

The *cis*-Acting Family of Repeats Can Inhibit as well as Stimulate Establishment of an *oriP* Replicon

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Previously we have shown that the establishment of an *oriP* replicon is dependent on its epigenetic modification, which occurs in only 1 to 10% of proliferating cells (E. R. Leight and B. Sugden, *Mol. Cell. Biol.* 21: 4149–4161, 2001). To gain insights into the *cis*-acting requirements for the establishment of *oriP* replicons, we monitored the replication of *oriP* plasmid derivatives for several weeks following their introduction into cells. In EBNA-1-positive 143B and H1299 cells, plasmids containing only the region of dyad symmetry (DS) of *oriP* replicated but were lost more rapidly from cells than were *oriP* plasmids, demonstrating that the family of repeats (FR) of *oriP* acts in *cis* to stimulate replication in these cells. Unexpectedly, we found that the DS plasmid was established efficiently in 293/EBNA-1 cells, being lost at a rate of only 8% per cell generation over 24 days posttransfection. However, plasmids containing the FR in addition to the DS of *oriP* replicated but were lost at a rate of approximately 30% per cell generation in 293/EBNA-1 cells, indicating that the FR inhibits *oriP*'s establishment in this cell line. FR's enhancement of transcription of a promoter in *cis* and FR's ability to inhibit replication fork movement do not account solely for *oriP*'s inefficient establishment. In addition, DNA looping between FR and DS neither stimulates nor inhibits replication. Deletion of 11 EBNA-1 binding sites in the FR or replacement of the FR with DS sequences, however, does overcome the inhibitory activity of the FR, thereby allowing efficient establishment of the *oriP* derivative in 293/EBNA-1 cells.

Epstein-Barr virus (EBV) is a gammaherpesvirus that causes infectious mononucleosis (12, 42) and is associated with several malignancies, including Hodgkin's disease, gastric carcinoma, and B-cell lymphomas in immunocompromised individuals (9, 18, 23, 26, 51). EBV also has been found to contribute causally to Burkitt's lymphoma and nasopharyngeal carcinoma in prospective epidemiological surveys (10, 17, 31). EBV's ability to induce and maintain proliferation of the B cells it infects likely underlies its contribution to these malignancies. Within these latently infected cells, the viral genome is present as a circular plasmid that is synthesized only once per cell cycle during S-phase (1, 61) and is efficiently partitioned to daughter cells (29, 36, 55, 56), akin to the genome of its host cell. We use the term replication to encompass these DNA synthesis and partitioning events. Only two viral components are required for the replication of the EBV genome, the latent origin *oriP* and its binding protein EBNA-1; all else is contributed by the cell (40, 59, 62).

oriP is composed of two *cis*-acting elements, the family of repeats (FR) and the region of dyad symmetry (DS), which are separated by approximately 1 kbp of DNA. The FR contains 20 imperfect copies of a 30-bp repeat to which EBNA-1 binds site specifically and with high affinity (5, 6, 44). The DS, which contains four low-affinity EBNA-1-binding sites, is the site at or near which DNA synthesis initiates (15). The FR, in con-

junction with EBNA-1, is thought to ensure faithful partitioning (or maintenance) of *oriP* replicons. This supposition is based partially on the findings that (i) plasmids lacking 14 or more EBNA-1 binding sites in the FR of *oriP* fail to support long-term replication in D98/Raji and Raji cells (8, 47, 57) and (ii) addition of the FR to a plasmid containing mammalian or viral autonomously replicating sequences allows long-term replication in EBNA-1-positive cells (30, 34).

Plasmids containing *oriP* support efficient replication in EBNA-1-positive cells selected to retain them, being lost at a rate of 2 to 4% per cell generation after removal of selection (29, 56), a rate of loss which resembles that of *ARS/CEN* plasmids in *Saccharomyces cerevisiae* (33). 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) (37). Upon investigation of these disparate observations, we have found that *oriP* replicons must be modified epigenetically for establishment, and this modification occurs in only 1 to 10% of transfected cells (37). Our observations of *oriP* plasmids are consistent with studies on EBV that demonstrate that EBV DNA is lost rapidly from proliferating cells following infection (45) and is established in approximately 1% of infected cells (27, 58).

We wished to gain insights into the *cis*-acting requirements for the establishment of an *oriP* replicon in different cell lines. We therefore monitored the replication levels of *oriP* plasmid derivatives in multiple EBNA-1-positive cell lines over several weeks following their introduction into cells. Here we report that cell lines differ in their ability to support the replication of

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a plasmid containing only the DS of *oriP*. In 143B and H1299 cells that express EBNA-1, DS plasmids support replication, albeit inefficiently, and are lost more rapidly from cells than are *oriP* plasmids. That is, EBNA-1-binding sites within the FR clearly stimulate replication in 143B and H1299 cells. Unexpectedly, however, plasmids containing only the DS of *oriP* were established efficiently in 293/EBNA-1 cells, providing efficient DNA synthetic and partitioning functions over 24 days posttransfection. Plasmids containing the FR in addition to the DS supported replication but were lost precipitously in 293/EBNA-1 cells and all other cell lines analyzed (27, 37, 45, 58), indicating that the FR inhibits establishment of *oriP* replicons in 293/EBNA-1 cells and likely inhibits establishment of *oriP* replicons in all other cell lines. We show that this inefficient establishment of *oriP* is not due to FR's enhancement of transcription from the *oriP* replicon or to DNA looping between the FR and DS.

In addition, the FR's ability to act as a replication fork barrier does not solely underlie the inefficient establishment of *oriP* replicons. Deletion of EBNA-1 binding sites within the FR or substitution of the FR with DS sequences, however, overcame the inhibitory activity of the FR, allowing efficient establishment of the *oriP* plasmid derivative in 293/EBNA-1 cells. Our findings indicate that the FR of *oriP* possesses inhibitory as well as stimulatory activities with respect to replication. By defining the mechanism by which an epigenetic event overcomes the inhibitory activity of the FR, therapeutic agents may be developed to prevent the establishment of EBV's plasmid replicon.

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 *Escherichia 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, generating a length polymorphism upon amplification with primers 1588 and 86, as described below. 2402.7, the test plasmid containing only the DS of *oriP*, was constructed by inserting the DS between the *EcoRV* and *Sall* sites of 2275. The test plasmid lacking the DS of *oriP*, 2331, was constructed by deleting DS from 2278 by digestion with *EcoRV* and religation.

The Δ TKp plasmid 2619 was constructed by addition of a *BglII* linker at the *EcoRI* site of 2278, followed by destruction of the herpes simplex virus thymidine kinase promoter by digestion with *BglII* and religation. 2278 was digested with *BstXI* and religated to remove the right-hand 11 EBNA-1 binding sites in the FR and 353 nucleotides between the FR and DS (nucleotides 7708 to 8378 of the B95-8 strain of EBV), generating plasmid 2617 (9FR+DS). Three EBNA-1-binding sites from the FR (nucleotides 7458 to 7548 of the B95-8 strain of EBV) were introduced between the *Bpu10I* and *XbaI* sites of 2278 to construct plasmid 2777 (3FR+DS). The DS was introduced between the *Bpu10I* and *Sall* sites of 2278 in order to replace the FR of *oriP*, generating plasmid 2776 (DS+DS).

Gerber et al. have defined a minimal *cis*-acting sequence within the murine ribosomal DNA (rDNA) locus (+642 to +747 of this locus) that is sufficient for inhibiting replication fork movement in vitro when HeLa protein extract is provided (16). This *cis*-acting sequence was introduced between the *Bpu10I* and *XbaI* sites of 2278 to construct plasmid 2785 (RFB→DS), in which RFB→ refers to the replication fork barrier (RFB) in the +642 to +747 orientation. Given that the rDNA RFB is orientation dependent, whereas the FR is not (11, 16), an additional rDNA RFB was introduced between the *BamHI* and *HindIII* sites of 2785 to generate plasmid 2789 (RFB→DS←RFB).

