Supporting Information

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SI Text

Plasmids. The plasmids used in short-term and long-term replication assays have a pPUR (Invitrogen) backbone with the FR element from EBV (p2895). Each plasmid contains the origin region to be tested and the DS element from EBV: the Raji ori fragment from EBV spans from 146 kbp to 160 kbp (p3043); the Raji middle fragment spans from EBV 67 kbp to 81 kbp (p3416). The plasmids used in replication assays after establishment have the same backbone (p3335) and origins to be tested as described above (p3338 and p3417) and a DS cassette containing the DS element from EBV, the TK gene from HSV, an IRES, and the mRFP gene flanked by loxP sites. The sequences of these vectors are available at: www.bioinfo.wisc.edu/bxaf4/bm/pcs.php.

Cell Lines. Raji is an EBV-positive Burkitt's lymphoma cell line, which was cultured in RPMI medium 1640 with 10% FBS, 200 units of penicillin, and 200 μg of streptomycin sulfate per milliliter. BJAB/EBNA1 (clone #8) (1) stably expresses EBNA1 and hygromycin B phosphotransferase. The clone of cells was derived from BJAB by transfection with p2512, an EBNA1 IRES hygromycin B phosphotransferase expression vector. BJAB/EBNA1 cells were grown in the same medium as Raji cells with 400 μg/ml hygromycin (GIBCO) for selection. The 293/EBNA1 is a cell clone derived from the 293 cell, which is a human embryonic kidney cell line (ATCC-CRL-1573) of neuroendothelial origin. The 293/EBNA1 cells stably express EBNA1 and neomycin phosphotransferase and were cultured in DMEM/HG with 10% FBS and 250 μg/ml G418 for selection.

Transfection. Raji cells and BJAB/EBNA1 cells were transfected by electroporation. The 293/EBNA1 cells were transfected by lipofectamine 2000 (Invitrogen). Ten micrograms of test DNA were transfected in each transfection along with 1 μg of an eGFP expression vector to monitor the efficiency of transfection.

Short-Term Replication Assay. Ten micrograms of test DNA were tranfected into Raji cells along with 1 μg of an eGFP expression vector to monitor the transfection efficiency by counting eGFP-expressing cells under the fluorescent microscope 2 days after transfection. Cells were cultured in RPMI medium 1640 with 10% FBS without selection. Cells (1×10^7) were harvested 4 days after transfection. Extrachromosomal DNA was purified by the alkaline lysis method (see below). During the purification, 5 ng of pPUR DNA was added into the preparation to serve as a control for recovery and digestion with DpnI. Ninety percent of the harvested DNA was digested with 50 units of DpnI and 25 units of a single-cut enzyme at 37°C overnight. Ten percent of the sample was digested with 10 units of a single-cut enzyme alone to serve as a control for DNA recovery. Plasmid 2895 (1 μg) was incubated with 1% of the digestion reaction in parallel and then checked on an agarose gel to confirm the completeness of the DpnI digestion. The DNAs were subsequently detected by Southern blot analysis.

Alkaline Lysis. Cells (1×10^7) were harvested and resuspended in 3 ml of 50 mM glucose, 25 mM Tris (pH 8.0), and 10 mM EDTA. The cells were lysed with 3 ml of 200 mM NaOH and 1% SDS and incubated on ice for 5 min. The samples were neutralized with 3 ml of ice-cold, 3 M potassium acetate and 11.5% glacial acetic acid and left on ice for 10 min. The samples were centrifuged at 15,000 rpm in a Ti50 rotor for 30 min at 4°C. The DNAs were extracted first with equal volumes of phenol/chloroform and then with chloroform. The DNAs were precipitated with 2.5 volumes of ethanol and resuspended in 200 μl of TE, treated with 20 μg RNaseA at 37°C for 1 h, treated with 20 μg protease K in the presence of 0.5% SDS at 37°C overnight, extracted again with 1:1 phenol/chloroform, and reprecipitated with ethanol.

