The plasmid replicon of EBV consists of multiple *cis*-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element

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Plasmids containing *oriP*, the plasmid origin of Epstein-Barr virus (EBV), are replicated stably in human cells that express a single viral *trans*-acting factor, EBNA-1. Unlike plasmids of other viruses, but akin to human chromosomes, *oriP* plasmids are synthesized once per cell cycle, and are partitioned faithfully to daughter cells during mitosis. Although EBNA-1 binds multiple sites within *oriP*, its role in DNA synthesis and partitioning has been obscure. EBNA-1 lacks enzymatic activities that are present in the origin-binding proteins of other mammalian viruses, and does not interact with human cellular proteins that provide equivalent enzymatic functions. We demonstrate that plasmids with *oriP* or its constituent elements are synthesized efficiently in human cells in the absence of EBNA-1. Further, we show that human cells rapidly eliminate or destroy newly synthesized plasmids, and that both EBNA-1 and the family of repeats of *oriP* are required for *oriP* plasmids to escape this catastrophic loss. These findings indicate that EBV's plasmid replicon consists of genetic elements with distinct functions, multiple *cis*-acting elements that facilitate DNA synthesis and viral *cis/trans* elements that permit retention of replicated DNA in daughter cells. They also explain historical failures to identify mammalian origins of DNA synthesis as autonomously replicating sequences.

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Introduction

Epstein-Barr Virus (EBV) maintains its genome as a plasmid in cells that it immortalizes. Only one viral element *in cis*, *oriP*, and one viral protein, EBNA-1, are required for viral plasmid replication (Yates *et al.*, 1984, 1985; Lupton and Levine, 1985; Reisman *et al.*, 1985; Kirchmaier *et al.*, 1995). EBV replication is unlike those of other viruses in that it faithfully mimics cellular replicons: EBV and small *oriP*/EBNA-1 plasmids are synthesized only once in each S-phase (Adams, 1987; Yates and Guan, 1991). This semi-conservative DNA synthesis is coupled to efficient partitioning such that EBV plasmids are distributed accurately to daughter cells during 95–98% of all mitoses (Sugden and Warren, 1988; Kirchmaier and Sugden, 1995).

oriP plasmids also differ from other viral replicons in that the origin-binding protein of EBV, EBNA-1, does not have an ATPase or helicase activity (Frappier and O'Donnell, 1991; Middleton and Sugden, 1992). One mechanism by which EBNA-1 could contribute to *oriP* replication, in parallel with those viral replicons requiring viral helicases such as SV40 (Stahl *et al.*, 1986), BPV-1 (Yang *et al.*, 1993) and HSV-1 (Bruckner *et al.*, 1991), would be for EBNA-1 to associate with a cellular helicase and tether it to DS, the site near or at which DNA synthesis from *oriP* initiates (Gahn and Schildkraut, 1989). We searched for cellular helicases or other replication factors which could associate with EBNA-1 bound specifically to *oriP* or DS in an extensive yeast one-hybrid screen and found no candidates with a primary sequence or known function consistent with any role in DNA synthesis.

oriP is also striking for its detection as an autonomously replicating sequence (ARS). *oriP* was identified early as an ARS in mammalian cells and, despite many attempts, no mammalian cellular origin has been detected subsequently as an ARS (DePamphilis, 1996). However, some cellular DNAs when tested in conjunction with the family or repeats (FR) of *oriP*, which binds EBNA-1, do behave as ARSs in the presence of EBNA-1 (Krysan *et al.*, 1989; Masukata *et al.*, 1993). This observation indicates that FR and EBNA-1 work together to maintain DNAs that are synthesized in proliferating cells.