Plasmid 2775 (FR*DS) was constructed by inserting the DS between the *Bpu10I* and *EcoRV* sites of 2331. The DS was inserted between the *Bpu10I* and *EcoRV* sites of 2777 to generate plasmid 2784 (3FR*DS).

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 (CMV) promoter and which 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, so that amplification by PCR yields a 656-bp product.

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 143B, a human osteosarcoma cell line (ATCC CRL-8303); C33A/EBNA-1, a human cervical carcinoma cell line (ATCC HTB-31) into which plasmid 1553, which expresses EBNA-1 and hygromycin B phosphotransferase, was integrated (2); H1299/1728#3, a p53-null human lung carcinoma cell line (ATCC CRL-5803) that maintains the 1728 *oriP*/EBNA-1 expression plasmid (37); 293/EBNA-1, a human embryonic kidney cell line that stably expresses EBNA-1 and neomycin phosphotransferase (ATCC CRL 10852); and the 293/1728#5 cell clone, which was selected to stably replicate the 1728 *oriP*/EBNA-1 expression plasmid (37). Cell lines were grown in Dulbecco's modified Eagle's medium with high glucose and supplemented with 10% fetal bovine serum and 200 U of penicillin and 200 μ g of streptomycin sulfate per ml. 143B cells were grown in medium containing calf serum instead of fetal bovine serum. 293/EBNA-1, C33A/EBNA-1, H1299/1728#3, and 293/1728#5 cell lines were also grown in the presence of G418 sulfate (200 μ g/ml) and 100, 300, and 200 μ g of hygromycin B/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 test plasmid (10 μ g of *oriP* plasmid) and prokaryotic backbone plasmid (6.5 μ g), 5 μ g of 2145, an expression vector for EGFP, and 10 μ g of 1728, an *oriP*-based EBNA-1 expression plasmid, or 10 μ g of 2048, an empty expression plasmid, were prepared and placed onto 15-cm dishes containing adherent cell lines (48). The 1728 and 2048 plasmids were not introduced into C33A/EBNA-1, H1299/1728#3, and 293/EBNA-1 cell lines. H1299/1728#3 cells were suspended by trypsinization, mixed with the precipitate, and plated onto a 15-cm dish. Medium was changed 5 to 8 h after addition of the precipitate.

DNAs were introduced into 143B cells by electroporation (32). At 2 days posttransfection, cells were harvested, and the percent EGFP-positive cells was enumerated 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 [34, 41].) 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. This assay allows us to track the fate of replicated plasmids in a population of cells in the absence of selection. We have shown previously that the growth rates of the untransfected and transfected cells are indistinguishable (37).

Quantitative competitive PCR assay. At the indicated time points posttransfection, cells were harvested, and low-molecular-weight DNA was extracted by the method of Hirt (25) and prepared as described (37).

A modified quantitative competitive PCR assay (28) 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. (The competitor DNA was linearized and quantified as described [37].) A total of 10^5 cell equivalents of digested, Hirt-extracted DNA were added to a tube containing competitor DNA, $1 \times$ *Taq* buffer (Roche), 0.2 mM each of a mix of deoxynucleoside triphosphates, 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 touchdown protocol in a Hybad Omn-E thermocycler for 22 cycles using the following conditions: 94°C for 60 s, 60°C for 30 s, and 72°C for 75 s for two cycles; in the remaining cycles, DNA was denatured at 94°C for only 30 s and the annealing temperature decreased by 1°C every second cycle until reaching 55°C. The primers used included 5'-GATCA AGAGACAGGATGAGGATCG-3' (primer 1588), which lies in the herpes simplex virus thymidine kinase promoter region, and the previously described primer 5'-ACGATTCCGAAGCCCAACCTTTCA-3' (primer 86) (28). 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 was electrophoresed through a 1.2% agarose gel using $0.5 \times$ TBE (Tris-borate-EDTA) buffer containing ethidium bromide (100 ng/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 was 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.

Cesium chloride density gradient analysis. Equimolar amounts of an *oriP* plasmid (10 μ g of *oriP*) and a plasmid containing only the DS of *oriP* (6.5 μ g of DS) were introduced independently into 293/EBNA-1 cells together with 5 μ g of 2145 (an expression vector for EGFP). At 2 days posttransfection, cells were harvested and the percent EGFP-positive cells was enumerated as a measure of the transfection efficiency. For each transfection, approximately 50% of the cells were EGFP positive. The cells were expanded on 15-cm dishes and labeled with 100 μ M bromodeoxyuridine and 200 μ M deoxycytidine for 17 h at 5 days posttransfection (61). Plasmid DNA was isolated by Hirt extraction of approximately 5×10^7 cells (as described above), linearized with *Xho*I, and mixed with CsCl to a density of 1.74 g/ml in a 5-ml volume (61). These samples were spun at 60,000 rpm for 19 h in a Beckman type NVT 65.2 rotor and fractionated into 200- μ l aliquots, and the refractive index of every fourth fraction was determined. One fourth of each fraction was denatured, transferred to Gene Screen Plus hybridization membrane (NEN Life Sciences) via dot-blotting (Schleicher and Schuell Minifold I), and hybridized to labeled DNAs encompassing the neomycin phosphotransferase gene. Signals were captured with a phosphorimager and analyzed with ImageQuant software (Molecular Dynamics).

RESULTS

Plasmids containing only DS of *oriP* support replication with various efficiencies in different cell lines. To gain insight into the *cis*-acting requirements for the establishment of an *oriP* replicon in different cell lines, we monitored the replication levels of *oriP* plasmid derivatives containing wild-type *oriP* (*oriP*), only the DS of *oriP* (DS), and only the FR of *oriP* (FR) in multiple EBNA-1-positive cell lines at early times posttransfection. The fate of these replicated plasmids was then monitored over 2 weeks following their introduction into cells. To do this, equimolar amounts of a test plasmid (*oriP*, DS, or FR) and prokaryotic backbone plasmid (which serves as an internal negative control) were introduced into 143B cells with or without an expression plasmid for EBNA-1 or into C33A/EBNA-1, H1299/1728#3, and 293/EBNA-1 cells that stably express EBNA-1. Cells were grown in the absence of selection. At different times following transfection, plasmid DNA was isolated by Hirt extraction and digested exhaustively with *Dpn*I, and the level of replicated, *Dpn*I-resistant DNA was determined by quantitative competitive PCR (28).

Under our conditions *Dpn*I cleaves input methylated plasmid prepared from *dam*⁺ *E. coli* and hemimethylated plasmid which has undergone one round of DNA synthesis in mammalian cells (3). In a representative experiment in 143B cells, the level of replicated *oriP* test plasmid detected at 4 days posttransfection in the presence of EBNA-1 was greater than 38 times the level detected in the absence of EBNA-1 (Fig. 1). The plasmid containing only the DS of *oriP* supported EBNA-1-dependent replication, albeit at 13% of the efficiency of the *oriP* plasmid. This replicated DS plasmid was undetectable (<0.1 molecule per cell) by 7 days posttransfection (data not shown). These experiments demonstrate that the DS of *oriP* is not sufficient for efficient replication in 143B cells and indicate that the EBNA-1-binding sites within the FR stimulate replication by promoting DNA synthesis at the DS and/or by maintaining the newly synthesized plasmid. Even though the FR is present in *cis*, replicated *oriP* plasmids are lost precipitously from the population of transiently transfected 143B cells and

143B cells

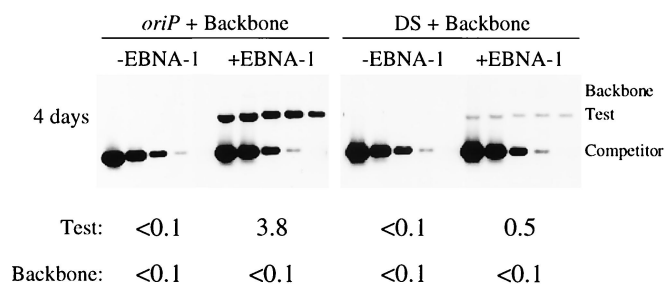


FIG 1. Plasmid containing only the DS of *oriP* supports EBNA-1-dependent replication, albeit inefficiently, at 4 days posttransfection in 143B cells. A test plasmid containing *oriP* (*oriP*) or only the DS of *oriP* (DS) was introduced into 143B cells together with an equimolar amount of prokaryotic backbone plasmid (Backbone) either with (+EBNA-1) or without (-EBNA-1) an expression plasmid for EBNA-1. At 4 days 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 6×10^5 cell equivalents and a competitor DNA standard curve (9 pg, 3 pg, 600 fg, 120 fg, and 24 fg). Numbers below each gel refer to the average number of molecules present per transfected cell.

are established in only 6 to 10% of these successfully transfected cells (37).