Southern Blot Analysis. DNA samples were separated in agarose gels in 0.8% 1× TAE containing 100 ng/ml ethidium bromide. The gel was treated with 0.2 M HCl for 30 min, 0.5 M NaOH, 0.5 M NaCl for 30 min, neutralized in 0.7 M Tris (pH 7.0), 1.5 M NaCl for 30 min, and then transferred by capillary action to GeneScreen Plus membrane (NEN Life Sciences) overnight in 10× SSC. The membrane was exposed to UV to cross-link the DNA and the membrane. The membrane was treated with 0.4 M NaOH for 3 min, 1 M Tris·HCl (pH 7.0) for 3 min, and 2× SSC for 3 min. The blot was hybridized with 25 ng of pPUR DNA, which was labeled with P^{32} by using a RediPrime II kit (Amersham Biosciences) and purified with Quickspin columns (Roche) in Ultrahyb buffer (Ambion) overnight. The blot was washed twice with 2× SSC, 1% SDS at 60°C for 15 min each, then twice with 0.1× SSC and 0.1% SDS at 58°C for 15 min each. The membrane was exposed to a storage phosphor screen (Molecular Dynamics) for at least 6 h, the signal was detected by a Storm 860 PhosphorImager, and band intensities were quantified by ImageQuant 5.2 (Molecular Dynamics).

Long-Term Replication Assay. To measure the ability of the plasmids to support long-term replication, Raji cells or BJAB/EBNA1 cells with transfected with the test plasmids along with an eGFP-expression vector. Green cells, which represented transfected cells, were counted 2 days after transfection. One, 3, 10, 100, and 500 green cells were plated per well into 96-well plates with 1 μg/ml puromycin for selection. The number of positive wells was counted 3 weeks after plating. From the percentage of negative wells, the efficiencies of colony formation were estimated by a Poisson distribution. To measure the ability of the plasmids to support long-term replication in 293/EBNA1, cells were transfected with the test plasmids along with an eGFP expression vector. Green cells were counted 2 days after transfection. One hundred, 300, 1,000 and 3,000 green cells were plated into 15-cm dishes along with 1 μg/ml puromycin for selection. The number of colonies formed was counted visually 3 weeks after plating.

Replication Assay After Establishment. Raji or BJAB/EBNA1 cells harboring established test plasmids were tranfected with 5 μg of a Cre IRES GFP espression vector. GFP-positive cells, which represent Cre-expressing cells, were sorted by FACS-Vantage SE with FACS-DIVA option (Becton Dickinson) into 96-well plates at 3, 10, 30, 100, and 500cells per well. Sorted cells were cultured under selection with 1 μg/ml puromycin for 3 weeks. The number of positive wells was counted, and the colonies were expanded and treated with 1 μg/ml ganciclovir to confirm the successful recombination by Cre recombinase. Cells in which recombination failed would express the herpes viral thymidine kinase and be susceptible to be killed by ganciclovir. From the percentage of negative wells, the efficiencies of colony formation were estimated by a Poisson distribution.

Nuclear Matrix Preparation. Raji cells (1×10^8) transfected with oriP plasmids were harvested 4 days after transfection. The cells

