml and 100 μg of hygromycin B per ml, respectively. Cells were grown at 37°C in a humidified 5% CO₂ atmosphere. The doubling time of these cell lines is 22 to 24 h.

For the time course experiments, calcium phosphate precipitates containing equimolar amounts of *oriP* test plasmid (10 μg) and prokaryotic backbone plasmid (6.5 μg), 5 μg of 2145, (an expression vector for EGFP), and 10 μg of 1728 (an *oriP*-based EBNA-1 expression plasmid) or 10 μg of 2048 (an empty expression plasmid) were prepared and placed onto 15-cm dishes containing adherent cell lines (37). Plasmids 1728 and 2048 were not introduced into 293/EBNA-1 and C33A/EBNA-1 cells. H1299 cells were suspended by trypsinization, mixed with the precipitate, and plated onto a 15-cm dish. The medium was changed 5 to 8 h after addition of the precipitate. DNAs were introduced into 143B cells by electroporation (22). At 2 days posttransfection, cells were harvested and the percentage of EGFP-positive cells was determined as a measure of the transfection efficiency. (The transfection efficiency of *oriP* plasmids should be equivalent to or greater than that of the EGFP expression vector, as the FR of *oriP* promotes plasmid retention [29, 24].) Cells were expanded on 15-cm dishes and harvested at 4 to 6 days posttransfection, at which time a dilution of cells was replated such that the dishes were near confluence at the next time point, and the remaining cells were prepared by Hirt extraction, as described below.

Quantitative competitive PCR assay. (i) **Hirt extraction and digestion of low-molecular-weight DNAs.** At the indicated time points posttransfection, cells were harvested and low-molecular-weight DNA was extracted by the method of

Hirt (17). The supernatant was transferred to a new tube, incubated with 100 μ g of RNase A per ml at 37°C for 2 h, and subsequently treated with 200 μ g of proteinase K per ml overnight at 37°C. Samples were extracted with phenol-chloroform and the DNA was precipitated with isopropanol. The DNA pellet was rinsed with 70% ethanol, dried, and resuspended at 5×10^5 cell equivalents per μ l in Tris-EDTA (TE) (10 mM Tris, pH 7.0, 1 mM EDTA). Fifty million cell equivalents were digested in a volume of 500 μ l overnight at 37°C with 200 U of *DpnI*, to cleave the input fully methylated DNAs and hemimethylated DNAs, and 100 U of *XhoI*, to linearize the *oriP* test and prokaryotic backbone plasmids. The next day, an additional 100 U of *DpnI* was added and incubated overnight. Samples were extracted with phenol-chloroform, ethanol precipitated, and resuspended at 5×10^4 cell equivalents per μ l in TE for analysis by quantitative competitive PCR or resuspended at 5×10^5 cell equivalents per μ l in TE for analysis by Southern blotting.

(ii) **Preparation of competitor DNA.** Twenty micrograms of 2264 was linearized with *MluI* for 2 h at 37°C, extracted with phenol-chloroform, ethanol precipitated, and resuspended in TE. This DNA was resolved on an agarose gel using 0.5 \times TBE buffer (0.45 M Tris-borate, 0.5 mM EDTA) containing 100 ng of ethidium bromide per ml. Images were captured with a charge-coupled device camera (IS-1000 digital imaging system; Alpha Innotech Corporation) and analyzed with ImageQuant software (Molecular Dynamics). The amount of linearized 2264 was determined by comparison to known quantities of lambda *HindIII* and phi X *HaeIII* DNA markers run in parallel.

(iii) **PCR assay and data analysis.** A modified quantitative competitive PCR assay (19) was used to measure the amount of replicated, *DpnI*-resistant plasmid DNA present at various times posttransfection. Five PCRs were performed per sample using decreasing amounts of competitor DNA: 9 pg (corresponding to approximately 3.12×10^6 molecules), 3 pg, 600 fg, 120 fg and 24 fg. One hundred thousand cell equivalents of digested, Hirt-extracted DNA was added to a tube containing competitor DNA, 1 \times *Taq* buffer (Roche), 0.2 mM each deoxynucleoside triphosphate, 10 pmol of each primer (0.17 μ M each), and 1.5 U of *Taq* DNA polymerase (Roche) in a total volume of 60 μ l. DNA was amplified by a touch-down protocol in a Hybaid Omn-E Thermocycler for 22 cycles using the following conditions: two cycles of 94°C for 60 s, 60°C for 30 s and 72°C for 75 s; in the remaining cycles, DNA was denatured at 94°C for only 30 s and the annealing temperature was decreased by 1°C every second cycle until it reached 55°C. The primers used included 5'-GATCAAGAGACAGGATGAGGATC G-3' (primer 1588), which lies in the herpes simplex virus thymidine kinase promoter region, and the previously described primer 5'-ACGATTCCGAAGC CCAACCTTCA-3' (primer 86) (19). The sizes of the amplified products generated from the primers are 656 bp for the competitor DNA, 931 bp for the *oriP* test plasmid, and 1,164 bp for the prokaryotic backbone plasmid.

One-third of the PCR mixture was electrophoresed through a 1.2% agarose gel using 0.5 \times TBE buffer containing 100 ng of ethidium bromide per ml. Signals were captured with a charge-coupled device camera (IS-1000 digital imaging system; Alpha Innotech Corporation) and analyzed with ImageQuant software (Molecular Dynamics). The number of molecules of replicated *oriP* test plasmid and prokaryotic backbone plasmid were determined by interpolation between known quantities of competitor DNA. This number was divided by the number of transfected cells analyzed to give the average number of molecules per transfected cell. Bromodeoxyuridine-labeling studies demonstrate that the *oriP* test plasmid is synthesized only once per cell cycle (Leight and Sugden, unpublished observations).

Southern blotting. Approximately 10^7 cell equivalents of linearized, *DpnI*-digested DNA were loaded per lane on a 0.7% agarose gel and electrophoresed in 0.5 \times TBE buffer containing 100 ng of ethidium bromide per ml. The DNA was denatured and transferred to Gene Screen Plus hybridization membrane (NEN Life Sciences) (37). A probe was prepared by nick translation of 1728 (Amersham Pharmacia Biotech nick translation kit N 5000) and hybridized to the membrane. Signals were captured by a PhosphorImager (Molecular Dynamics) and analyzed using ImageQuant software (Molecular Dynamics).

Measurement of cell growth rates. 2446, an *oriP* plasmid expressing luciferase, and 2145, a plasmid expressing EGFP, were cotransfected into 293/EBNA-1 cells by calcium phosphate precipitation (37). At 2 days posttransfection, cells were harvested, the transfection efficiency was determined (as described above), and cells were plated at two cells, one cell, and one-half cell per well onto two 24-well dishes. The cells were allowed to grow in the absence of selection for 14 days, after which the number of wells containing cells was determined. For dishes plated at one cell per well and one-half cell per well, 24 of the 48 wells and 17 of the 48 wells contained cells, respectively. Using the Poisson distribution, we calculated that the colonies present on the dishes plated at one cell per well and one-half cell per well had a 69 and 80% probability, respectively, of arising from a single cell. The colonies on these dishes were triturated and allowed to grow an

additional 3 days (to ensure even cell growth on the dish), after which the number of cells present per well and the luciferase activity present per well were determined as previously described (30).

Measurement of establishment efficiency and isolation of *oriP*-positive cell clones. 1728, an *oriP* plasmid expressing EBNA-1 and hygromycin phosphotransferase, and 2145, a plasmid expressing EGFP, were cotransfected into H1299, 293, and 143B cells as described above. At 2 days posttransfection, the percentage of EGFP-positive cells was determined and cells were plated at 10^5 , 10^4 , and 10^3 cells per 15-cm dish in media containing hygromycin B (H1299, 300 μ g/ml; 293, 200 μ g/ml; 143B, 150 μ g/ml). Selection was applied for 3 to 4 weeks, at which time the drug-resistant colonies were enumerated. Several of these colonies were isolated and expanded in the presence of selection, giving rise to the H1299/1728#3, 293/1728#1, and 293/1728#5 cell clones. *oriP* test and prokaryotic backbone plasmids were then introduced into these cell clones, and replication was monitored over 2 weeks posttransfection as described above.