To determine if the DS plasmid supports inefficient replication in other cell lines, we monitored its replication in parallel to the *oriP* plasmid in three cell lines that stably express EBNA-1 (C33A/EBNA-1, H1299/1728#3, and 293/EBNA-1 cell lines). In C33A/EBNA-1 cells, as shown in a representative experiment (Fig. 2), the DS plasmid supports replication as efficiently as the *oriP* plasmid at 6 days posttransfection (Wilcoxon rank sum test: P [two-sided] = 0.28; $n = 3$), whereas a plasmid containing only the FR of *oriP* supports replication with a decreased efficiency compared to the *oriP* plasmid (Wilcoxon rank sum test: p [one-sided] = 0.04; $n = 2$). The replicated DS plasmid pool was lost precipitously from the cell population, so that the level of replicated DNA detected at 16 days posttransfection was 4% of the level detected at 6 days posttransfection. This rapid loss of replicated DS plasmids is analogous to the loss of *oriP* plasmids from the majority of transiently transfected cells (37), and is indicative of the inefficient establishment of these replicons.

In the H1299/1728#3 cell line, the DS plasmid likewise supported replication with a similar efficiency as the *oriP* plasmid at 5 days posttransfection. However, the level of replicated DS plasmid decreased by a factor of greater than 80 between 5 and 15 days posttransfection, whereas the level of replicated *oriP* plasmid decreased by a factor of only 25 during this time (data not shown). That is, in both the C33A/EBNA-1 and H1299/1728#3 cell lines, plasmids containing only the DS of *oriP* replicate but are lost precipitously from transfected cells, as are *oriP* plasmids. This precipitous loss of the replicated DS plasmid population could result from inefficient synthesis, inefficient maintenance, or both.

We and others have observed that plasmids containing only the DS of *oriP* support replication as efficiently as *oriP* plasmids at 2 to 4 days posttransfection in 293 cells which harbor EBNA-1 (37, 60). We therefore monitored the fate of repli-

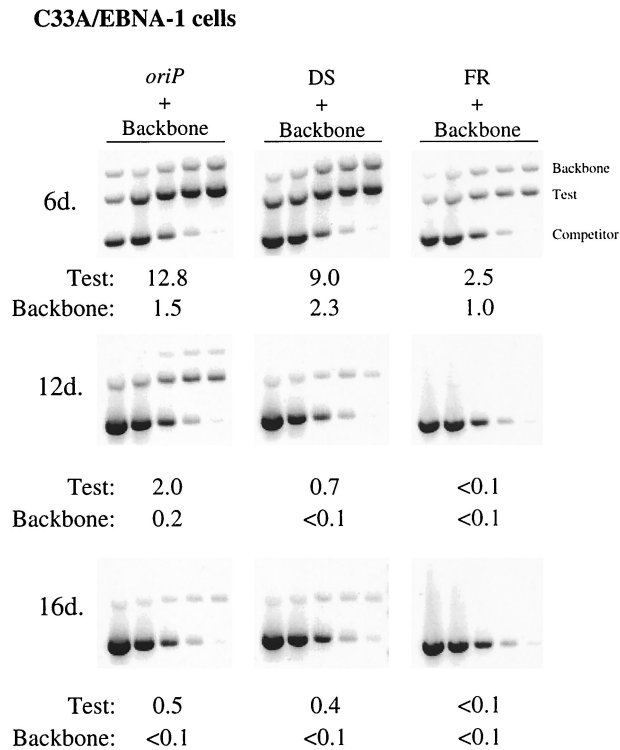


FIG. 2. Plasmid containing only the DS of *oriP* replicates within C33A/EBNA-1 cells but is lost precipitously during 2 weeks posttransfection, as is the *oriP* plasmid. Equimolar amounts of an *oriP* test plasmid (*oriP*), a test plasmid containing only the DS of *oriP* (DS), and a test plasmid containing only the FR of *oriP* (FR) were introduced independently into C33A/EBNA-1 cells together with a prokaryotic backbone plasmid (Backbone). At the indicated times 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 each gel refer to the average number of molecules present per transfected cell. 6d., day 6 posttransfection.

cated DS plasmids over 3 weeks following their introduction into 293 cells that express EBNA-1 stably (293/EBNA-1). Surprisingly, we found that while *oriP* plasmids were lost precipitously from the majority of transfected cells during this time (approximately a 33% rate of loss per cell generation between 6 and 18 days posttransfection; $n = 7$ [37]), the DS plasmid was established efficiently in transfected cells, being lost at a rate of only 8% per cell generation (Fig. 3B) ($n = 7$). That is, in one representative experiment (Fig. 3A), the level of replicated *oriP* plasmid detected at 13 days posttransfection was 2% of the level detected at 6 days posttransfection. However, the DS plasmid supported replication more efficiently than the *oriP* plasmid at 6 days posttransfection (Wilcoxon rank sum test: P [two-sided] = 0.009; $n = 7$), and this replicated DS plasmid pool decreased by a factor of only 3 between 6 and 16 days posttransfection.

A plasmid containing only the FR of *oriP* supported replication with only 6% of the efficiency of an *oriP* plasmid at 6 days posttransfection, and this replicated DNA was lost precipitously, as was the *oriP* plasmid (Fig. 3A). These experiments clearly demonstrate that plasmids containing only the

DS of *oriP* can be efficiently synthesized and maintained each cell cycle in 293/EBNA-1 cells. The FR, whose stimulatory function is not required in 293/EBNA-1 cells, acts in *cis* to promote the rapid loss of *oriP* plasmids in these cells.

To determine if the stability of the DS plasmid was restricted to the 293/EBNA-1 cell clone analyzed, we monitored the fate of the *oriP* and DS plasmids in the 293/1728#5 cell clone that stably maintains the 1728 *oriP*/EBNA-1 expression plasmid (37). The replicated DS plasmid pool remained stable between 5 and 16 days posttransfection (Fig. 4). However, addition of the FR in *cis* resulted in the precipitous loss of the replicated *oriP* plasmid population in the 293/1728#5 cell clone (37), as seen in 293/EBNA-1 cells. Given that *oriP* plasmids and EBV itself are established inefficiently in all proliferating cell lines analyzed to date (27, 37, 45, 58), the inhibitory activity of the FR observed in 293/EBNA-1 cells and 293/1728#5 cells likely underlies the inefficient establishment detected in all of these cell lines. We therefore pursued experiments to characterize the inhibitory activity of the FR in 293/EBNA-1 cells.

FR of *oriP* does not limit DNA synthesis at the DS. Why does a plasmid containing only the DS of *oriP* support stable replication within 293/EBNA-1 cells, yet addition of FR to this plasmid promotes the replicon's precipitous loss from the majority of transfected cells? This observation is counterintuitive, because the FR has been shown to act in *cis* to promote the maintenance of plasmids containing a putative viral or mammalian origin in 293 and 143B cell lines (30, 34). Given that the DS plasmid supported replication more efficiently than the *oriP* plasmid at 6 days posttransfection (Wilcoxon rank sum test: P [two-sided] = 0.009; $n = 7$), we postulated that the FR may impose licensing on the DS of *oriP* so that it is synthesized only once per cell cycle. In the absence of the FR, the DS plasmid would be amplified each cell cycle, as is the replicon of bovine papillomavirus (BPV), and these DS replicons would be maintained if four EBNA-1-binding sites within the DS are sufficient for plasmid maintenance in 293/EBNA-1 cells.