were washed twice with 40 ml of 5 mM Tris·HCl (pH 7.4), 0.5 mM EDTA-KOH (pH 7.5), 125 nM spermidine, 50 nM spermine, and 20 mM KCl in the presence of 1% thiodiglycol and 0.5 mM PMSF. The nuclei were spun down, 1 μl of the nuclei were diluted 1:100 in 1% SDS, and the absorbance was determined with a spectrotometer at 260 nm. Ten A260 units of nuclei were resuspended in 0.2 ml of 5 mM Tris·HCl (pH 7.4), 125 nM spermidine, 50 nM spermine, and 20 mM KCl in the presence of 1% thiodiglycol, 0.5 mM PMSF, and 1 mM CuSO4. The nuclei were incubated on ice for 10 min. An extraction buffer containing 20 mM Hepes, 2 mM EDTA (pH 7.5), 100 mM Li-acetate, 0.1% digitonin, and 10 mM LIS (lithium 3′, 5′-diiodosalycilate) was added slowly to the resuspended nuclei that were then incubated at room temperature for 5 min. The histone-depleted nuclei were recovered by centrifugation for 20 min at room temperature. The pellet was washed by adding 10 or 15 ml of digestion buffer, 20 mM Tris (pH 7.5), 20 mM KCl, 70 mM NaCl, 10 mM MgCl2, 125 nM spermidine, 50 nM spermine, and 20 mM KCl in the presence of 1% thiodiglycol, 0.5 mM PMSF, and 1% digitonin three to four times. After the last centrifugation, the haloes were gently removed from the bottom of the tube into a minimal volume of digestion buffer (200 μl). They were digested with 200 units of PvuII at 37°C for 3 h. The digested DNAs were separated from the matrix by diluting the reaction to a final volume of 500 μl with digestion buffer and centrifuging at 2, 500 $\times$ *g* for 10 min at 4°C. The supernatant was saved. The pellet was washed with another 300 μl of digestion buffer and vortexed or pipetted well, and the centrifugation was repeated. The matrix was in the pellet, which could be analyzed for DNA content as well. The matrix-bound DNA was extracted by adding 40 mM EDTA, 1% SDS, and 1 mg/ml protease K. The supernatants containing solubilized DNA were combined and adjusted to 0.2% SDS, 40 mM EDTA, 0.2 mg/ml protease K. The protease digestions were incubated overnight at 37°C. The DNA was purified by standard phenol-chloroform extraction and ethanol precipitation. The recovered DNA was subjected to Southern blot analysis.

Fluorescent *in Situ* Hybridization (FISH). FISH was performed based on the protocol of Lawrence *et al.* (2) with the following modifications. Raji cells transfected with DS or Raji ori plasmids were harvested on 8, 12, and 16 days after transfection. The cells were treated with 0.075 M KCl for 20 min at 37°C, fixed in methanol/acetic acid (3:1) for 30 min at room temperature, and spread on ice-cold slides. The slides were air-dried and treated with 4× SSC (1× SSC; 0.15 M NaCl, 0.015 M sodium citrate) containing 0.5% (vol/vol) nonidet P-40 (Sigma) for 30 min at 37°C, dehydrated in a cold ethanol series (70%, 80%, 90%) for 2 min each, air dried, and denatured in 70% formamide–2× SSC for 2 min at 72°C. The slides were dehydrated in a cold ethanol series again and air dried. The hybridization probes for detection of transfected plasmids were generated by nick translation using biotin-11-dUTP (Roche). Twenty micrograms of a probe was ethanol-precipitated in the presence of 6 μg of salmon sperm DNA (Eppendorf) and 4 μg of human Cot-I DNA (Invitrogen), resuspended in CEP hybridization buffer (Vysis), and incubated for 10 min at 70°C, cooled for 5 min at 4°C, and incubated at 37°C for 1 h. A hybridization mix containing 5 ng of probe was placed on each sample and incubated overnight at 37°C in a moist chamber. The slides were washed twice in 2× SSC containing 50% formamide for 30 min at 50°C. The hybridized probes were revealed by incubation with 30 μl of detection solution containing streptavidin conjugated to Cy3 (Cytocell) for 20 min at 37°C. The slides were washed twice in 4× SSC containing 0.05% Triton X-100 (Sigma) for 5 min at room temperature. The chromosomes were counterstained by mounting medium containing diamidino-2-phenylindole (DAPI) (Vector). The images were acquired by an inverted fluorescence microscope (Axiovert 200M, Zeiss) equipped with a digital CCD camera (Axiocam HRm; Zeiss) and a z-motor. Cy3 and DAPI were visualized by using specific, individual filter sets for Texas-red and DAPI. Images were collected by a magnification ×63, 1.4 N.A. oil-immersion objective lens (Plan Prochromaro; Zeiss) with 5–10 slices of Z-stacks, with exposures of 0.01 s and 1 s to detect Cy3 and DAPI, respectively. Axiovison software (Zeiss) was used for acquisition and analysis of the images.

1. Wang J, Lindner SE, Leight ER, Sugden B (2006) Essential elements of a licensed, mammalian plasmid origin of DNA synthesis. *Mol Cell Biol* 26:1124–1134.

2. Lawrence, JB, Singer RH (1985) Quantitative analysis of in situ hybridization methods for the detection of actin gene expression. *Nucleic Acids Res* 13:1777–1799.