The cloning efficiency refers to the percentage of cells that are competent to plate and form a colony in the absence of selection and was determined by plating one cell per well onto a 96-well dish and enumerating those wells without cell growth after approximately 2 weeks in the absence of selection. The cloning efficiency was calculated using the Poisson distribution, where $f(0)$, the probability of no cell growth in a well, is equal to e^{-m} (m is the number of viable cells). For example, if 73 of 96 wells plated contain no cell growth, $f(0) = 0.76$, and m therefore equals 0.27. Given that only one cell was plated per well, the cloning efficiency (number of viable cells/number of cells plated) is merely m . These data were compiled to determine the establishment efficiency, which is defined as (no. of drug-resistant colonies/no. of transfected, clonable cells plated) \times 100%.

RESULTS

Replicated *oriP* plasmids are lost precipitously from cells during 2 weeks posttransfection. To further our understanding of the replication of *oriP* plasmids soon after their introduction into human cells, we assayed the pool of replicated *oriP* plasmids in the absence and presence of EBNA-1 in various cell lines during approximately 2 weeks posttransfection. Equimolar amounts of *oriP* test plasmid and prokaryotic backbone plasmid (which serves as an internal negative control) were introduced into H1299, 293, and 143B cells with or without an expression plasmid for EBNA-1. At different time points posttransfection, plasmid DNA was isolated by Hirt extraction and digested exhaustively with *DpnI*, and the level of replicated, *DpnI*-resistant DNA was determined by quantitative competitive PCR (19). Under our conditions, *DpnI* cleaves input methylated plasmid prepared from *dam*⁺ *E. coli* and hemimethylated plasmid which has undergone one round of DNA synthesis in mammalian cells (3). At 4 days posttransfection in a representative experiment with H1299 cells, the level of replicated *oriP* test plasmid detected in the presence of EBNA-1 was 28-fold greater than the level detected in the absence of EBNA-1 (Fig. 1A). This replicated *oriP* test plasmid was lost precipitously during the subsequent 10 days, such that the level of replicated *oriP* test plasmid detected at 14 days posttransfection was less than 1% of the level detected at 4 days posttransfection. That is, the *oriP* test plasmid replicated but was rapidly lost from the majority of cells in the H1299 cell line during 2 weeks posttransfection. Clearly, this loss could reflect inefficient DNA synthesis, inefficient partitioning, or both.

To determine if the precipitous loss of *oriP* plasmids from EBNA-1-expressing cells during 2 weeks posttransfection was peculiar to the H1299 cell line, we monitored the fate of *oriP* test plasmids in the 293 and 143B human cell lines which historically have been used to monitor replication of *oriP* plasmids (35, 52). In a representative experiment with 293 cells at 5 days posttransfection, the replication efficiency of the *oriP* test plasmid increased sixfold when EBNA-1 was provided in

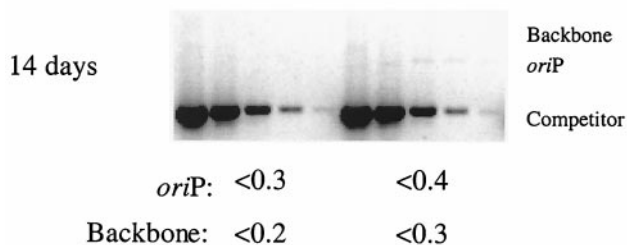
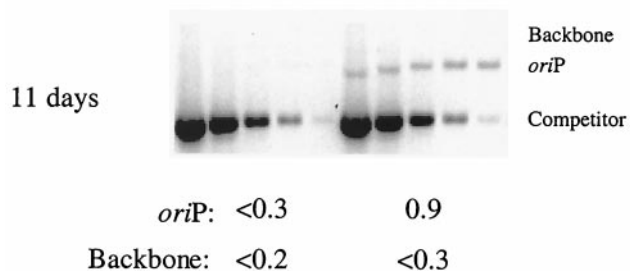
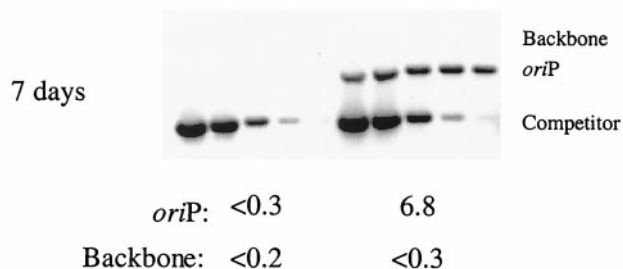
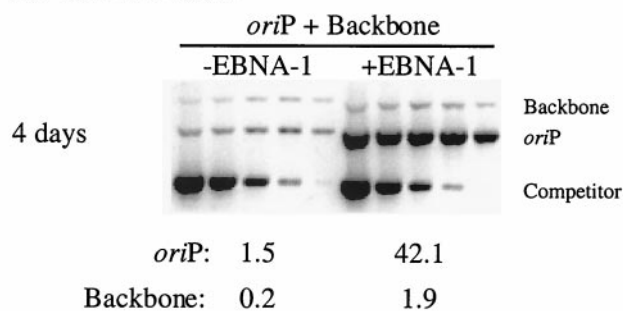
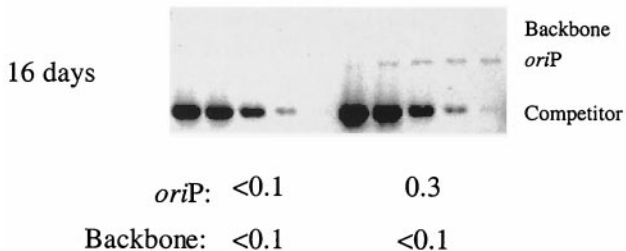
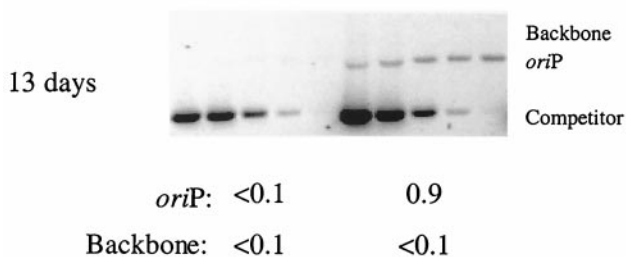
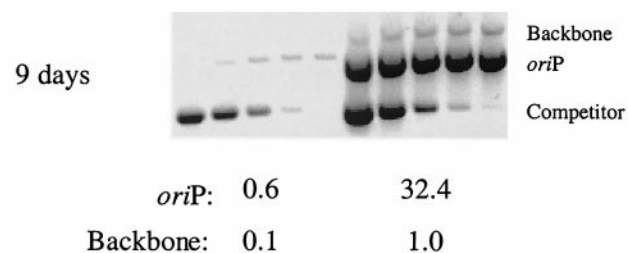
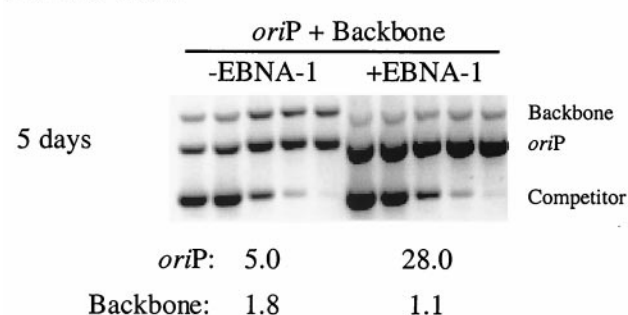
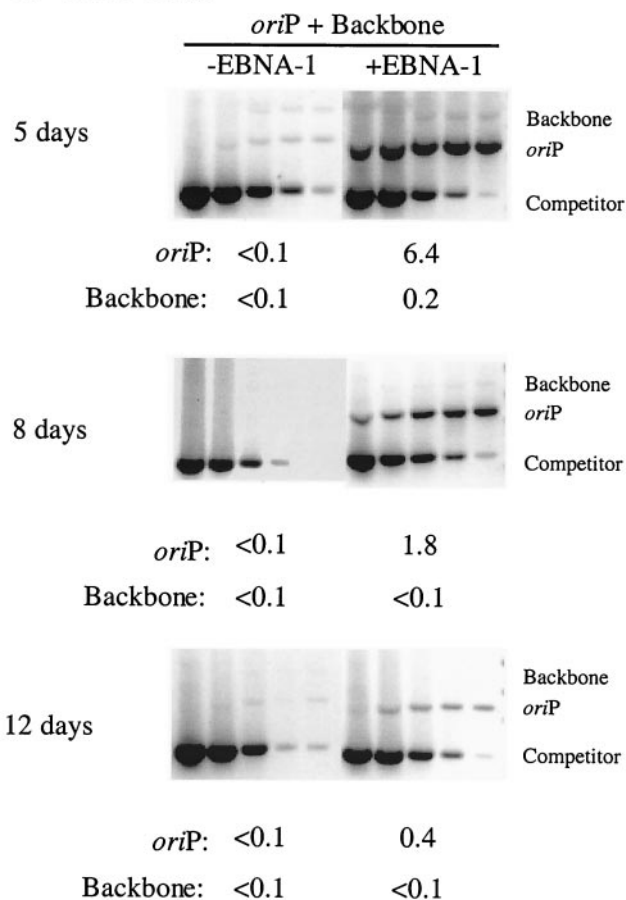
A. H1299 cells**B. 293 cells**