These singular features of EBV's replicon led us to test the possibility that EBNA-1 acts after DNA synthesis and that the initiation of DNA synthesis at *oriP* can be mediated solely by cellular enzymes. We have found that plasmids containing *oriP* or just its FR or DS elements are synthesized efficiently in cells within 48 h in the absence of EBNA-1. We have also found that these newly synthesized plasmids are lost rapidly from cells in the absence of FR and/or EBNA-1, indicating that mammalian cells have a mechanism to degrade or eliminate newly synthesized plasmids. We conclude that one of EBNA-1's roles in *oriP* replication is to prevent the degradation or elimination of newly synthesized *oriP* plasmids. Plasmids that support transcription are lost similarly in the absence of FR and EBNA-1. It is likely that the cell's capacity to destroy or eliminate newly synthesized plasmids has doomed to failure the historical attempts to identify mammalian origins as ARSs.

Results

EBNA-1 does not assemble DNA synthetic machinery at *oriP*

We used a yeast one-hybrid screen to identify human proteins localized to *oriP* or DS through EBNA-1 (Li and Herskowitz, 1993). In this assay, only yeast that contain a fused cDNA whose product can activate transcription

Fig. 1. Quantitation of plasmid DNAs synthesized in C33A cells 48 h post-transfection. The presence or absence of a co-transfected EBNA-1 expression plasmid is indicated above each set of quantitative PCRs. PCRs were performed using *DpnI*-digested Hirt extracts from 2×10^4 transfected cells. The positions of amplified fragments from the competitor DNA are indicated by 'C', and the amounts of competitor DNA used in each PCR is indicated below each lane. The amplified fragments from the *oriP* plasmid and the prokaryotic backbone plasmid are indicated by 'oriP' and 'backbone'. The numbers below each set of PCRs indicate the quantified number of plasmid molecules present per transfected cell.

Table I. Proteins that interact with EBNA-1 bound to *oriP* or DS in *S.cerevisiae* one-hybrid screens

Yeast strain	cDNA library	No. of transformants screened	cDNAs recovered (DDBJ/EMBL/GenBank NID)	No. of isolates
<i>oriP</i> -HIS3	EBV-immortalized B-lymphocytes	6×10^6	gC1q-R (g472955) EBNA-1 DNA-binding domain (g59074)	173 11
DS-HIS3	EBV-immortalized B-lymphocytes	5.1×10^6	p75 nuclear phosphoprotein (g402147) CpG island DNA	1 1
<i>oriP</i> -HIS3	K562 erythro-leukemia cells	4.3×10^6	gC1q-R (g472955) p75 nuclear phosphoprotein (g402147) Rch1 a-karyopherin (g791184) Histone H1 (g184071) Human homolog of lipocalin (g211502) Nucleolar GTP-binding protein (g179284) ORF similar to yeast SCD6 (g939713) CpG island DNA D38491 human ORF of unknown function (g559327)	24 2 2 1 1 1 1 2 1

of HIS3 through an interaction with EBNA-1 bound at *oriP* or DS can grow in the absence of histidine. Libraries of cDNAs from B-lymphocytes or K562 cells fused to the GAL4 activation domain were screened in *oriP*-HIS3 and DS-HIS3 yeast strains that expressed EBNA-1. Out of 1.53×10^7 primary transformants, 221 encoded cDNAs that scored positively, and these are listed in Table I. In light of the functions of other viral origin-binding proteins, it seemed likely that EBNA-1 bound to *oriP* would interact with components of the cellular DNA synthetic machinery. Surprisingly, the cDNAs identified do not encode proteins with known or predicted functions in DNA synthesis. This result raised the possibility that the contribution of EBNA-1 to replication of *oriP* plasmids is not during DNA synthesis. We therefore tested whether EBNA-1 is required for *oriP* DNA synthesis, by comparing the synthesis of *oriP* plasmids in cells that do not express EBNA-1 with that in cells which express EBNA-1.