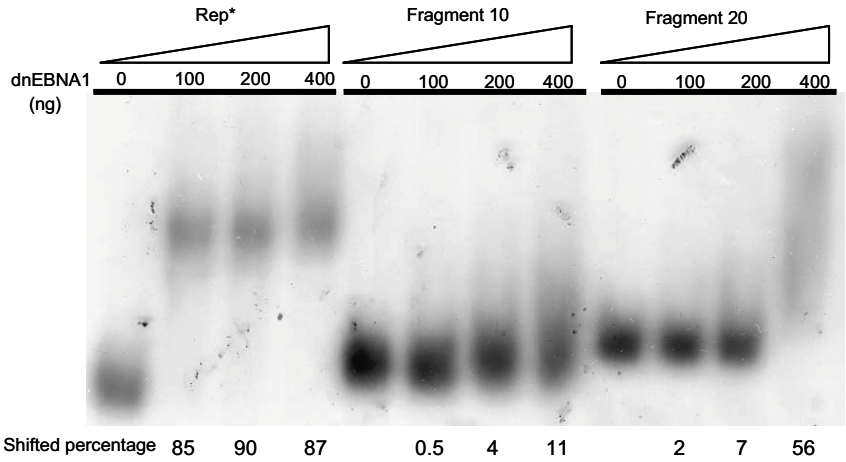

Fig. S1. No EBNA1-binding site can be detected in Raji ori. We assayed Raji ori to determine whether EBNA1 binds it as EBNA1 binds *oriP* and Rep*. We tested 36 overlapping fragments (listed in Table S1), which are ≈600 bp in length on average, encompass the 14 kbp of Raji ori, and were obtained either by digestion of cloned Raji EBV DNA or PCR. The Raji ori fragments were end-labeled and incubated with increasing amounts of the purified derivative, His-tagged DNA-binding and dimerization domain of EBNA1(dnEBNA1). Electrophoretic mobility shift assays (EMSA) were performed to identify EBNA1-binding sites. The 600-bp Rep* fragment, an EBNA1-dependent origin that has two EBNA1-binding sites, was used as a positive control (1). No sites with 10% of Rep*'s efficiency to bind to EBNA1 could be detected throughout the entire Raji ori region, whereas 50% of Rep* can be shifted by 50 ng (data not shown), and 85% of Rep* can be shifted by 100 ng of dnEBNA1 in the EMSA. Two sample fragments, 10 and 20, are shown in the gel. Because at least three copies of Rep* are required to support extrachromosomal replication ≈83% as efficiently as DS (2), we conclude that DNA replication in Raji ori is independent of EBNA1's direct binding. Raji ori is more likely to function akin to cellular origins, such as β-globin, in an EBNA1-independent manner.

1. Wang J, Lindner SE, Leight ER, Sugden B (2006) Essential elements of a licensed, mammalian plasmid origin of DNA synthesis. *Mol Cell Biol* 26:1124–1134.

2. Kirchmaier AL, Sugden B (1995) Plasmid maintenance of derivatives of oriP of Epstein–Barr virus. *J Virol* 69:1280–1283.