FIG. 1. Newly introduced *oriP* plasmids replicate within EBNA-1-positive cells but are lost precipitously during 2 weeks posttransfection. Equimolar amounts of *oriP* test plasmid (*oriP*) and prokaryotic backbone plasmid (Backbone) were introduced into H1299 (A), 293 (B), and 143B (C) cells with (+EBNA-1) or without (-EBNA-1) an expression plasmid for EBNA-1 and into 293/EBNA-1 cells (D) which stably express EBNA-1. At the indicated time points posttransfection, plasmid DNA was isolated by Hirt extraction and digested with *Xho*I and *Dpn*I, and the level of replicated, *Dpn*I-resistant DNA was determined by quantitative competitive PCR. PCRs were performed using 10^5 cell equivalents and a competitor DNA standard curve (9 pg, 3 pg, 600 fg, 120 fg, and 24 fg). Numbers below gels refer to the average number of molecules present per transfected cell. The 143B time course was conducted in duplicate, and gels were rearranged to present a single experiment. The amount of replicated *oriP* test plasmid present at 6 days posttransfection in 293/EBNA-1 cells was quantified from a separate gel in which 3.3×10^3 cell equivalents were assayed.

trans. (Replication efficiency refers to the average number of *Dpn*I-resistant molecules present per transfected cell and encompasses both DNA synthetic and partitioning events.) This augmentation by EBNA-1 was not as dramatic as that detected in H1299 and 143B cells (compare Fig. 1 A, B, and C at 4 to 5 days posttransfection) and reflects the high permissivity of 293 cells for replication of any introduced DNAs in the absence of EBNA-1 at early time points posttransfection (see also refer-

ence 50). As in H1299 cells, in 293 cells the replicated *oriP* test plasmid detected in the presence of EBNA-1 was lost at such a precipitous rate that the level of replicated *oriP* test plasmid detected at 16 days posttransfection was 1% of the level detected at 5 days posttransfection (Fig. 1B). Furthermore, in a representative experiment with 143B cells, the level of replicated *oriP* test plasmid detected at 5 days posttransfection in the presence of EBNA-1 was 64-fold greater than the level

C. 143B cells



D. 293/EBNA-1 cells

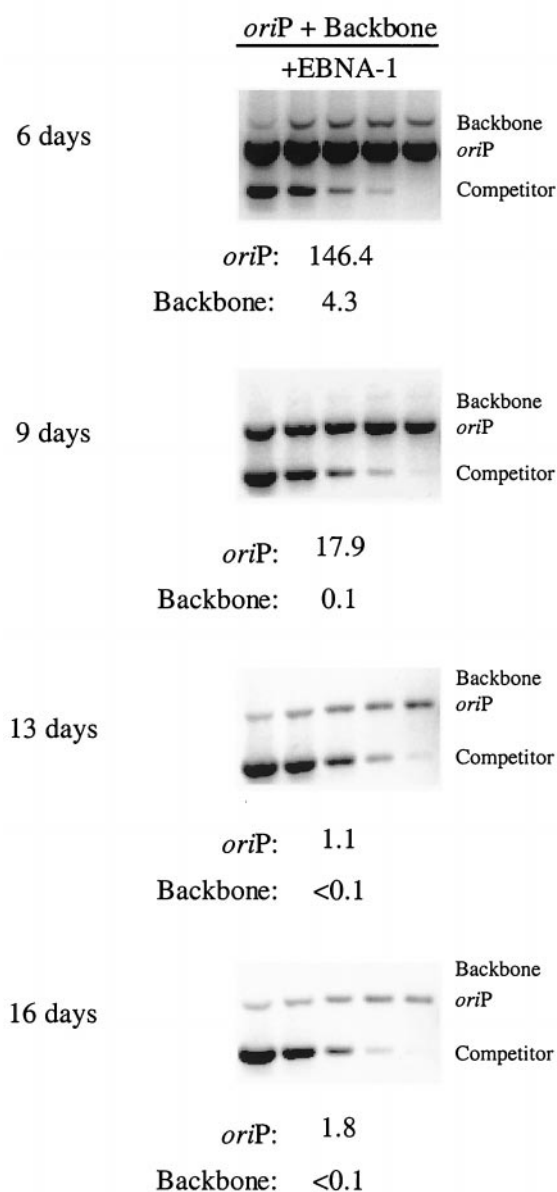


FIG. 1—Continued.

detected in the absence of EBNA-1. However, this replicated *oriP* test plasmid decreased by a factor of sixteen between 5 and 12 days posttransfection even though EBNA-1 was provided in *trans* (Fig. 1C). These experiments demonstrate that in multiple human cell lines an *oriP* plasmid is competent to support replication; however, this replicated DNA is lost rapidly from the majority of the cell population even though EBNA-1 is provided in *trans*.

In the experiments presented in Fig. 1 A, B, and C, EBNA-1 was expressed from an *oriP* plasmid provided in *trans* (1728, an *oriP*/EBNA-1 expression plasmid). We wanted to determine if this plasmid was also lost rapidly, as the loss of this plasmid would result in a loss of EBNA-1 expression that could affect the observations for the *oriP* test plasmid. Equimolar amounts

of the *oriP* test plasmid and prokaryotic backbone plasmid were introduced into 293 cells together with the *oriP*/EBNA-1 expression plasmid, and the level of replicated plasmid present at 5, 9, 13 and 16 days posttransfection was determined by Southern blotting. The *oriP*/EBNA-1 expression plasmid supported replication but was lost precipitously from the population of cells during the time course, as was the *oriP* test plasmid (Fig. 2). The replicated *oriP*/EBNA-1 expression plasmid was lost at the same rate as the replicated *oriP* test plasmid, indicating that declining levels of EBNA-1 could contribute to the loss of these replicated plasmids.

We therefore investigated whether newly introduced *oriP* plasmids were lost precipitously in cell lines which expressed EBNA-1 stably. The *oriP* test plasmid and prokaryotic back-