Initiation of DNA synthesis at *oriP* is mediated solely by cellular proteins

A form of PCR shown to be quantitative (Kirchmaier and Sugden, 1997) was used to measure the number of newly synthesized, *DpnI*-resistant, *oriP* plasmids present 48 h after their transfection into C33A cells. The *oriP* plasmids were introduced along with an EBNA-1 expression plasmid, or a control expression plasmid. As shown in

Table II. Synthesis of *oriP* plasmids in the presence and absence of EBNA-1^a

Hours post-transfection	(+ EBNA-1		(-) EBNA-1	
	<i>oriP</i>	Backbone	<i>oriP</i>	Backbone
48 h ^b	6.8 ± 1.5	0.7 ± 0.4	10.4 ± 4.6	0.5 ± 0.2
72 h ^b	5.7 ± 0.8	≤ 0.6	2.5 ± 1	≤ 0.6
96 h ^b	9.6 ± 1.1	≤ 0.7	1.2 ± 1	≤ 0.15

^aNumbers reflect the number of *DpnI*-resistant plasmid molecules present per transfected cell.

^bData represents the average of three experiments performed in C33A cells.

Figure 1, and summarized in Table II, at 48 h post-transfection there were approximately equal numbers of newly synthesized *oriP* plasmids in the presence or absence of EBNA-1: 7–10 molecules of *oriP* plasmids were present per transfected cell, whereas control plasmids that consist of the prokaryotic backbone into which *oriP* was cloned were present at 0.3–1 molecule per transfected cell. This synthesis of *oriP* DNA in the absence of EBNA-1 is striking, and has been confirmed with five different *oriP* plasmids in the human cell lines C33A, 293 and 143B.

DpnI-resistant *oriP* plasmids synthesized in the absence of EBNA-1 can be detected by direct Southern hybridization (Figure 2). An *oriP* plasmid was introduced into

Fig. 2. Detection of *DpnI*-resistant 994, an *oriP* plasmid, synthesized in 143B cells in the absence of EBNA-1, 48 h post-transfection by Southern hybridization. *DpnI*-digested extracts of 143B cells transfected with 994 were digested with *HindIII* to linearize the *DpnI*-resistant newly synthesized DNA. *HindIII*-digested Hirt extract from 5×10^6 cells was electrophoresed alongside the indicated amounts of *HindIII*-digested 994, and detected by Southern hybridization. The position of the *DpnI*-resistant, linearized, newly synthesized *oriP* DNA is indicated. Products of *DpnI* digestion migrate at the bottom of the gel. There is ~10–15 pg of newly synthesized 994 DNA detected, corresponding to 2–3 copies of 994 per transfected cell.

143B cells, with a control plasmid but without an EBNA-1 expression plasmid, 2 days prior to Hirt extraction and analysis by Southern blot after *DpnI* digestion. Two to three *DpnI*-resistant plasmid molecules were detected per transfected cell 48 h post-transfection, whereas the control plasmid was not detected. The newly synthesized, linearized, *DpnI*-resistant plasmids were of unit length and comigrated with linearized standards, indicating that all 29 *DpnI* sites were unmethylated on each strand and, therefore, that both strands were synthesized fully (see below). The level of intact *oriP* DNA detected in 143B cells by Southern hybridization is similar to the level of *oriP* DNA quantified using competitive PCR 48 h post-transfection in these cells (Figure 6, data not shown). This result confirms that *oriP* plasmids are synthesized efficiently in the absence of EBNA-1.

***oriP* plasmids undergo at least two rounds of synthesis without EBNA-1**

DNA synthesis of *oriP* plasmids is semi-conservative (Yates and Guan, 1991), and the transfected C33A cells divide approximately every 24 h (data not shown). Because the rate at which transfected DNA enters a replicating pool is unknown but probably asynchronous, it is likely that newly synthesized *oriP* plasmids detected 2 days after transfection will be present in hemi-methylated and unmethylated pools. Fully unmethylated DNA results from two or more rounds of DNA synthesis. We characterized the specificity of digestion by *DpnI* in our assay conditions and found that chemically synthesized hemi-methylated substrates analogous to products of one round of semi-conservative synthesis are digested efficiently, whereas unmethylated substrates are not (Table III). Thus, the presence of *DpnI*-resistant *oriP* plasmids at 2 days post-transfection indicates that EBNA-1 is not required for at least two rounds of *oriP* plasmid synthesis.