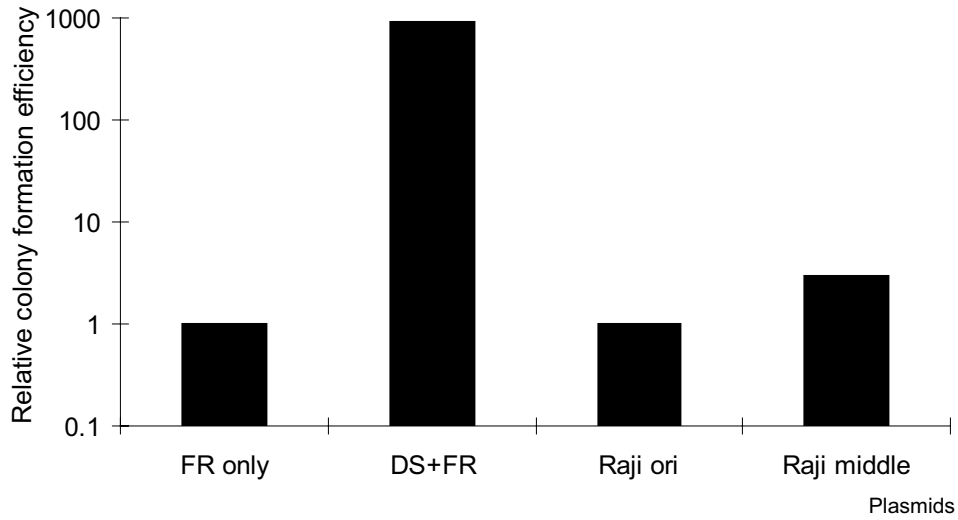

Fig. S2. Raji ori and Raji middle cannot be established as extrachromosomal replicons in 293/EBNA1 cells. Raji ori or Raji middle plasmids were transfected into 293 cells stably expressing EBNA1. The transfected cells were selected with puromycin for 3 weeks. The activity of the plasmid origins to support long-term replication was determined by the ability of the transfected cells to give rise to drug-resistant colonies. The efficiency of cells transfected with FR plasmids giving rise to drug-resistant colonies that result from integration was set to be 1. Cells transfected with either Raji ori or Raji middle plasmids did not give rise to colonies more efficiently than cells transfected with the FR plasmids.

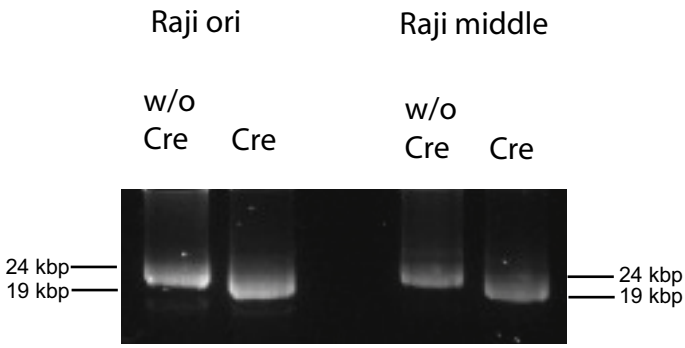

Fig. S3. Raji ori and Raji middle plasmids maintain extrachromosomal status after removal of the DS cassette. To test whether Raji ori- and Raji middle-containing plasmids maintain their extrachromosomal status after the removal of the DS cassette, cell clones harboring either Raji ori or Raji middle plasmids were expanded for 3 weeks after the introduction of Cre recombinase. Extrachromosomal DNAs were harvested by the alkaline lysis method. Harvested DNAs were transformed into *E. coli*, and plasmid DNAs were recovered. Agarose-gel eletrophoresis was use to evaluate the presence of plasmid DNAs and the successful deletion of the DS cassette as monitored by a change in the length of the plasmids (24 kbp with the DS cassette; 19 kbp after deletion of the DS cassette).