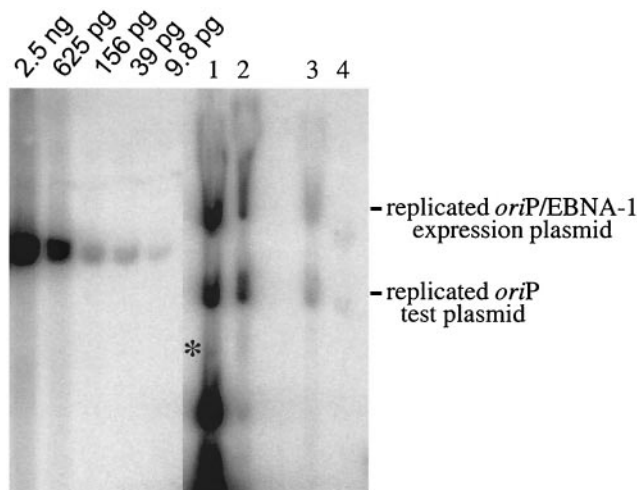


FIG. 2. The *oriP*/EBNA-1 expression plasmid supports replication but is lost precipitously during 2 weeks posttransfection, as is the *oriP* test plasmid. Equimolar amounts of *oriP* test plasmid and prokaryotic backbone plasmid were introduced into 293 cells together with 1728, an *oriP*-based expression plasmid for EBNA-1 (*oriP*/EBNA-1 expression plasmid). At 5, 9, 13, and 16 days posttransfection (lanes 1 to 4, respectively), plasmid DNA was isolated by Hirt extraction and digested with *Xho*I and *Dpn*I and the level of replicated, *Dpn*I-resistant DNA was determined by Southern blotting. Approximately 10^7 cell equivalents were loaded per lane and electrophoresed beside a standard curve of linearized 1728 DNA. The Hirt DNAs ran aberrantly in the gel due to the presence of contaminating chromosomal and mitochondrial DNA. Approximately 5% of chromosomal DNA is present in the Hirt extract (3 μ g per 10^7 cells). The replicated backbone plasmid (indicated by an asterisk) cannot be detected clearly because it migrates with the *Dpn*I-sensitive material near the bottom of the gel.

bone plasmid were introduced into 293/EBNA-1 cells, a cell line which expresses EBNA-1 stably, and the level of replicated plasmid was monitored over 16 days. At 6 days posttransfection in a representative experiment, the level of replicated *oriP* test plasmid was 34-fold greater than the level of replicated prokaryotic backbone plasmid. This replicated *oriP* test plasmid was rapidly lost over the subsequent 10 days such that the level of replicated *oriP* test plasmid present at 16 days posttransfection was 1% of the level detected at 6 days posttransfection (Fig. 1D). This *oriP* test plasmid was also lost rapidly in C33A/EBNA-1 cells, which express EBNA-1 stably, such that the level of replicated plasmid detected at 16 days posttransfection was less than 7% of the level detected at 6 days posttransfection (data not shown). That is, even when EBNA-1 is expressed stably in cells, *oriP* plasmids undergo replication but still are lost precipitously from the majority of the cell population.

A graphical representation of multiple, independent time course experiments conducted with H1299, 293, and 143B cells, in which EBNA-1 was provided in *trans*, and 293/EBNA-1 cells, in which EBNA-1 was expressed stably, is presented in Fig. 3. For each of these cell lines, the loss of *oriP* plasmids from the population of cells over 2 weeks posttransfection varied dramatically from the loss of *oriP* plasmids in an established *oriP*-positive cell clone. That is, in established *oriP*-positive cell clones, *oriP* plasmids are lost at a rate of 2 to 4% per cell generation after removal of selection (20, 47), whereas

during 2 weeks posttransfection, *oriP* plasmids are lost at an average rate of 26 to 37% per cell generation from the majority of the cell population. Why then are *oriP* plasmids stable in the established *oriP*-positive cell clones yet unstable upon introduction into a population of EBNA-1-expressing cells? We examined two general explanations for these disparate experimental observations. First, the precipitous loss of replicated *oriP* plasmids could result from a selective growth advantage of the untransfected cells relative to the transfected cells, such that in the absence of selection for the *oriP* plasmid, the transfected *oriP*-positive cells are overgrown by untransfected cells. Alternatively, given no selective growth advantage of the untransfected cells, our observations would suggest that the establishment of an *oriP* replicon is inefficient such that *oriP* plasmids are replicated efficiently in only a minority of transfected cells.

Precipitous loss of *oriP* plasmids does not result from a selective growth advantage of the untransfected cells relative to the transfected cells. To test the first scenario of varying growth advantages, we measured the proliferation of transfected and untransfected cells as depicted in Fig. 4A. An *oriP* plasmid expressing luciferase and a plasmid expressing EGFP were introduced into 293/EBNA-1 cells, and the transfection efficiency was determined at 2 days posttransfection by calculating the percentage of EGFP-positive cells. Cells were then plated at two cells, one cell, and one-half cell per well onto 24-well dishes and allowed to grow in the absence of selection for 14 days, at which time the number of wells containing cells was enumerated. Based on the Poisson distribution, the colonies present on the dishes plated at one and one-half cell per well had a 69 and 80% probability, respectively, of arising from a single cell. The colonies on these dishes were triturated and allowed to grow an additional 3 days (to allow even growth in the wells), after which the cells present in each well were enumerated and the average luciferase activity present per cell was measured. Luciferase activity serves as an indicator for the presence of *oriP* plasmids. During 17 days, 293/EBNA-1 cells are expected to undergo 20 to 21 doublings, giving rise to 1×10^6 to 2×10^6 cells per well. If the untransfected and transfected cell populations have similar growth kinetics, 1×10^6 to 2×10^6 cells should be present per well and few wells should exhibit high luciferase activity, indicating a loss of *oriP* plasmids from the cell population. Alternatively, if the untransfected cell population has a selective growth advantage, only those untransfected cells should give rise to 1×10^6 to 2×10^6 cells per well after 17 days. In addition, the transfected cells (57% of the population) should give rise to 1 to 2% the number of cells as the untransfected population (based on the 50- to 100-fold decrease in replicated *oriP* plasmids during 2 weeks posttransfection as shown in Fig. 3) and should exhibit high luciferase activity per cell. Our experimental observations are consistent with the former of these two outcomes. The numbers of transfected and untransfected cells per well which accumulated after 17 days were similar, with a variance of only 43% (average of $1.35 \times 10^6 \pm 5.9 \times 10^5$ cells) (Fig. 4B). With respect to luciferase activity, at 2 days posttransfection, an average of 3×10^3 relative light units (RLU) per transfected cell was detected, while 10^{-4} RLU per untransfected cell was measured. After 17 days, $<10^{-3}$ RLU/cell was detected in 80% of the wells, between 10^{-1} and 10^{-2} RLU/cell was detected in