To test this hypothesis, we monitored the amount of plasmid that underwent one or additional rounds of DNA synthesis during one cell division cycle. Equimolar amounts of an *oriP* plasmid and a plasmid containing only the DS of *oriP* were introduced independently into 293/EBNA-1 cells, and the cells were labeled with bromodeoxyuridine for 17 h at 5 days posttransfection. Plasmid DNA was isolated by Hirt extraction, linearized, and separated on a CsCl gradient. Southern blotting was then used to quantify the amount of plasmid DNA that underwent zero (light-light [LL]), one (heavy-light [HL]), or multiple (heavy-heavy [HH]) rounds of DNA synthesis. Contrary to our hypothesis, the DS plasmid supported DNA synthesis only once per cell cycle, as did the *oriP* plasmid (Fig. 5). Namely, the FR of *oriP* does not limit DNA synthesis at the DS; rather, the DS alone possesses the *cis*-acting sequences required for licensing. This finding is consistent with the observation of Shirakata et al. that DS plasmids must traverse early G₁ phase prior to their synthesis in S-phase (50).

In addition to its synthetic function, the four EBNA-1-binding sites within the DS of *oriP* must also allow efficient plasmid maintenance in 293/EBNA-1 cells, given that other replicons that are efficiently synthesized once per cell cycle do not support stable replication in 293 cells (21, 34). That is, Krysan et al. have shown that plasmids containing putative mammalian

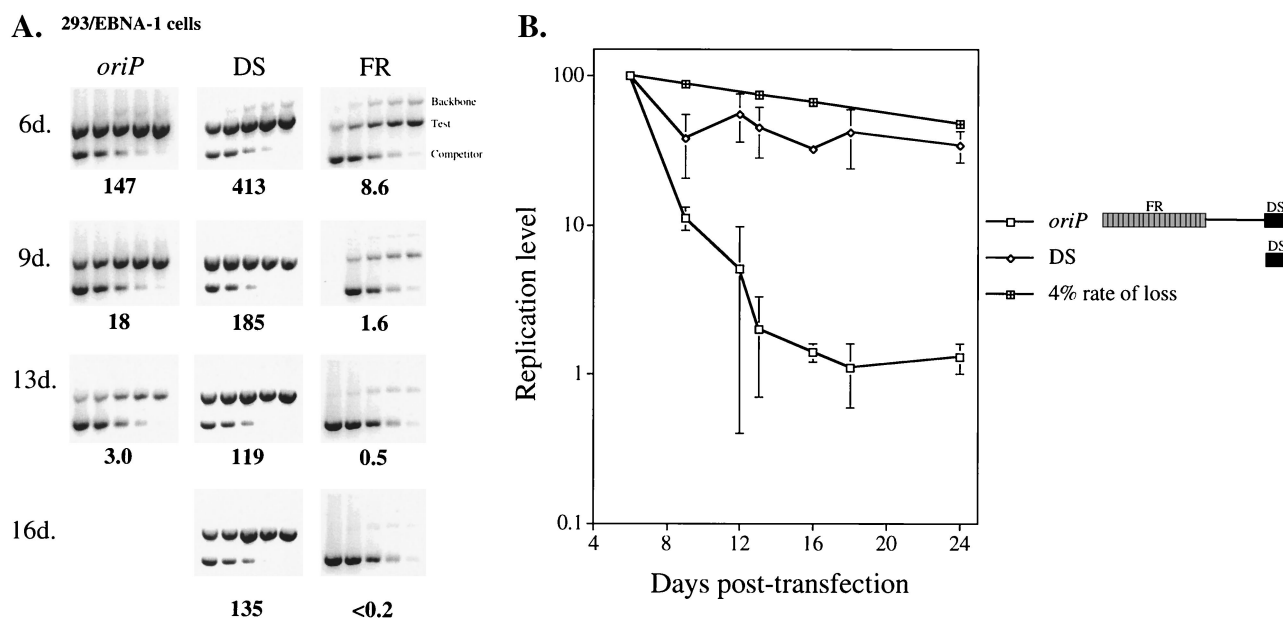


FIG. 3. (A) Plasmid containing only the DS of *oriP* supports efficient replication over 16 days posttransfection in 293/EBNA-1 cells, while replicated *oriP* plasmids are lost precipitously. Equimolar amounts of an *oriP* test plasmid (*oriP*), a test plasmid containing only the DS of *oriP* (DS), and a test plasmid lacking the DS of *oriP* (FR) were introduced independently into 293/EBNA-1 cells together with a prokaryotic backbone plasmid (Backbone). At the indicated times posttransfection, plasmid DNA was isolated by Hirt extraction and digested with *XhoI* and *DpnI*, and the level of replicated, *DpnI*-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), except for the day 9 time point (9d.) for the FR experiment, in which 9 pg of competitor was not used. Numbers below each gel refer to the average number of molecules present per transfected cell for each test plasmid (*oriP*, DS, and FR). The amount of replicated *oriP* plasmid present at 6 days posttransfection and the amount of replicated DS plasmid present at each time point were quantified from separate gels in which 3.3×10^3 cell equivalents were assayed. The amount of replicated backbone plasmid detected at 6 days posttransfection in the *oriP*, DS, and FR experiments was 3.0, 3.6, and 0.8 molecules per transfected cell, respectively, and was undetectable at the latter times (<0.2 molecule/transfected cell). The 16-day time point for the *oriP* test plasmid is not shown because the cells were lost to contamination after the day 13 harvest. (B) DS plasmids are stable during 24 days posttransfection in 293/EBNA-1 cells, while *oriP* plasmids are lost precipitously. Shown is a graphic representation of experiments conducted as described above. The replication level of each plasmid was plotted versus the days posttransfection. For each independent experiment, the level of replicated test plasmid detected at 6 days posttransfection was set to 100% and the replication level at later time points was set relative to this point (100% = 439 ± 251 *oriP* and 754 ± 324 DS molecules per transfected cell). The data points are representative of the number of independent experiments listed: 6 days, $n = 7$; 9 days, $n = 3$; 12 days, $n = 4$; 13 days, $n = 3$; 16 days, $n = 2$; 18 days, $n = 4$; 24 days, $n = 4$. Three experiments in which the replication of the *oriP* test plasmid was monitored at 6, 9, 13, and 16 days posttransfection were presented previously (37). Note that the level of replicated *oriP* plasmid decreased approximately 100-fold from the cell population between 6 and 18 days posttransfection, then remained stable between 18 and 24 days. This finding is consistent with our previous observation that *oriP* plasmids are established in only 1% of transfected 293 cells (37). The 4% rate-of-loss curve, depicted by black cross-hatched boxes, is theoretical and is based on previous studies of established, drug-resistant cell clones (29, 56).

origins support efficient replication at 4 days posttransfection in 293 cells; however, these plasmids were lost unless EBNA-1 and its binding sites in the FR were provided (34). Likewise, we have observed that a plasmid containing an efficient DNA synthetic element comprised of eight copies of the viral Rep* element (30) is lost at twice the rate of a DS plasmid in 293/EBNA-1 cells (Leight and Sugden, unpublished observations), indicating that the DS must possess efficient DNA synthetic and maintenance functions in these cells.

Efficient transcriptional activation from an *oriP* replicon does not promote its precipitous loss. The FR clearly inhibits the establishment of an *oriP* replicon in 293/EBNA-1 cells, and an epigenetic event is required to overcome this inhibitory activity (37). But how does the FR inhibit establishment? The FR possesses two features that are not shared by the DS. First, the FR acts as a potent transcriptional enhancer, activating transcription of a promoter present in *cis* by 10- to 100-fold when EBNA-1 is provided in *trans* (4, 35, 46, 57). Second, when EBNA-1 dimers are bound to the 20 binding sites in the FR, the DNA synthesis machinery cannot proceed efficiently

through this region (11, 15). The FR therefore scores as a replication fork barrier (RFB) in two-dimensional gel electrophoresis studies (15). We examined whether either of these attributes of the FR underlies its inhibitory activity.