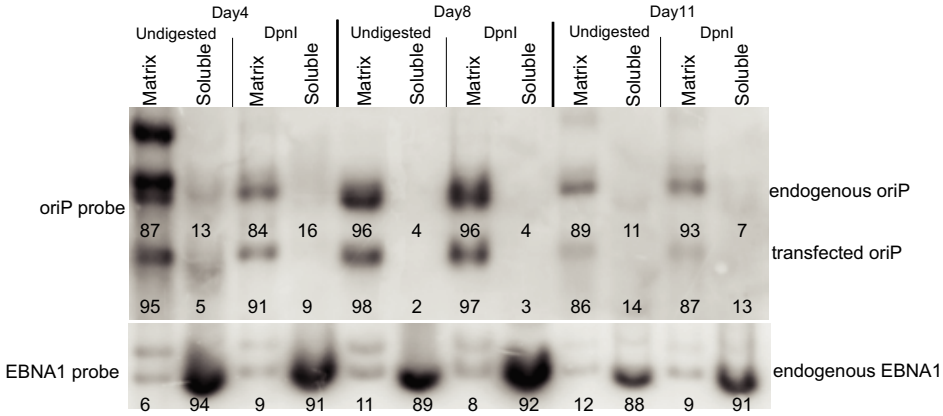

Fig. S4. All newly introduced plasmids localize to the nuclear matrix. *oriP* plasmids were transfected into Raji cells. The nuclear matrix was prepared from the transfected cells 4, 8, and 11 days after transfection. DNAs that were copurified with the nuclear matrix were detected by Southern blot analysis with probes for *oriP* and EBNA1. The detection of endogenous *oriP* and EBNA1 sequences served as internal controls because *oriP* has been shown to be in fractions containing the nuclear matrix, whereas the EBNA1 gene is not (1). The numbers below each lane indicate the percentage of the signal present in either the matrix or soluble fractions when compared with the total signal. On days 4, 8, and 11, 85–98% of the replicated DNA was detected in the matrix fraction. The percentage of the replicated DNA in the matrix fraction did not increase through out the time course. This data shows that established plasmids localize to the nuclear matrix; however, localization to the nuclear matrix is not a rate-limiting step for establishment.

1. Jankelevich S, Kolman JL, Bodnar JW, Miller G (1992) A nuclear matrix attachment region organizes the Epstein–Barr viral plasmid in Raji cells into a single DNA domain. *EMBO J* 11:1165–1176.

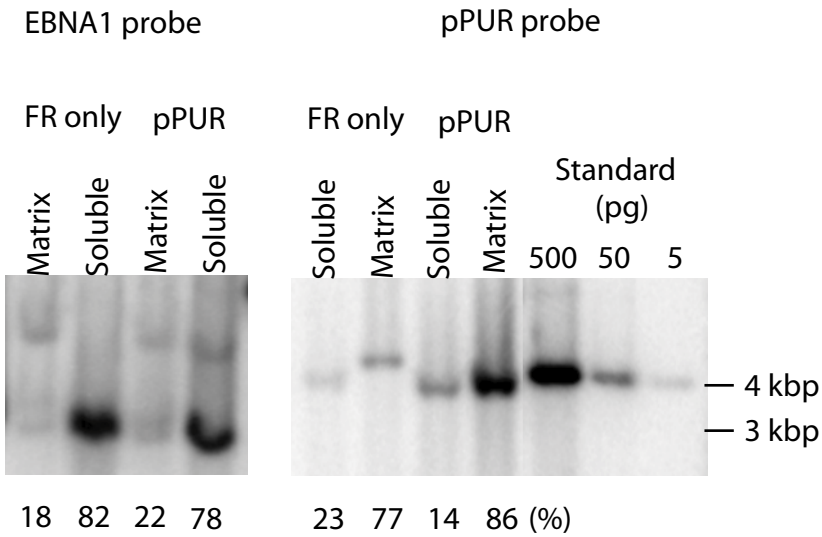

Fig. S5. Newly introduced plasmids localize to the nuclear matrix independently of their ability to support DNA synthesis. pPUR- and FR-only plasmids, which contain no initiation sites for DNA synthesis in mammalian cells, were transfected into Raji cells. The nuclear matrix was prepared from the transfected cells 4days after transfection. DNAs that were copurified with the nuclear matrix were detected by Southern blot analysis with a pPUR probe and an EBNA1 probe. The detection of EBNA1 sequences serves as the internal control for viral sequences that do not localize to the nuclear matrix (1). The numbers below the blot indicate the percentage of the signal present in either the matrix or the soluble fraction when compared with the total. Seventy-five percent to 85% of the introduced DNAs were detected in the matrix fraction. This data indicates that newly introduced plasmids localize to the nuclear matrix independent of their ability to support DNA synthesis.

1. Jankelevich S, Kolman JL, Bodnar JW, Miller G (1992) A nuclear matrix attachment region organizes the Epstein–Barr viral plasmid in Raji cells into a single DNA domain. *EMBO J* 11:1165–1176.