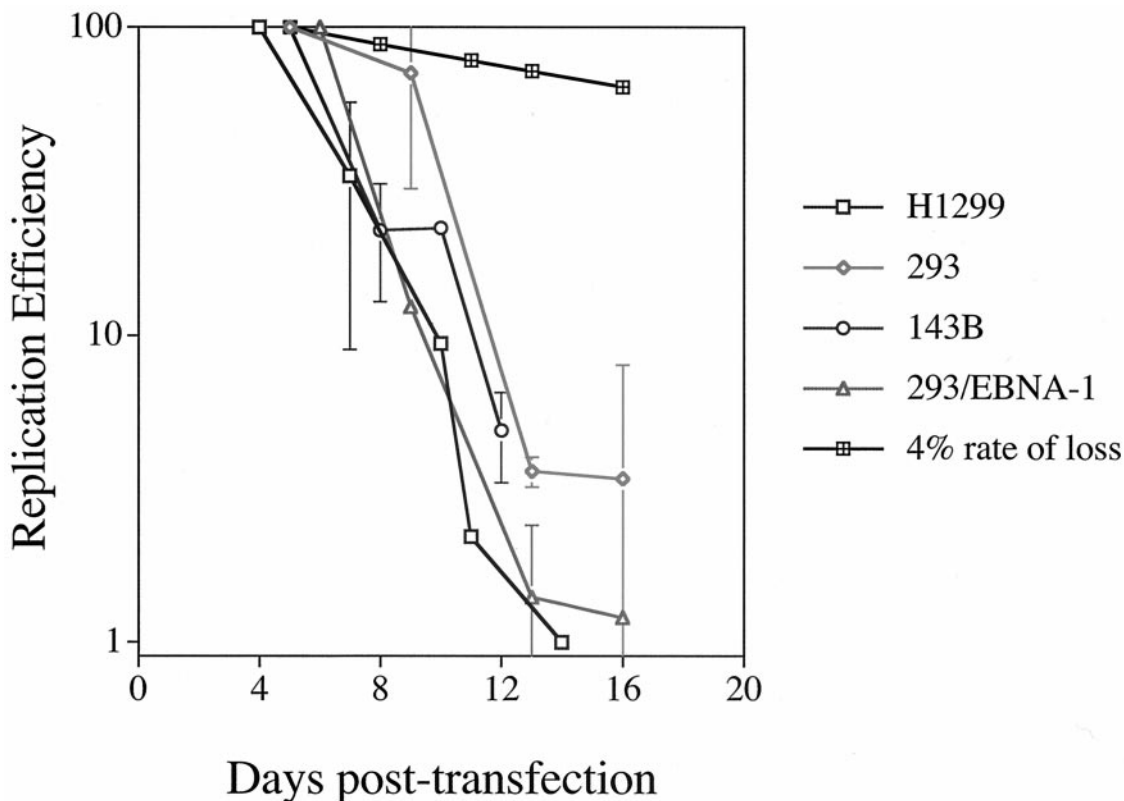
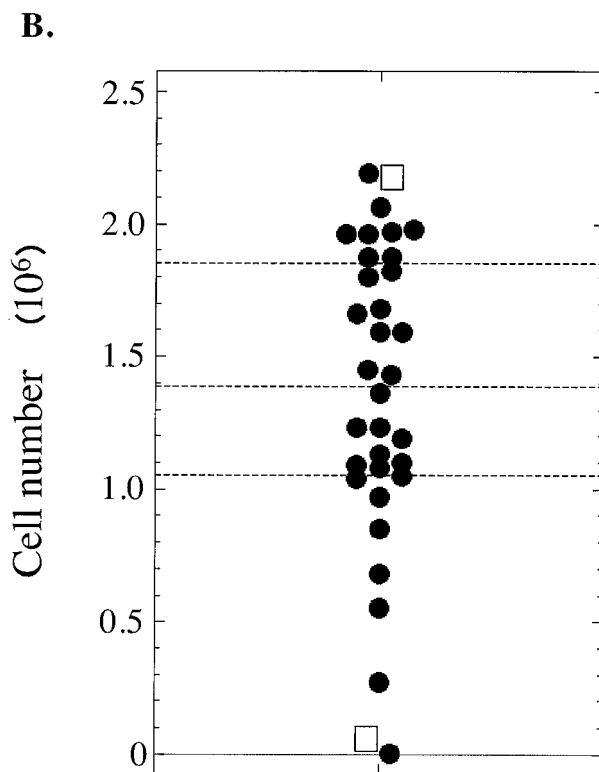
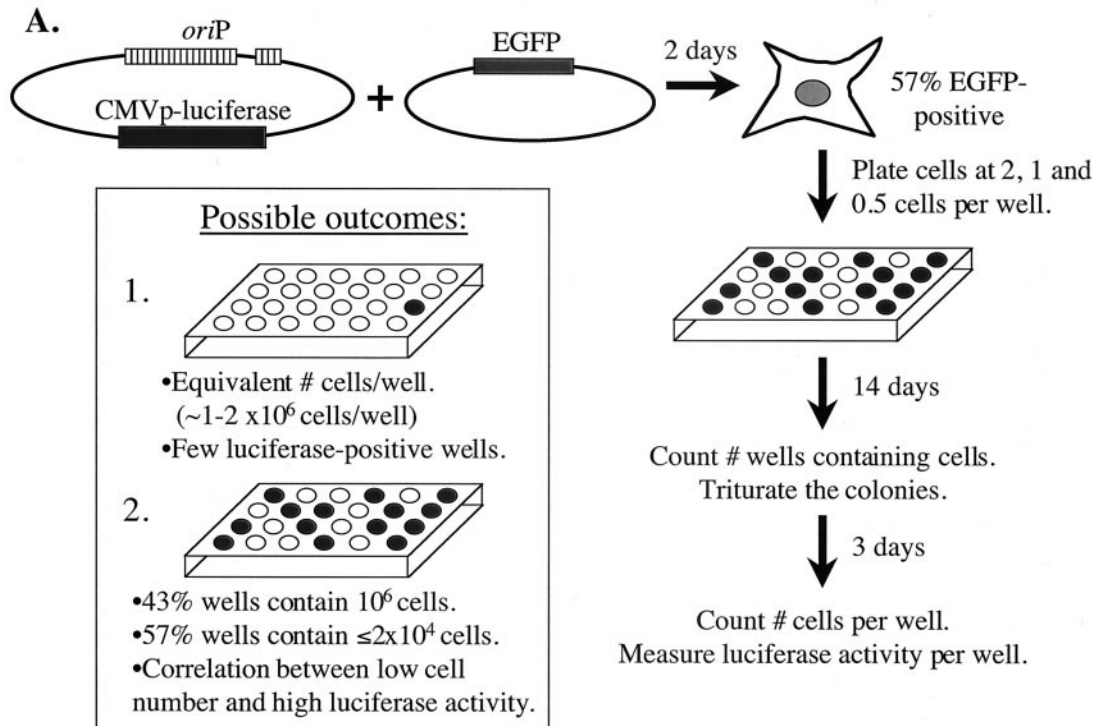


FIG. 3. The loss of *oriP* plasmids from a population of cells during 2 weeks posttransfection varies dramatically from an established *oriP*-positive cell clone. A graphical representation of multiple, independent time course experiments with H1299 (open boxes), 293 (diamonds), and 143B (circles) cells in which EBNA-1 is provided in *trans* and 293/EBNA-1 cells (triangles) in which EBNA-1 is expressed stably is shown. Experiments were conducted as described in the legend to Fig. 1. The replication efficiency is plotted versus the days posttransfection. For each independent experiment, the level of replicated *oriP* test plasmid detected at the first time point posttransfection was set to 100% and the replication efficiency of this plasmid at later time points was set relative to this initial time point. Data for H1299 cells represent the average \pm standard deviation of two experiments, except for the 10-day time point, which represents a single experiment, and the 11- and 14-day time points, which represent a second independent experiment. For 293 cells, data represent the average \pm standard deviation of three experiments, except for the 13-day time point, which represents two experiments. Data for 143B cells represent the average \pm standard deviation of two experiments for the 8- and 12-day time points and a single experiment for the 10- and 16-day time points (16-day data were not plotted but were less than 7% of the replicated *oriP* test plasmid detected at the 5-day time point). For 293/EBNA-1 cells, data represent the average \pm standard deviation of two experiments, except for the 16-day time point, which represents one experiment. A 4% rate of loss, measured for selected cell clones, is depicted by cross-hatched boxes.

17% of the wells, and 2 RLU/cell was detected in the well possessing the highest luciferase activity. That is, the luciferase activity of the transfected cells decreased rapidly during the time course, indicating loss of the *oriP* plasmid. Most importantly, there was no correlation between low cell number and high luciferase activity according to Kendall's rank correlation test (P [two sided] = 0.79). These findings demonstrate that the rapid loss of replicated *oriP* plasmids does not result from a selective growth advantage of the untransfected cells relative to the transfected cells.

Inefficient establishment of *oriP* plasmids in multiple cell lines. That the rapid loss of replicated *oriP* plasmids is not the result of a selective growth disadvantage indicates that the majority of transiently transfected cells support *oriP*'s replication inefficiently. Yet *oriP*-positive cell clones in which *oriP* plasmids are replicated (i.e., synthesized and partitioned faithfully) in 96 to 98% of cell cycles can be selected (20, 47). Given these disparate results, we predicted that the establishment of an *oriP* replicon in human cell lines would be inefficient. To test this prediction, an *oriP* plasmid expressing EBNA-1 and

hygromycin phosphotransferase and a plasmid expressing EGFP were cotransfected into H1299, 293, and 143B cells. At 2 days posttransfection, the percentage of EGFP-positive cells was determined and cells were plated at 10^5 , 10^4 , and 10^3 cells per 15-cm dish in media containing hygromycin B. Selection was applied for 3 to 4 weeks, after which the drug-resistant colonies were enumerated. In addition, the cloning efficiency of each cell line was determined by plating one cell per well onto a 96-well dish and counting those wells with and without cell growth after approximately 2 weeks in the absence of selection. Compilation of these data allowed calculation of the establishment efficiency, which is defined as follows: (no. of drug-resistant colonies/no. of transfected, clonable cells plated) \times 100%. For H1299 and 293 cells, approximately 1% of clonable cells transfected with the *oriP* plasmid established a drug-resistant colony which supported *oriP*'s efficient replication (Table 1 and Fig. 5). In 143B cells, only 6 to 10% of transfected, clonable cells gave rise to drug-resistant colonies. These experiments demonstrate that the establishment of an *oriP* replicon is inefficient in multiple human cell lines. This



inefficient establishment of EBV's *oriP* replicon correlates with the precipitous loss of *oriP* plasmids during 2 weeks posttransfection. Together these observations support a model in which replicated *oriP* plasmids are lost rapidly from the majority of EBNA-1-expressing cells but a minority of transfected cells