The Calos laboratory has shown an inverse correlation between the transcription and replication of a plasmid containing autonomously replicating sequences (22). We therefore postulated that an inverse relationship between replication and transcription of an *oriP* plasmid exists. The *oriP* plasmid analyzed in our studies contains both replicative (*oriP*) and transcriptional (FR enhancer and the thymidine kinase promoter) elements. We therefore addressed whether elimination of transcriptional activation from the thymidine kinase promoter by its deletion could rescue the replication defect of the *oriP* plasmid. The levels of replicated *oriP* plasmid (*oriP*) and *oriP* plasmid lacking the thymidine kinase promoter (Δ TKp) were monitored over 16 days following their introduction into 293/EBNA-1 cells. The Δ TKp plasmid supported replication but was lost precipitously, as was the *oriP* plasmid (Fig. 6), demonstrating that FR's potent transcriptional activation of the

293/1728 #5 cell clone

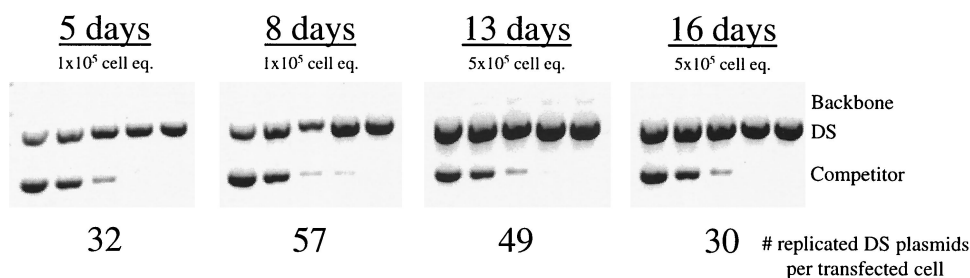


FIG. 4. Replicated DS plasmids are stable during 16 days posttransfection in the 293/1728#5 cell clone, as seen in the 293/EBNA-1 cell clone. The 293/1728#5 cell clone was isolated as described (37). This cell line was selected to stably replicate the 1728 plasmid, which contains *oriP* and an EBNA-1 expression cassette. The levels of replicated DS plasmid (DS) and prokaryotic backbone plasmid (Backbone) were monitored over 16 days posttransfection as described in the legend to Fig. 3A. Numbers below each gel refer to the average number of replicated DS plasmids present per transfected cell. The replicated backbone plasmid was present at less than one copy per transfected cell for each time point. These data were confirmed by Southern blot analysis (data not shown).

thymidine kinase promoter does not underlie the replicon's precipitous loss. There is not an inverse relationship between transcription and replication of an *oriP* plasmid as is seen with plasmids containing autonomously replicating human sequences (22).

Multiple EBNA-1 binding sites in FR of *oriP* promote inefficient establishment of plasmid replicon. Given that the FR's enhancement of transcription from the *oriP* replicon does not underlie the replicon's precipitous loss, we addressed whether the FR, by acting as a potent RFB, promotes this inefficient establishment. *oriP* plasmids that initiate DNA synthesis during the last 3 h of S-phase may not be completely synthesized by the end of S-phase, as 3 or more hours are devoted to DNA synthesis through the FR (given that the FR is detected as an RFB in EBV and assuming that DNA elongation occurs at a rate of 1 kbp/min) (15). These plasmids may be eliminated from the cell if a checkpoint is not imposed to ensure duplication of the *oriP* plasmid prior to entry into G₂ phase. The FR, by acting as an RFB, may thereby promote the loss of plasmids whose origin fires late during S-phase.

Platt et al. have shown that the number of EBNA-1-binding sites is critical for RFB formation (40). In their studies, the DS is not detected as an RFB, yet when it is trimerized and introduced in place of the FR, it scores as an RFB (43). To test our hypothesis, we therefore determined whether deletion of EBNA-1-binding sites within the FR or substitution of the FR with low-affinity binding sites from the DS could rescue the replication defect. *oriP* plasmid derivatives containing only nine and three EBNA-1 binding sites from the FR (9FR+DS and 3FR+DS, respectively) and a derivative in which the FR was replaced by the DS (DS+DS) were introduced independently into 293/EBNA-1 cells, and the level of replicated plasmid DNA was monitored over 24 days posttransfection in parallel to the *oriP* and DS plasmids. The 9FR+DS, 3FR+DS, and DS+DS plasmids supported stable replication over the time course, as did the plasmid containing only the DS of *oriP* (Fig. 7A). That is, deletion of EBNA-1-binding sites within the FR or substitution of the FR with DS sequences overcomes the inhibitory activity of the FR, allowing efficient establishment of the replicon.

While these findings are consistent with a model in which disruption of RFB formation overcomes the inhibitory activity of the FR, they do not address whether an RFB per se can act in *cis* to promote the loss of a plasmid containing only the DS of *oriP* in 293/EBNA-1 cells. We therefore asked whether an RFB, when introduced into a plasmid containing only the DS of *oriP* (RFB→DS), could promote the replicon's inefficient establishment. We chose the rDNA RFB for this purpose

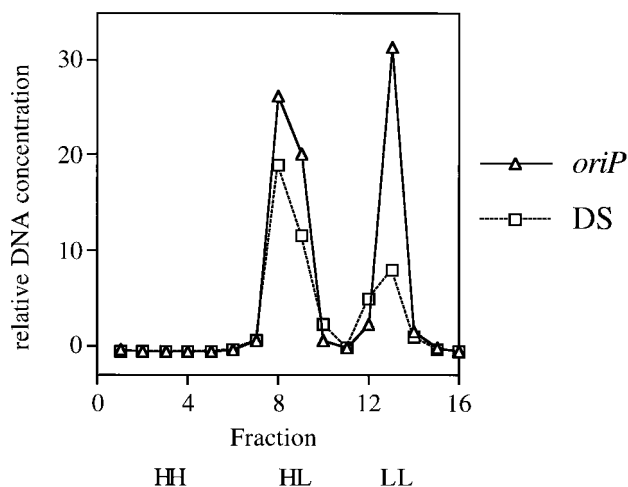


FIG. 5. Plasmid containing only the DS of *oriP* is synthesized once per cell cycle, as is an *oriP* replicon (1, 61). Equimolar amounts of an *oriP* plasmid (*oriP*) and a plasmid containing only the DS of *oriP* (DS) were introduced independently into 293/EBNA-1 cells, and the cells were labeled with bromodeoxyuridine for 17 h at 5 days posttransfection. Plasmid DNA was isolated by Hirt extraction of approximately 5×10^7 cells, linearized with *XhoI*, and separated on a CsCl density gradient. The refractive index of every fourth fraction was determined, one-fourth of each fraction was transferred to a nylon membrane, and plasmid DNA present in the LL, HL, and HH peaks was determined by Southern blotting (48). The relative DNA concentration was plotted versus fraction number. No plasmid DNA was detected at the density at which HH DNAs should migrate. The limit of detection was approximately 1 molecule per transfected cell (25 pg).