Density labeling of newly synthesized *oriP* plasmids

To corroborate the observation that EBNA-1 is not required for *oriP* plasmid synthesis, we measured DNA synthesis from *oriP* by incorporation of a density label. Newly synthesized plasmids were labeled with bromodeoxyuridine (BrdU) to distinguish between light:light (LL) unreplicated DNA, heavy:light (HL) products from one or two rounds of synthesis, and heavy:heavy (HH) products from two or more rounds of synthesis. The profiles of total cellular DNA, *oriP* DNA and control DNA recovered from cells labeled with BrdU for 60 h in the presence or absence of EBNA-1 and separated by equilibrium density centrifugation in CsCl are shown in Figure 3.

Between 55 and 65% of the transfected cells divided once within the 60 h labeling period as measured by the ratio of HL:LL genomic DNA. It is known that treatment with BrdU even in the presence of deoxycytidine can retard the cell cycle (Meuth and Green, 1974). The HH:HL genomic DNA ratio indicates that between 25 and 35% of the cells that underwent DNA synthesis once, underwent it a second time. *oriP* plasmids were present in HL and HH fractions even in the absence of EBNA-1, confirming that EBNA-1 is not required for *oriP* synthesis. Furthermore, *oriP* plasmids and genomic DNA were distributed similarly in both gradients, demonstrating that semi-conservative replication of *oriP* plasmids (Yates and Guan, 1991) does not require EBNA-1. Prokaryotic backbone plasmids were synthesized to lower levels than *oriP* plasmids such that they are present at ~10% the level of *oriP* plasmids in HH fractions. The inefficient replication of prokaryotic plasmids has been observed previously in mammalian cell lines (Wysokenski and Yates, 1989). The presence of *oriP* increases replication of plasmid DNAs substantially relative to that of the prokaryotic backbone. When the LL plasmid DNAs in Figure 3 observed in the absence of EBNA-1 are used for normalization, there are 8.7-fold more *oriP* plasmids in HH fractions than prokaryotic backbone plasmids.

Finally in the density labeling experiment, we compared the distribution of *oriP* plasmids in the presence and absence of EBNA-1. When the LL DNAs are used for normalization, there is 2.5-fold more *oriP* DNA in the HH fractions in the presence of EBNA-1 than in its absence. This analysis indicates that EBNA-1 increased by 1.6-fold the frequency with which *oriP* plasmids were synthesized in each of the first two cell cycles. If this increased frequency of synthesis were constant for two subsequent cell cycles, then 25% of the *oriP* DNA replication detected at 48 h would be present at 96 h in the absence of EBNA-1. We tested this prediction.

EBNA-1 prevents catastrophic loss of *oriP* plasmids

Newly synthesized, *DpnI*-resistant *oriP* plasmids present in C33A cells 3 or 4 days after transfection were quantified to measure the level of replicated *oriP* plasmids present after additional cell cycles. *oriP* plasmids continued to be synthesized and maintained in cells that expressed EBNA-1, such that they were present at ~7–10 copies per transfected cell at both time points tested (Figure 4). In sharp contrast, in the absence of EBNA-1, levels of *oriP* plasmids were drastically reduced, such that 10% of the

Fig. 3. Density gradient profiles of *oriP* plasmids labeled with BrdU in 293 cells. *oriP* plasmids were transfected into 293 cells along with the prokaryotic backbone plasmid. The presence or absence of a co-transfected EBNA-1 expression plasmid is indicated within each graph. From the beginning of the transfection procedure, the 293 cells were labeled with BrdU for 60 h, after which total cellular DNA from 10⁷–2×10⁷ cells was separated on a CsCl gradient. The amounts of total DNA, *oriP* DNA and prokaryotic backbone DNA present in each fraction were determined by spectroscopy (total DNA) or Southern hybridization and plotted against the fraction number to produce each of the two graphs. The distribution profiles corresponding to total DNA, *oriP* DNA and prokaryotic backbone DNA are indicated in the graph legend. Note that the amounts of genomic DNA are plotted on a linear scale, while the amounts of plasmid DNA are plotted on a logarithmic scale. The amounts of DNA present in LL, HL and HH fractions are indicated below each graph. The length of the isolated cellular DNAs is at least 10-fold that of the plasmids, resulting in less diffusion and smaller peak width for the cellular DNA.