FIG. 4. The precipitous loss of newly introduced *oriP* plasmids does not result from a selective growth advantage of the untransfected cell population relative to the transfected cell population. (A) The experiment depicted was conducted as described in Results. Given the 57% transfection efficiency, 14 of every 24 wells (plated at one cell/well) should contain the *oriP* plasmid expressing luciferase, as indicated by dark wells. The inset illustrates two possible outcomes. For outcome 1, with no selective growth advantage of the untransfected cell population, the cells would be expected to undergo 20 to 21 doublings, resulting in 1×10^6 to 2×10^6 cells per well, with approximately 1% of the wells exhibiting high luciferase activity, indicating the loss of *oriP* plasmids from the majority of the cell population. In outcome 2, the untransfected cell population has a selective growth advantage, so only those untransfected cells (43% of the population) would give rise to 1×10^6 to 2×10^6 cells per well after 17 days. In this scenario, the transfected cells (57% of the population) would give rise to $\leq 2 \times 10^4$ cells/well and would exhibit high luciferase activity per cell. (B) Shown is a dot plot depicting the number of cells which accumulated after 17 days from 34 separate wells. The quartiles are indicated by dashed lines. The transfected and untransfected cells which accumulated after 17 days were similar, with a variance of only 43% (average of $1.35 \times 10^6 \pm 5.9 \times 10^5$ cells). Two wells contained few cells, with only 3×10^3 and 5×10^4 cells present. The luciferase activity from each well was normalized to the number of cells present in the well. The two wells containing the highest luciferase activity were indicated by boxes (0.4 RLU/cell for the well containing 2.2×10^6 cells; 2.3 RLU/cell for the well containing 5.3×10^4 cells). According to Kendall's rank correlation test, there is no correlation between low cell number and high luciferase activity (P [two-sided] = 0.79).

supports efficient replication of *oriP* plasmids and gives rise to established, drug-resistant colonies.

Newly introduced *oriP* plasmids replicate but are also lost precipitously in established *oriP*-positive cell clones. Why is a minority of the cell population competent to support efficient replication of *oriP* plasmids but the majority rapidly loses replicated *oriP* plasmids? A heritable alteration in the minority

TABLE 1. Establishment of an *oriP* replicon in multiple cell lines

Cell line	Avg no. of drug-resistant colonies ^a	No. of transfected cells plated (10 ³) ^b	Cloning efficiency ^c (%)	Establishment efficiency ^d (%)
H1299	76	5.0	79	1.9
	93	13		0.9
293	12.5	5.8	31	0.7
	16.5	4.6		1.2
	18 ^e	5.6		1.1
143B	132	2.2	60	9.9
	56.5	1.5		6.2

^a Numbers reflect the average of 2 dilutions of cells plated.

^b Transfection efficiency is based upon the percentage of EGFP-positive cells present at 2 days posttransfection.

^c Cloning efficiency refers to the percentage of cells which are competent to plate and form a colony in the absence of selection and is defined by the variable *m* in the Poisson distribution, as described in Materials and Methods. Percentages are based on two independent experiments.

^d Establishment efficiency refers to the percentage of cells which take up the *oriP* plasmid and support its stable replication, thereby generating a drug-resistant colony. The establishment efficiency is defined by the following equation: (no. of drug-resistant colonies/no. of transfected, clonable cells plated) × 100%.

^e Number reflects a single dilution of cells plated.

of the cell population, manifested at the genetic or epigenetic level, may ensure faithful replication of *oriP* plasmids. Alternatively, a heritable alteration in the newly introduced *oriP* plasmid may be required for its establishment. To distinguish between these possibilities, we determined the fate of newly introduced *oriP* plasmids in drug-resistant cell clones which harbor established *oriP* replicons. If a heritable alteration within a cell clone is responsible for the establishment of *oriP* plasmids, newly introduced *oriP* plasmids should be efficiently replicated within them. However, if modification of the newly introduced *oriP* plasmid is required for its establishment, newly introduced *oriP* plasmids should replicate but should be lost from the majority of cells as they are from other recipients. For this experiment, an *oriP* plasmid expressing EBNA-1 and hygromycin phosphotransferase was introduced into the H1299 and 293 cell lines and hygromycin-resistant cell clones, referred to as H1299/1728#3, 293/1728#1, and 293/1728#5, were isolated. These cell clones maintained 1728 as a plasmid and expressed EBNA-1 functionally (data not shown). Equimolar amounts of *oriP* test plasmid and prokaryotic backbone plasmid were introduced into these cell clones, the clones were grown in the absence of selection for the resident *oriP* plasmid (1728), and the fate of the plasmids was monitored over 2 weeks posttransfection by Southern blotting or quantitative competitive PCR.

A Southern blot analysis was conducted to monitor the fate of the resident 1728 plasmid in comparison to the newly introduced *oriP* test plasmid in the 293/1728#5 cell clone. The level of replicated, resident plasmid 1728 remained stable over the time course, whereas the level of replicated, newly introduced plasmid 2278 decreased by a factor of 12 over the 8-day time period (Fig. 5). In parallel with the Southern blot analysis, the levels of replicated, newly introduced *oriP* test plasmid and prokaryotic backbone plasmid were determined by quantitative competitive PCR. At 5 days posttransfection in the 293/1728#5 cell clone, the level of replicated *oriP* test plasmid was at least 27-fold greater than the level of replicated backbone

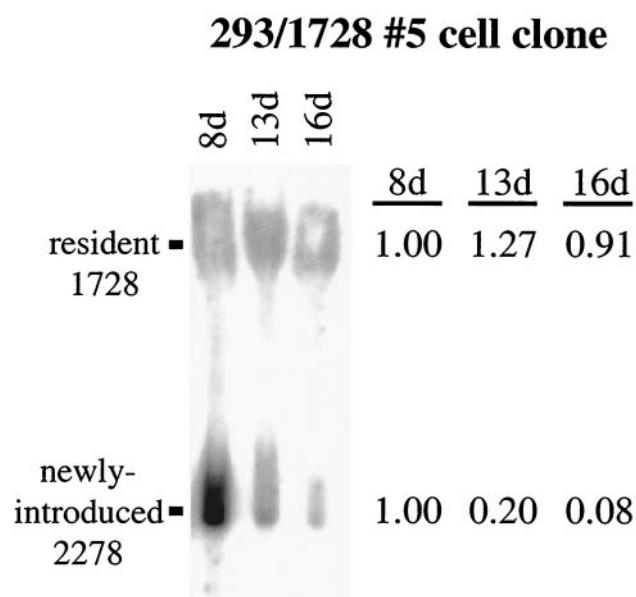


FIG. 5. Replicated, newly introduced *oriP* plasmids are lost precipitously from an established, *oriP*-positive cell clone, while resident *oriP* plasmids are stable. Equimolar amounts of *oriP* test plasmid (2278) and prokaryotic backbone plasmid (2276) were introduced into the established *oriP*-positive cell clone 293/1728#5, which harbors the 1728 *oriP*/EBNA-1 expression plasmid. Plasmid DNA was isolated by Hirt extraction at the indicated time points posttransfection and digested with *Xho*I and *Dpn*I. The levels of replicated, *Dpn*I-resistant 1728 (resident 1728) and 2278 (newly introduced 2278) were determined by Southern blotting. The level of replicated DNA detected at 8 days posttransfection was set to 1 for each of the two plasmids. The Hirt DNAs contain aberrantly in the gel due to the presence of contaminating chromosomal and mitochondrial DNA. Approximately 5% of chromosomal DNA is present in the Hirt extract (3 μ g per 10⁷ cells).

plasmid (Fig. 6A). As also seen by Southern analysis, the replicated, newly introduced *oriP* test plasmid was rapidly lost such that the level of replicated *oriP* test plasmid present at 16 days posttransfection was 10% of the level present at the 5-day time point. The replicated, newly introduced *oriP* test plasmid similarly was lost in the H1299/1728#3 and 293/1728#1 cell clones such that the level of replicated plasmid decreased by factors of 25 and 42, respectively, during a period of 10 days (Fig. 6B), while the resident plasmid 1728 remained stable (data not shown). That is, in multiple established *oriP*-positive cell clones, the newly introduced *oriP* test plasmids supported replication but were lost precipitously while the resident *oriP* plasmids remained stable. These experiments demonstrate that the establishment of *oriP* plasmids within selected *oriP*-positive cell clones is not due to heritable alteration(s) in the host cell clones.