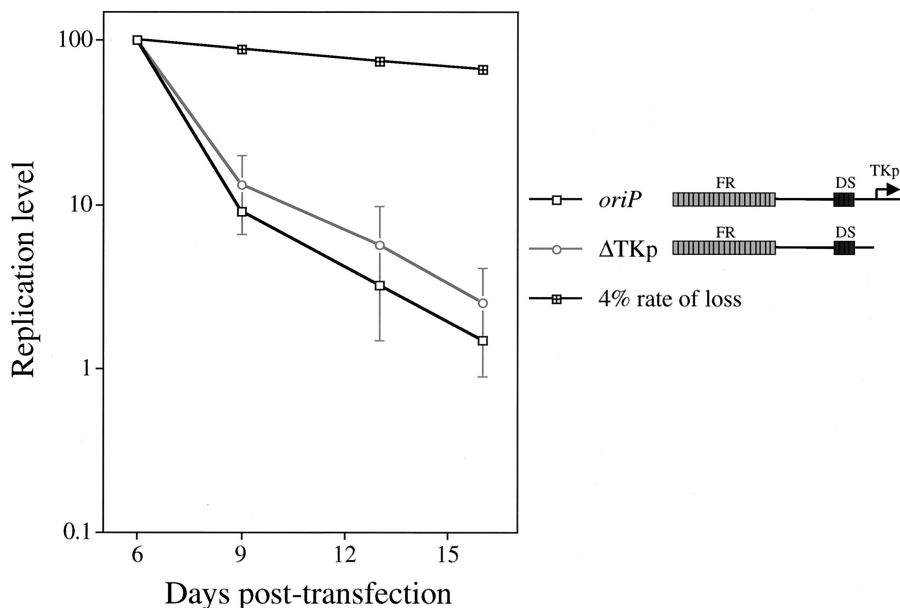


FIG. 6. Efficient transcriptional activation from an *oriP* plasmid does not promote its precipitous loss. The level of replicated *oriP* test plasmid (*oriP*) and *oriP* test plasmid lacking the thymidine kinase promoter (Δ TKp) was monitored over 16 days posttransfection in 293/EBNA-1 cells as described in the legend to Fig. 3A. The replication level of these plasmids was plotted versus the days posttransfection. For each independent experiment, the level of replicated test plasmid detected at 6 days posttransfection was set to 100%, and the replication level at later time points was set relative to this point (100% = 403 *oriP* and 318 ± 175 Δ TKp molecules per transfected cell). The loss of the Δ TKp plasmid is representative of three independent experiments conducted in parallel to the *oriP* test plasmid. The 4% rate-of-loss curve, depicted by black cross-hatched boxes, is theoretical and is based on previous studies of established, drug-resistant cell clones (29, 56).

because this RFB has been conserved in organisms ranging from *S. cerevisiae* to humans (7, 39) and can function efficiently even in the presence of EBNA-1 (39). In addition, this conserved site of RFB formation can also function in the context of a plasmid (16). Given that the rDNA RFB is a directional (or polar) barrier while the FR is not (11, 16), a plasmid containing two copies of the rDNA RFB in a tail-to-tail orientation (RFB \rightarrow DS \leftarrow RFB) was also analyzed (Fig. 7B). These plasmids were introduced independently into 293/EBNA-1 cells, and the level of replicated plasmid DNA was monitored over 24 days posttransfection in parallel to the *oriP* and DS plasmids.

The RFB \rightarrow DS and RFB \rightarrow DS \leftarrow RFB plasmids were efficiently established, supporting efficient DNA synthesis and maintenance each cell generation over the 24-day period (Fig. 7B). This experiment demonstrates that an RFB alone does not impose instability on a DS plasmid. Rather, multiple EBNA-1-binding sites in the FR of *oriP* possess an independent inhibitory function that promotes the inefficient establishment of plasmid replicons.

Spacing between FR and DS does not affect establishment of an *oriP* replicon. EBNA-1 dimers bound to the FR can associate with EBNA-1 dimers bound to the DS, resulting in the formation of intramolecular "looped" DNA complexes and intermolecular "linked" DNA complexes, as visualized by electron microscopy (14, 54). DNA looping and linking between the FR and DS are not required for replication in that, in 293/EBNA-1 cells, plasmids containing only the DS of *oriP* are established efficiently. We wished to address whether intramolecular DNA looping between the FR and DS could contribute to the inefficient establishment of *oriP* replicons. Given that it

is less energetically favorable for a *trans*-acting factor to loop two *cis* elements when the distance between those elements is decreased (20), we constructed an *oriP* plasmid derivative in which the 963 bp separating FR and DS were deleted (FR*DS) (Fig. 8). DNA looping is unlikely to occur in the FR*DS plasmid, as Su et al. did not detect looped DNA complexes in electron microscopy studies with a DNA fragment containing the FR, and the length of the loops with an *oriP* DNA fragment detected ranged from 1,000 to 1,780 bp (54). DNA loops between the juxtaposed FR and DS would be less than 800 bp.

This FR*DS plasmid was introduced into 293/EBNA-1 cells in parallel to the *oriP* and DS plasmids, and the level of replicated DNA was monitored over 24 days posttransfection. The FR*DS plasmid was lost precipitously from cells between 6 and 18 days posttransfection (Fig. 8), as was the *oriP* plasmid, demonstrating that DNA looping between the FR and DS does not underlie the inefficient establishment of an *oriP* replicon. To ensure that juxtaposition of the FR and DS does not impose a novel replication defect, we monitored the replication of an *oriP* plasmid derivative containing three EBNA-1 binding sites from the FR juxtaposed to the DS (3FR*DS). This plasmid supported efficient replication over 24 days posttransfection, as did the 3FR+DS plasmid (Fig. 7A and 8), confirming that juxtaposition of the FR and DS does not impose a novel replication defect.

These experiments demonstrate that the spacing between FR and DS does not affect the establishment of an *oriP* replicon. This conclusion is also supported by the studies of the DS+DS, 9FR+DS, and 3FR+DS plasmids. DNA looping is expected to occur efficiently between distant EBNA-1-binding sites in these plasmids, given that single EBNA-1-binding sites