Table III. Activity of *DpnI* on methylated, hemi-methylated and unmethylatedDNAs

Substrate ^a	Input digested (%)
5' gtcGATCgtcactGATCagtccaGATCgtaacgGATCtccgatGATC CTAGcagtgaCTAGtcaggtCTAGcattgctCTAGaggctaCTAGgcg 5'	97
5' gtcGATCgtcactGATCagtccaGATCgtaacgGATCtccgatGATC CTAGcagtgaCTAGtcaggtCTAGcattgctCTAGaggctaCTAGgcg 5'	95 ^b ; 97 ^c
5' gtcGATCgtcactGATCagtccaGATCgtaacgGATCtccgatGATC CTAGcagtgaCTAGtcaggtCTAGcattgctCTAGaggctaCTAGgcg 5'	3

^aSubstrates and digestion conditions are described in Materials and methods. *dam* methylase recognition sites are shown in upper case and N⁶-methylated deoxyadenosines are underlined.
^bDigestion of the methylated strand.
^cDigestion of the unmethylated strand.

oriP DNA detected at 48 h is detected by 96 h post-transfection in C33A cells. Loss is also indicated in that <25% of the *oriP* DNA detected at 48 h was present one cell cycle later at 72 h. This rate of loss is more dramatic in 143B cells, where only 1–5% of the *oriP* DNA detected at 48 h in the absence of EBNA-1 can be detected at 96 h post-transfection (Figure 6 and data not shown). The reduction in levels of replicated *oriP* plasmids in 143B cells and C33A cells is more rapid than that predicted by measuring the 1.6-fold effect of EBNA-1 on *oriP* synthesis during the first two cell cycles, or the dilution to 25% expected over two cell cycles if plasmids were no longer

synthesized, but distributed to daughter cells. The rate at which replicated *oriP* DNA is lost in the absence of EBNA-1 varies in different cells. In contrast to the rapid loss observed in 143B and C33A cells, newly synthesized *oriP* plasmids are lost from transfected 293 cells at a rate of ~50% per day (data not shown). At least 143B and C33A cells, and presumably most human cell lines, must actively eliminate or destroy plasmids introduced into them. In such cell lines, the mechanism by which EBNA-1 maintains *oriP* plasmids enables them to evade this active process of loss.
This conclusion has been verified independently by

Fig. 4. Quantitation of DNAs synthesized in C33A cells 72 h (A) and 96 h (B) after transfection. The presence or absence of a co-transfected EBNA-1 expression plasmid is indicated above each set of quantitative PCRs. PCRs were performed using the equivalent of 2.5×10^4 transfected cells. The images are labeled as in Figure 1.

measuring the rates of loss of luciferase activity in different cells transfected with replicating and non-replicating plasmids that express luciferase. Because the luciferase mRNA and protein have short half-lives (Thompson *et al.*, 1991), detection of luciferase activity reflects the presence of plasmid DNAs that can express luciferase. Luciferase activity dependent upon transcription from the plasmid RSVL, which lacks EBNA-1-binding sites, is lost rapidly over two cell divisions in 143B, C33A and 293 cells, with a decreasing order of rates similar to the rates observed for loss of replicated *oriP* in the absence of EBNA-1 (Figure 5). In contrast, luciferase activity from a vector containing *oriP*, 1033, is maintained when an EBNA-1 expression plasmid is co-transfected with the reporter plasmid. This observation supports our contention that EBNA-1 functions to prevent the loss of *oriP* plasmids in proliferating human cells.