Establishment of an *oriP* replicon is dependent upon its epigenetic modification. Given that establishment of an *oriP* replicon is not due to a heritable alteration in the drug-resistant cell clones, a heritable alteration in the newly introduced *oriP* plasmid must be required for its establishment. Such a heritable modification may be manifested at the genetic or epigenetic level. We therefore investigated whether established cell clones harbor intact *oriP* plasmids in which the DS is of wild-type sequence. (The DS is the site at which DNA

A. 293/1728 #5

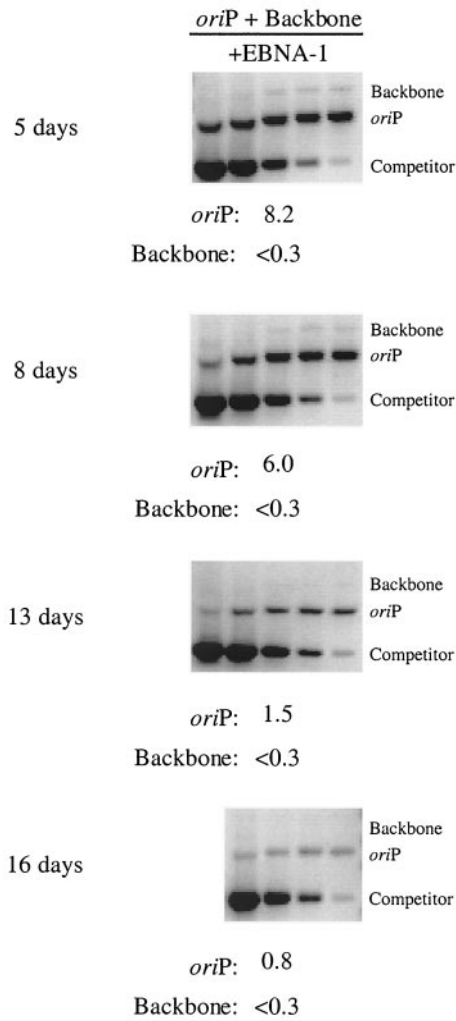
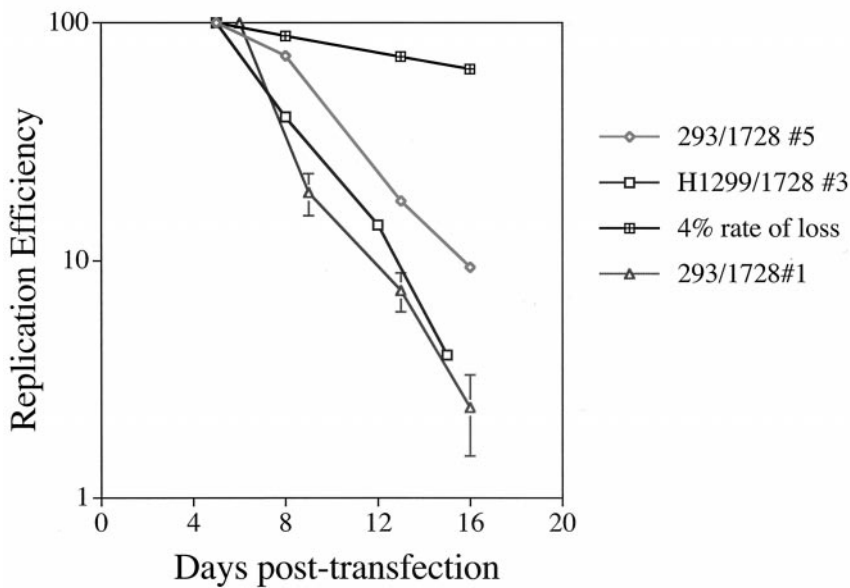


FIG. 6. Newly introduced *oriP* plasmids support replication but are lost precipitously in *oriP*-positive cell clones. (A) The experiment was conducted as described in the legend to Fig. 5. The level of replicated, *DpnI*-resistant *oriP* test plasmid (*oriP*) and prokaryotic backbone plasmid (Backbone) was determined by quantitative competitive PCR. PCRs were performed using 10^5 cell equivalents and a competitor DNA standard curve (9 pg, 3 pg, 600 fg, 120 fg, 24 fg). Numbers below each gel refer to the average number of molecules present per transfected cell. Note that for the 16-day time point, a PCR mixture containing 9 pg of competitor was not analyzed. (B) Graphical representation of time course experiments in the selected, *oriP*-positive cell clones 293/1728#5 (diamonds), H1299/1728#3 (boxes), and 293/1728#1 (triangles). Experiments were conducted as described above. For each independent experiment, the level of replicated *oriP* test plasmid detected at the first time point posttransfection was set to 100%. Data represent a single experiment for the 293/1728#5 and H1299/1728#3 cell clones and three independent experiments for the 293/1728#1 cell clone. A 4% rate of loss is depicted by black cross-hatched boxes.

B.



synthesis initiates within *oriP* [12].) The DS of *oriP* with flanking sequences was PCR amplified from three separate Hirt extractions of the 293/1728#5 cell clone and one Hirt extraction of the H1299/1728#3 cell clone and sequenced. In these cell clones, 1728 is present as an extrachromosomal element at approximately one copy per cell as determined by Southern blotting (data not shown). These 500 nucleotides of *oriP* were wild type in sequence (data not shown). These results are consistent with previous studies in which plasmids containing two copies of *oriP* were rescued from established cell clones and introduced into *E. coli*. Both copies of *oriP* were shown to be intact by restriction endonuclease digestion, and each functioned to support long-term replication (47).

While *oriP* is intact in established cell clones, genetic alteration(s) in the plasmid backbone may promote the replicon's establishment. Hence, we addressed whether *oriP* plasmids isolated from an established cell clone functionally behave as do native *oriP* plasmids. If genetic modification of an *oriP* plasmid were required for its establishment, the plasmids rescued from a drug-resistant cell clone should be established efficiently when reintroduced into mammalian cells. The 293/p220#2 drug-resistant cell clone was utilized for this experiment because it harbors 20 to 50 established p220 replicons per cell (Jun Komano, personal communication). p220 is a derivative of p201, a plasmid used historically to identify EBNA-1 as the sole viral *trans*-acting factor required for the stable replication of *oriP* plasmids (52). p220 contains *oriP*, the beta-lactamase gene and origin of replication from pBR322, and encodes hygromycin B phosphotransferase and EBNA-1. p220 was rescued from the 293/p220#2 established cell clone and introduced into *E. coli*. Plasmid DNA was isolated from a population of approximately 2,000 colonies to ensure representation of the repertoire of established *oriP* plasmids from the cell clone. This rescued p220 DNA was then reintroduced into 293 cells and the establishment efficiency was measured (Table 2). The rescued p220 plasmid population was established in only 5.5% of transfected, clonable cells, in a manner analogous to the establishment of the native p220 plasmid (P [two sided] = 0.44, Wilcoxon rank sum test). The plasmid p220ΔDS, in which the DS of *oriP* was deleted from p220, served as a control plasmid that cannot support efficient, EBNA-1-dependent DNA synthesis. pHEBo, the parental plasmid of p220 that lacks EBNA-1-encoding sequences, was used as a control *oriP* plasmid that cannot support long-term replication in the absence of EBNA-1. The establishment efficiency of these plasmids was significantly different from that of the native p220 plasmid (P [two sided] = 0.04 when each plasmid is compared to p220; Wilcoxon rank sum test). These experiments demonstrate that genetic alteration(s) of an *oriP* plasmid does not contribute to its establishment. Rather, the establishment of an *oriP* replicon is dependent upon an epigenetic event with respect to the replicon, which occurs infrequently and therefore is detected in only a minority of transfected cells.