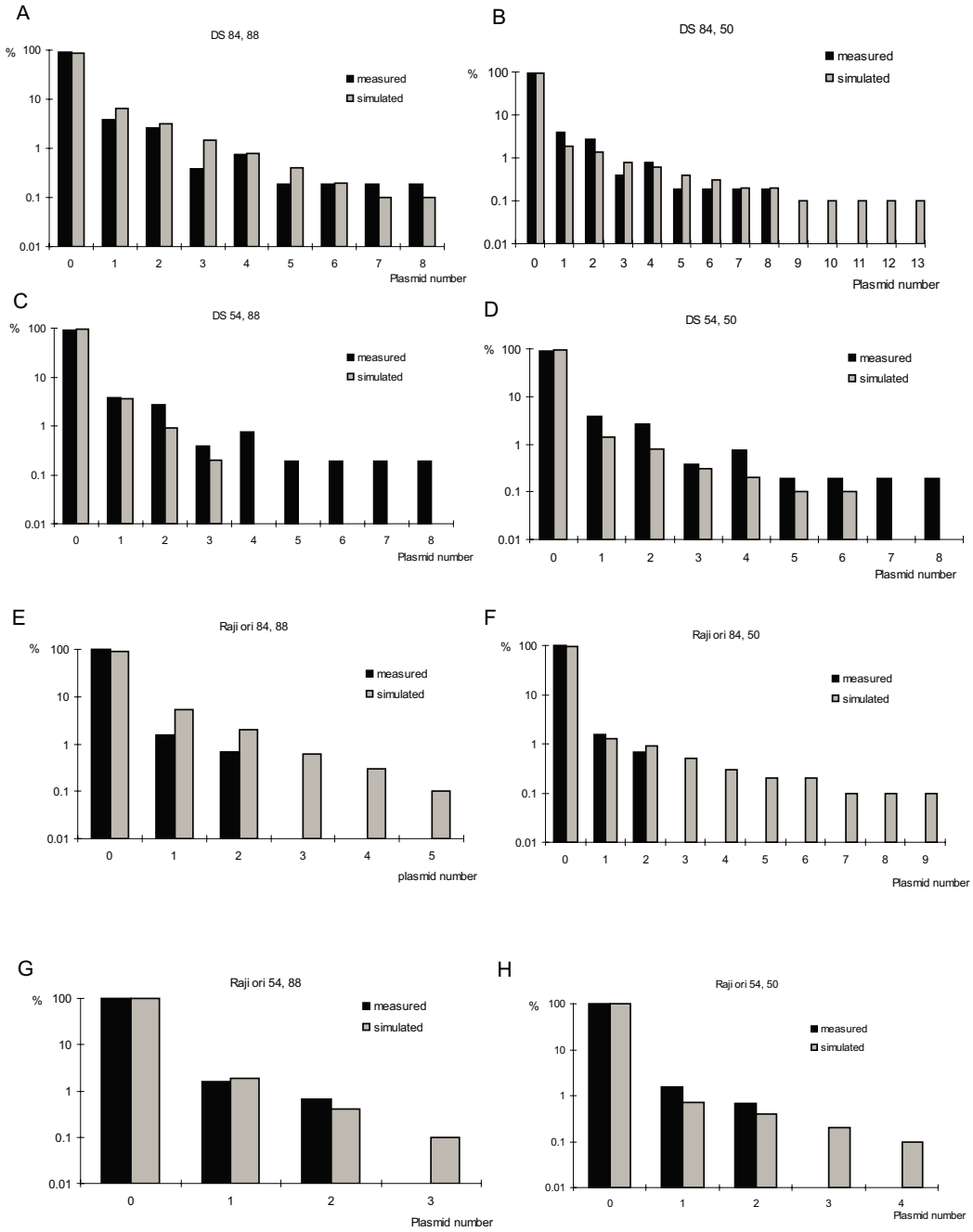

Fig. S6. Shown are comparisons of the measured and computer simulated distributions of plasmids with DS or Raji ori in cells 16 days after transfection. The simulations in *A–D* are based on the distribution of DS plasmids measured 8 days after transfection (Fig. 4), with the efficiency of DNA synthesis being (in *A*) set to 84% per plasmid per S phase and faithful partitioning being set to 88% [the values determined experimentally for DS (1)]; (in *B*) the efficiency of DNA synthesis being set to 84% per plasmid per S phase and the faithful partitioning being set to 50%; (in *C*) the efficiency of DNA synthesis being set to 54% per plasmid per S phase and the faithful partitioning being set to 88%; (in D) the efficiency of DNA synthesis being set to 54% per plasmid per S phase and the faithful partitioning being set to 50%. The simulations in *E–H* are based on the distribution of Raji ori plasmids measured 8 days after transfection (Fig. 4) with the efficiency of DNA synthesis being (in *E*) set to 84% per plasmid per S phase and the faithful partitioning being set to 88%; (in *F*) the efficiency of DNA synthesis being set to 84% per plasmid per S phase and the faithful partitioning being set to 50%; (in *G*) the efficiency of DNA synthesis being set to 54% per plasmid per S phase and the faithful partitioning being set to 88%; (in *H*) the efficiency of DNA synthesis being set to 54% per plasmid per S phase and the faithful partitioning being set to 50%. The Y-axes represent the percent of cells with a given plasmid number. The measured distributions are simulated best with the DS plasmids being synthesized approximately with an efficiency of 84% per plasmid per S phase and being partitioned faithfully 88% of the time. The measured distributions for Raji ori plasmids are simulated best when they are synthesized with an efficiency of 54% per plasmid per S phase and partitioned faithfully 88% of the time. The simulations were conducted with the program described in ref. 1 and are available at http://mcardle.oncology.wisc.edu/sugden/simulation.txt.