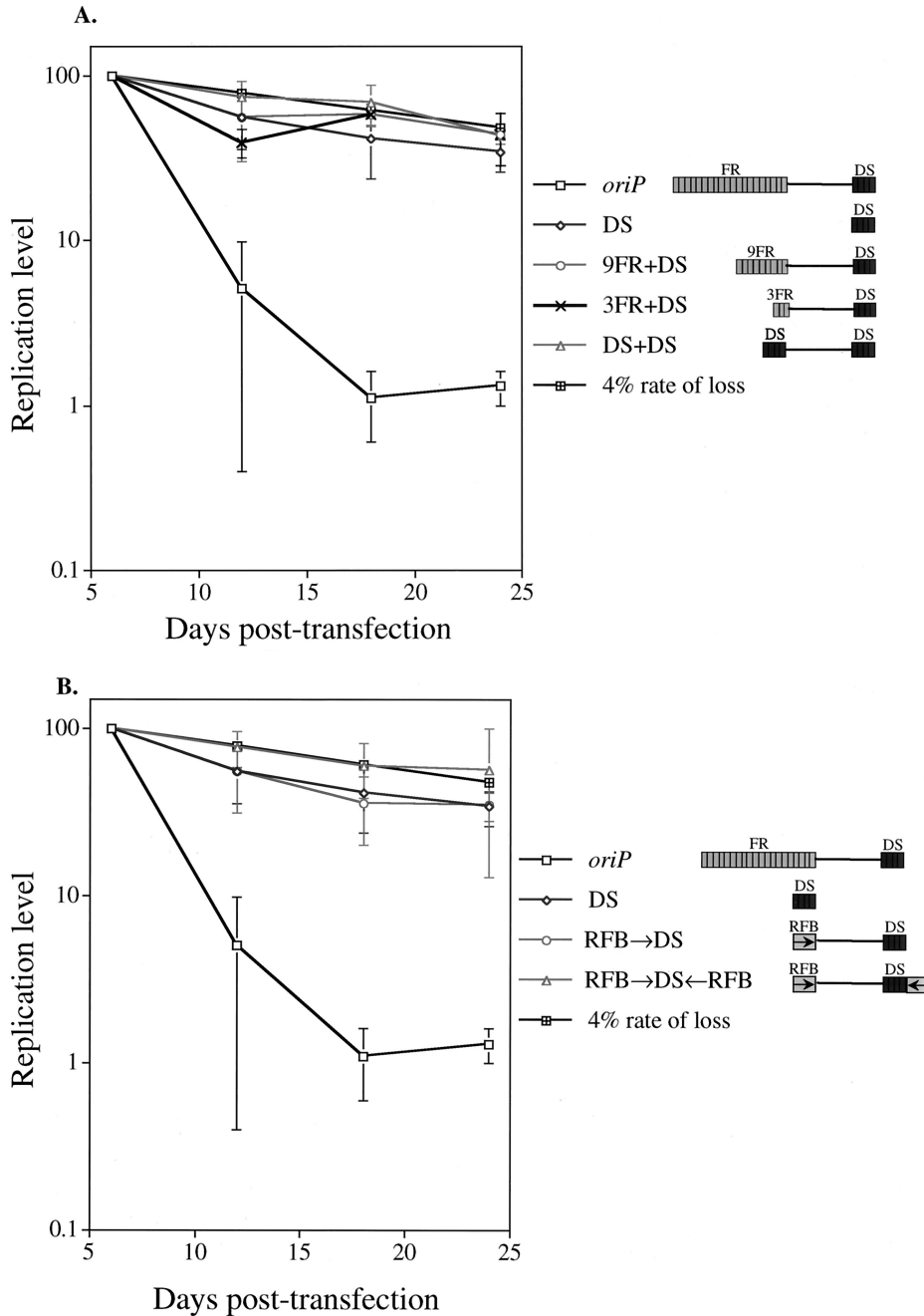


FIG. 7. (A) Multiple EBNA-1-binding sites within the FR of *oriP* inhibit the replicon's establishment. *oriP* plasmid derivatives containing permutations in the number and type (high versus low affinity) of EBNA-1-binding sites were introduced into 293/EBNA-1 cells, and the level of replicated plasmid DNA was monitored over 24 days posttransfection as described in the legend to Fig. 3A. The *oriP* and DS plasmids were analyzed in four independent experiments conducted in parallel to the *oriP* plasmid derivatives shown. (These data are also presented in Fig. 7B and 8, as all *oriP* plasmid derivatives were analyzed simultaneously.) The replication level of these plasmids was plotted versus the days posttransfection. For each of four independent experiments, the level of replicated test plasmid detected at 6 days posttransfection was set to 100%, and the replication level at later time points was set relative to this point (100% = 625 ± 121 *oriP*, 967 ± 258 DS, 876 ± 175 9FR+DS, 1149 ± 405 3FR+DS, and 1,498 ± 487 DS+DS molecules per transfected cell). The 4% rate-of-loss curve, depicted by black cross-hatched boxes, is theoretical and is based on previous studies of established, drug-resistant cell clones (29, 56). (B) An RFB from the rDNA locus, when introduced in place of the FR of *oriP*, does not inhibit establishment of the replicon. *oriP* plasmid derivatives containing one (RFB→DS) or two (RFB→DS←RFB) copies of the rDNA RFB (16) in place of the FR of *oriP* were introduced into 293/EBNA-1 cells, and the level of replicated plasmid DNA was monitored over 24 days posttransfection as described in the legend to Fig. 3A. The *oriP* and DS plasmids were analyzed in four independent experiments conducted in parallel to the *oriP* plasmid derivatives shown. (The data are also presented in Fig. 7A and 8, as all *oriP* plasmid derivatives were analyzed simultaneously.) The replication level of these plasmids was plotted versus the days posttransfection. For each of four independent experiments, the level of replicated test plasmid detected at 6 days posttransfection was set to 100%, and the replication level at later times was set relative to this point (100% = 625 ± 121 *oriP*, 967 ± 258 DS, 1,043 ± 462 RFB→DS, and 843 ± 215 RFB→DS←RFB molecules per transfected cell). The 4% rate-of-loss curve, depicted by black cross-hatched boxes, is theoretical and is based on previous studies of established, drug-resistant cell clones (29, 56).

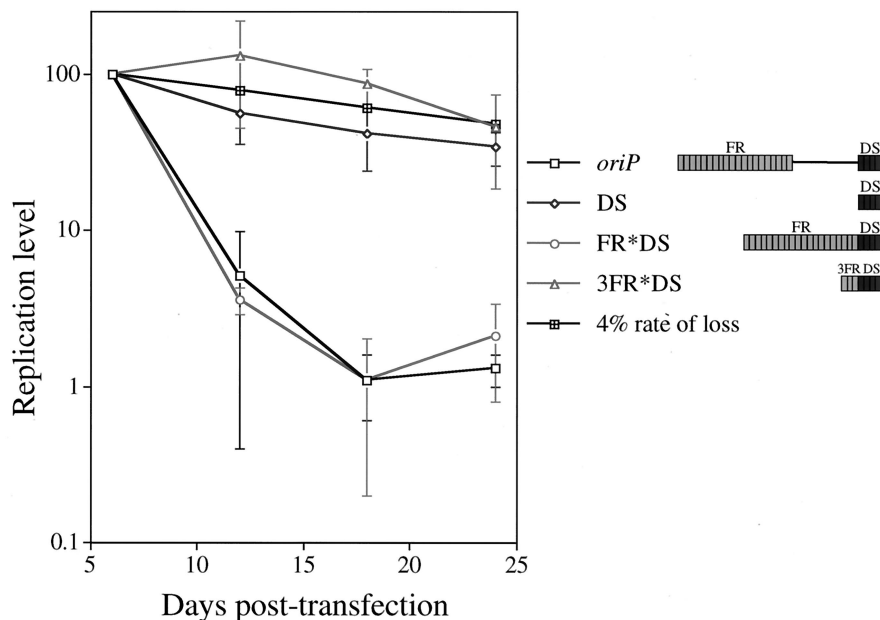


FIG. 8. Spacing between the FR and DS of *oriP* has no effect on establishment of the replicon. The 963 nucleotides separating the FR and DS of *oriP* were deleted in the context of the *oriP* test plasmid and the 3FR+DS plasmid, the resulting plasmids (FR*DS and 3FR*DS, respectively) were introduced into 293/EBNA-1 cells, and the level of replicated DNA was monitored over 24 days posttransfection as described in the legend to Fig. 3A. The *oriP* and DS plasmids were analyzed in four independent experiments conducted in parallel to the *oriP* plasmid derivatives shown. (The data are also presented in Fig. 7A and 7B, as all *oriP* plasmid derivatives were analyzed simultaneously.) The replication level of these plasmids was plotted versus the days posttransfection. For each of four independent experiments, the level of replicated test plasmid detected at 6 days posttransfection was set to 100%, and the replication level at later times was set relative to this point (100% = 625 ± 121 *oriP*, 967 ± 258 DS, 439 ± 222 FR*DS, and 817 ± 576 3FR*DS molecules per transfected cell). The 3FR*DS data are representative of three independent experiments except for the day 24 time point, which is representative of two experiments. The 4% rate-of-loss curve, depicted by black cross-hatched boxes, is theoretical and is based on previous studies of established, drug-resistant cell clones (29, 56).

that are separated by 930 nucleotides loop with the same frequency as an *oriP* DNA fragment when EBNA-1 is present in *trans* (19). These plasmids were established efficiently, however, demonstrating that DNA looping between distal EBNA-1 binding sites does not underline the inefficient establishment of *oriP* replicons.

DISCUSSION

Cell lines vary in their ability to support replication of *oriP* plasmid derivatives. We have monitored the fate of plasmids containing *oriP* or only the DS of *oriP* for 24 days following their introduction into various EBNA-1-positive cell lines. While *oriP* plasmids are lost precipitously and established inefficiently in all 22 cell lines analyzed to date (27, 37, 45, 58), surprisingly we have found that cell lines vary dramatically in their ability to support the replication of a DS plasmid. DS plasmids were eliminated rapidly from 143B cells, so that the level of replicated DS plasmid detected at 4 days posttransfection was only 10% of the level of wild-type *oriP* and dropped below the level of detection (<0.1 molecule/cell) by 7 days posttransfection. Replicated DS plasmids were lost at an intermediate rate in C33A/EBNA-1 cells. That is, the DS plasmid supported replication as efficiently as the *oriP* plasmid at 6 days posttransfection (Wilcoxon rank sum test: P [two-sided] = 0.28; $n = 3$) and was lost at a rate of approximately 28% per cell generation between 6 and 16 days following transfection, as was the *oriP* plasmid. In 293/EBNA-1 cells, however, the DS plasmid was established efficiently, being lost at a rate of only

8% per cell generation, while the *oriP* plasmid was lost at a rate of 33% per cell generation between 6 and 18 days posttransfection. This replicated DS plasmid was likewise stable in the 293/1728#5 cell clone.