DISCUSSION

Epigenetic modification of an *oriP* replicon is required for its establishment. We have monitored the fate of *oriP* plasmids from approximately 4 days posttransfection to more than 2

TABLE 2. Establishment of rescued *oriP* plasmid populations in 293 cells

Plasmid	Avg no. of drug-resistant colonies ^a	No. of transfected cells plated (10 ³) ^b	Cloning efficiency (%) ^b	Establishment efficiency (%) ^b
p220	57.5	3.3	31	5.6
	49.0	2.7		5.9
	60.5	3.5		5.6
rescued p220	36.0	2.4	31	4.8
	58.0	2.7		6.9
	67.5	3.4		6.4
	46.0	2.8		5.2
	39.0	3.0		4.2
p220ΔDS	51.0	3.1	31	5.3
	3.0 ^c	3.2		0.3
	3.0 ^c	3.3		0.3
pHEBo	1.0 ^c	3.4	31	0.09
	1.0	3.2		0.1
	3.0	2.9		0.3

^a Numbers reflect the average of 2 dilutions of cells plated.

^b Transfection, cloning, and establishment efficiencies are as defined in footnotes *b*, *c*, and *d* of Table 1.

^c Number reflects a single dilution of cells plated.

weeks posttransfection in the absence and presence of EBNA-1 in multiple cell lines. We have found that the level of replicated *oriP* plasmid present at 4 to 6 days posttransfection in the absence of EBNA-1 is approximately fivefold greater than the level of replicated prokaryotic backbone plasmid, consistent with the findings of Aiyar et al. (3). (These plasmids are isogenic except for the presence of *oriP* and a length polymorphism for distinction by PCR.) However, when EBNA-1 is provided *in trans*, the level of replicated *oriP* plasmid increased up to 64-fold. Unexpectedly, we found that these replicated *oriP* plasmids were lost at a rate of approximately 30% per cell generation during the time course in cell lines in which an *oriP* plasmid expressing EBNA-1 was cointroduced (H1299, 293, and 143) and in cell lines in which EBNA-1 was expressed stably (293/EBNA-1 and C33A/EBNA-1). This precipitous loss of *oriP* plasmids from recipient cells was not due to a growth disadvantage of the transfected cells, as equivalent numbers of transfected and untransfected cells accumulated after 17 days in culture. The rapid loss of *oriP* plasmids from the transiently transfected cell population contrasts with the loss of *oriP* plasmids from established cell clones, in which *oriP* plasmids are lost at a rate of 2 to 4% per cell generation after removal of selection (20, 47). These discrepant findings indicated that only a fraction of cells transfected with an *oriP* plasmid might support its efficient replication. Indeed, we found that only 1 to 10% of those cells transfected with an *oriP* plasmid expressing EBNA-1 and hygromycin phosphotransferase gave rise to drug-resistant clones in which an *oriP* replicon was established. When *oriP* plasmids were introduced into established *oriP*-positive cell clones, the newly introduced *oriP* plasmids supported replication but were lost precipitously while the resident *oriP* plasmids were stable. This experiment demonstrates that a heritable alteration in the cell clones is not responsible

for establishment of an *oriP* replicon. In addition, genetic alteration(s) of the plasmid does not promote the replicon's establishment. Rather, our experiments reveal that an infrequent, epigenetic event is required for the establishment of an *oriP* replicon. This epigenetic event must allow efficient DNA synthesis and/or partitioning, perhaps in a manner analogous to one of the epigenetic events required for origin function in *S. cerevisiae* and for faithful partitioning by *CEN* elements in *S. pombe* (11, 14, 41, 42). By defining this event, we will gain insight into the regulatory events involved in the stable replication of *oriP* plasmids, and perhaps into those events essential for replication fidelity in mammalian cells.

Why is an epigenetic event required for the establishment of a stable *oriP* replicon? The study of plasmids containing derivatives of *oriP* has provided some clues. *oriP* is composed of two *cis*-acting elements—the family of repeats (FR) and the DS. The FR, which contains 20 imperfect 30-bp repeats recognized by EBNA-1 dimers (5, 33), is thought to contribute to the partitioning of plasmids. For example, plasmids containing the FR and a putative chromosomal or viral origin support long-term replication in EBNA-1-expressing cells (21, 24). The DS contains four EBNA-1 binding sites of lower affinity (5, 33) and is the site at or near which DNA synthesis initiates (12). While both the FR and DS are required for efficient replication in some cell lines (35), we and others have found that plasmids containing only the DS of *oriP* support replication as efficiently as *oriP* plasmids at 2 to 4 days posttransfection in 293, HeLa, and C33A cells when EBNA-1 is provided in *trans* (15, 38, 50; Leight and Sugden, unpublished observations). These findings indicate that the DS is competent to support DNA synthetic and partitioning functions in some cell lines. We therefore monitored the replication of plasmids containing only the DS over 2 weeks posttransfection in 293/EBNA-1 cells that stably express EBNA-1. Surprisingly, we observed that while replicated *oriP* plasmids were lost precipitously, the replicated DS plasmids were stable during the time course and were present at greater than 100 copies per transfected cell, indicative of efficient synthesis and partitioning each cell generation (E. Leight and B. Sugden, unpublished data). That is, DS plasmids are established efficiently in some cell lines. Based on these findings, we propose that an epigenetic event is required to overcome an inhibitory activity of the FR of *oriP*. An epigenetic event may modulate the timing of origin firing to ensure completion of DNA synthesis despite the presence of a replication fork barrier imposed by EBNA-1 dimers bound to the FR (9, 12). Alternatively, an epigenetic event may promote efficient partitioning of *oriP* plasmids by favoring the formation of intermolecular interactions between *oriP* and mitotic chromosomes to the formation of intramolecular interactions (i.e., looping) between EBNA-1 dimers bound to the FR and DS (27, 28, 40, 44). Given that global methylation has no effect on the establishment of an *oriP* replicon (B. Sugden, unpublished observations), we favor a mechanism in which chromatin structure, as opposed to covalent modification, affects the replication efficiency of *oriP* plasmids.

Implications for studies of EBV. We have found that *oriP* plasmids are not intrinsically stable in transfected cells but rather must be modified epigenetically in order to support efficient DNA synthesis and partitioning each cell cycle. But is such modification required for the establishment of EBV it-

self? Two lines of evidence demonstrate that EBV DNA is not intrinsically stable in newly infected cells, indicating that an epigenetic event is required for the establishment of EBV's genome. Firstly, Reisman and Sugden have shown that upon infection of the EBV-positive B-lymphoblast cell line TG8 with the B95-8 strain of EBV, 85% of the superinfecting EBV DNA is lost over 7 days, while the resident EBV DNA (P3HR1) remains stable (34). The B95-8 strain of EBV established a transient infection in these experiments in that 20 to 30% of the B95-8 genomes were circularized by 2 days postinfection. (This circularized DNA was undetectable by 7 days postinfection.) In addition, while EBNA-1 is undetectable in TG8 cells, 60% of the cells were EBNA-1 positive at 2 days postinfection with the B95-8 strain of EBV. By 10 to 24 days postinfection, only 1% of cells remained EBNA-1 positive. The findings of Reisman and Sugden (34) mirror the precipitous loss of *oriP* plasmids observed in our studies and indicate that inefficient establishment occurs upon viral infection as well as transfection of an *oriP* replicon.

A second line of evidence demonstrates that EBV is established inefficiently in cells, as are other *oriP* replicons. In these studies a recombinant Akata strain of EBV (carrying the neomycin resistance gene) was used to infect epithelial and B-cell lines. The infection efficiency was monitored by detection of EBNA-1 at 3 to 5 days postinfection, and the resulting drug-resistant colonies were enumerated. The establishment efficiency of 15 epithelial cell lines ranged from 0.06 to 14.4%, with an average of 4.0% (18). Likewise, when the recombinant Akata strain of EBV was used to infect an EBV-negative Burkitt's lymphoma cell line (Akata), 1% of infected cells gave rise to drug-resistant, EBV-positive cell clones (48). These observations with infecting EBV are consistent with our data demonstrating an inefficient establishment of transfected *oriP* plasmids and indicate that establishment of EBV DNA may be dependent upon its epigenetic modification.

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