1. Nanbo A, Sugden A, Sugden B (2007) The coupling of synthesis and partitioning of EBV's plasmid replicon is revealed in live cells. *EMBO J* 26:4252–4262.

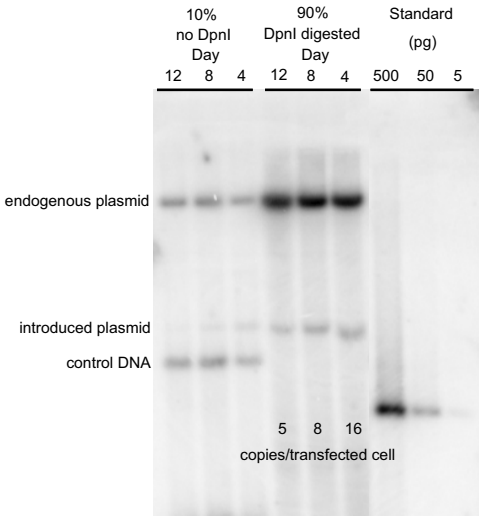

Fig. S7. Plasmids are synthesized more efficiently after establishment than before. Raji cells that already harbor an established *oriP* plasmid were transfected with another *oriP* plasmid having a different size than the endogenous one. Cells were cultured without selection after transfection. Four, 8, and 12 days after transfection, extrachromosomal DNAs were harvested and treated with DpnI to remove unreplicated DNAs. The replicated DNAs were then detected by Southern blot analysis. The signals detected on the Southern blot were corrected for recovery and quantified by comparing them with the standard DNA signals. The numbers under the lanes of the blot are the average numbers of plasmids present in each transfected cell at the given time point. The results show that newly introduced *oriP* plasmids are lost with a rate of 15% per cell generation, which is faster than the rate of loss of the endogenous *oriP* plasmids (8% per cell generation).

Table S1. Digestion of cloned fragments of Raji EBV DNA with restriction endonucleases or PCR were performed to obtain 36 overlapping fragments of Raji ori, 600 bp in length on average; the corresponding positions on the EBV genome are listed

Fragment no.	Position on EBV genome	Fragment no.	Position on EBV genome
1	145336–145991	19	152095–152713
2	145345–145717	20	152357–153186
3	145667–146320	21	152975–153656
4	145717–146357	22	152–975-153656
5	146320–146871	23	153024–153908
6	146422–146900	24	153767–154446
7	146787–147337	25	153432–154113
8	147241–147676	26	154121–154895
9	147624–148287	27	154654–155446
10	147676–148704	28	155397–155816
11	148287–148742	29	155478–155985
12	148704–149142	30	155891–155845
13	149108–149369	31	156655–157438
14	149323–149760	32	157869–158341
15	149690–150354	33	157673–158224
16	150312–151154	34	157869–158341
17	150947–151767	35	157997–158823
18	151448–152274	36	158345–159415