Variations between cell lines were also observed in historical studies in which the level of replicated *oriP* and DS DNA was monitored at 2 to 5 days posttransfection. In these experiments, the DS plasmid supported replication with less than 6% of the efficiency of the *oriP* plasmid in Raji cells and EBNA-1-expressing 143B cells (30, 57), whereas the DS plasmid supported replication as efficiently as the *oriP* plasmid in D98/Raji cells and EBNA-1-positive HeLa and 293 cells (24, 49, 60). Interestingly, cell line differences are not limited to the replication of DS plasmids. Stillman and Gluzman have shown that 293 cell extracts support efficient DNA synthesis of simian virus 40 DNA while HeLa cell extracts support only 12 to 25% of that level (53). In addition, while human papillomavirus replicates efficiently in 293 cells, it replicates poorly in HeLa cells due to limiting levels of cyclin E/CDK2 (38).

Clearly, cell lines vary in their support of replication of plasmids containing only four EBNA-1 binding sites from the DS. But what underlies this differential replication of DS plasmids? Previously, Aiyar et al. have shown that cell lines vary in their ability to eliminate replicated DNAs and transcribed DNAs (3). These DNAs were lost at a rate of 50% per cell generation in 293 cells, but were lost more rapidly in C33A and 143B cells (70 and 80% rate of loss per cell generation, respectively) (3). These differences in the maintenance of newly syn-

thesized plasmids, however, cannot solely account for our observations in 293/EBNA-1 cells. That is, if the efficient replication of DS plasmids in 293/EBNA-1 cells is due to efficient DNA synthesis at the DS coupled with a maintenance function provided by the cell, all plasmids containing an efficient origin should replicate efficiently over 24 days posttransfection. On the contrary, we have observed that a plasmid containing an efficient DNA synthesis element comprised of eight copies of the viral Rep* element (30) is lost at twice the rate of a DS plasmid in 293/EBNA-1 cells (Leight and Sugden, unpublished observations). Likewise, plasmids containing putative mammalian origins are lost from 293 cells unless EBNA-1 and its binding sites in the FR are provided (34). These findings demonstrate that the DS alone possesses efficient DNA synthesis and maintenance functions in 293/EBNA-1 cells but not in C33A/EBNA-1, H1299/1728#3, or EBNA-1-positive 143B cells. This feature of 293/EBNA-1 cells allowed us to unveil the inhibitory activity of the FR—an inhibitory activity that likely underlies the inefficient establishment of *oriP* replicons shared by 293/EBNA-1 cells and the other 21 cell lines analyzed to date (27, 37, 45, 58).

We propose that the differences observed in cell lines are due to variations in the levels of cellular *trans*-acting factors involved in EBNA-1-mediated DNA synthesis and/or maintenance functions. These factors may be abundant in 293 cells, so that four DNA-bound EBNA-1 dimers are sufficient for their recruitment. In 143B cells, these factors would be limiting, and additional DNA-bound EBNA-1 dimers (and therefore additional EBNA-1-binding sites from the FR) must be employed for their recruitment. A prediction for this model is that a cDNA(s) from 293 cells should complement the replication defect of the DS plasmid in EBNA-1-positive 143B cells.

FR can stimulate and inhibit replication of *oriP* replicons. EBNA-1 binding sites from the FR clearly contribute to DNA synthesis and/or maintenance of *oriP* plasmids in some cell lines. That is, we and others have shown that *oriP* plasmid derivatives containing fewer than seven EBNA-1 binding sites from the FR fail to support short-term replication in Raji, D98/Raji, and EBNA-1-positive 143B cells (8, 30, 47, 57). However, even with the intact FR, *oriP* plasmids are established in only 1 to 10% of transfected cells (37). By monitoring the replication of *oriP* plasmid derivatives in multiple cell lines, we unexpectedly found that the FR also acts in *cis* to promote the rapid loss of replicated *oriP* plasmids from the population of transfected 293/EBNA-1 cells and 293/1728#5 cells. This inhibitory activity of the FR likely underlies the inefficient establishment of *oriP* replicons in all cells, as *oriP* replicons are established inefficiently in all 21 cell lines analyzed to date (27, 37, 45, 58). Previously we have shown that *oriP* plasmids must be modified epigenetically for establishment (37). (Note that in Fig. 3B the level of replicated *oriP* plasmid decreased approximately 100-fold from the cell population between 6 and 18 days posttransfection, then remained stable between 18 and 24 days. This is consistent with our previous observation that *oriP* plasmids are established in only 1% of transfected 293 cells [37].) Our findings indicate that this epigenetic event is required to overcome an inhibitory activity of the FR.

How does the FR inhibit the establishment of *oriP* replicons? This still remains an enigma. Our studies, however, have defined parameters for FR's inhibitory activity in 293/EBNA-1

cells. We have shown that DNA looping between FR and DS neither stimulates nor inhibits replication (Fig. 8). Additionally, the FR does not limit DNA synthesis at the DS, as plasmids lacking the FR of *oriP* are licensed analogously to *oriP* plasmids (Fig. 5) (1, 61). Likewise, the FR does not inhibit activities of the DS by competing for EBNA-1. Namely, only 10% of EBNA-1 molecules are bound to the FR at 6 days posttransfection (given that there are approximately 3×10^4 EBNA-1 molecules per cell [52; Wang and Sugden, unpublished observations], 100 replicated *oriP* plasmids per cell, and 20 binding sites for EBNA-1 dimers within the FR), and previous studies have shown that the FR enhances EBNA-1's binding to the DS (13, 54). Furthermore, two attributes of the FR that are not shared by the DS, FR's enhancement of transcription of a promoter in *cis* and FR's ability to inhibit replication fork movement, do not solely account for *oriP*'s inefficient establishment. Deletion of 11 EBNA-1-binding sites in the FR or replacement of the FR with DS sequences, however, does overcome the inhibitory activity of the FR, allowing efficient establishment of the *oriP* derivative in 293/EBNA-1 cells (Fig. 7A).

Why do 20 EBNA-1-binding sites from FR inhibit replication whereas 9 EBNA-1-binding sites do not? There are two likely answers. First, an inhibitory factor may bind the 11 right-hand binding sites from FR, and deletion of these sites relieves inhibition. Alternatively, the 20 binding sites in the FR may act cooperatively to allow binding of an inhibitory factor. Such an inhibitory factor may be involved in modulation of chromatin structure or covalent modification of the FR. We find the first possibility unlikely, as the right-hand and left-hand EBNA-1-binding sites in the FR behave similarly in supporting transcription and replication (8, 57), and the nine left-hand EBNA-1-binding sites from the FR show >95% sequence identity with the 11 right-hand EBNA-1-binding sites (based on the Smith-Waterman algorithm). Identification of cellular factors that bind to the FR of *oriP* in transiently transfected cells, in which *oriP* plasmids are lost at ~30% per cell generation, and in established cell clones, in which *oriP* plasmids are lost at ~2 to 4% per cell generation (29, 56), should provide insights into the epigenetic event that underlies the establishment of *oriP* replicons and EBV's plasmid replicon.

Clearly the FR has both inhibitory and stimulatory functions with respect to *oriP*'s replication, but given that seven EBNA-1 binding sites from the FR are sufficient for plasmid maintenance in Raji and D98/Raji cells (47, 57) and are not even required in 293 cells, why has EBV evolved to retain all 20 EBNA-1-binding sites in the FR? We can only speculate that these 20 binding sites contribute to an essential function of the virus, such as efficient transcription of latent gene promoters within the established host cell. Even though EBV has kept these 20 binding sites in the FR at the expense of its establishment in proliferating cells, it has accomplished an astonishing feat—assimilation by the host cell as an extrachromosomal replicon that is synthesized only once per cell cycle and faithfully partitioned to daughter cells (1, 29, 56, 61).

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