EBNA-1 and FR can maintain an origin distinct from DS

When stably maintained in cells that express EBNA-1, DNA synthesis from *oriP* plasmids initiates at or near the DS (Gahn and Schildkraut, 1989). We have demonstrated that at least one other region of EBV close to the DS, Rep*, supports DNA synthesis albeit to a lower extent than *oriP*, such that plasmids that contain a trimer of Rep* and FR are stably maintained in proliferating cells that express EBNA-1 (Kirchmaier and Sugden, 1998). These plasmids are lost at 96 h in the absence of EBNA-1 (Kirchmaier and Sugden, 1998; Figure 6). We tested whether a plasmid with the FR and a trimer of Rep* replicates at 48 h, and found that it does (Figure 6). Therefore, a plasmid with FR and a DNA element other than DS replicates in the absence of EBNA-1, is lost by 96 h in the absence of EBNA-1, but is maintained in

proliferating cells in the presence of EBNA-1. This origin, Rep*, supports DNA synthesis without itself binding EBNA-1 (Kirchmaier and Sugden, 1998). That FR *in cis* and EBNA-1 *in trans* act to maintain plasmid replication with either DS or Rep* present *in cis* is consistent with the findings that FR and EBNA-1 also maintain plasmids containing cellular DNAs which support DNA synthesis (Krysan *et al.*, 1989; Masukata *et al.*, 1993). The levels of *oriP* and Rep* DNAs synthesized in the absence of EBNA-1 by 48 h are within 2-fold of that synthesized in its presence at 96 h, and stably thereafter, indicating that DNA synthesis *per se* is similar under both conditions (Table II, Figures 1, 3, 4 and 6; Kirchmaier and Sugden, 1995, 1997, 1998).

Distinct elements within *oriP* individually facilitate DNA synthesis

Plasmids containing FR or DS, and sequences between FR and DS as described in Materials and methods, were tested for their support of DNA synthesis in the presence or absence of EBNA-1. Both individually supported DNA synthesis in the absence of EBNA-1 48 h after their introduction into cells. Neither supported continued replication at 96 h in the presence or absence of EBNA-1 (Figure 7, data not shown). These findings underscore that *oriP* consists of multiple elements, FR, DS and probably Rep*, which individually can facilitate DNA synthesis when introduced into a prokaryotic backbone. It is in this context that we describe *oriP* as having multiple elements that facilitate DNA synthesis. DNA synthesis of plasmids is dependent on these elements, occurs in the absence of EBNA-1 and can be mediated solely by cellular factors.

Discussion

Although *oriP* functions as a cell cycle-regulated ARS in human cells expressing EBNA-1, the contributions of EBNA-1 to its synthesis and maintenance have been enigmatic. Unlike several other viral origin-binding proteins, EBNA-1 does not function as a helicase, and in a yeast one-hybrid screen fails to interact directly with cellular helicases or other obvious replication factors.

We have now demonstrated, using two independent techniques, that *oriP* is recognized directly by the human DNA synthetic machinery, indicating why *oriP*, but not most other viral origins, is replicated once per cell cycle, and in synchrony with cellular chromosomes in proliferating cells. We note that the level of *oriP* DNA synthesized in the absence of EBNA-1 at 48 h post-transfection is within 2-fold of the levels of *oriP* DNA synthesized in cells that stably express EBNA-1 at 96 h post-transfection (Kirchmaier and Sugden, 1997), or in stably transfected cells (Kirchmaier and Sugden, 1995), indicating that levels of DNA synthesis from *oriP* plasmids are independent of EBNA-1. Akin to chromosomal origins, *oriP* also contains multiple regions within it that independently facilitate DNA synthesis *in cis*. These regions may be the sites at which DNA synthesis initiates, or they may support it elsewhere *in cis*. We have therefore referred to these sites as elements that facilitate DNA synthesis. However, one of these sequences, the DS, is an origin of DNA synthesis (Gahn and Schildkraut, 1989), and it is therefore likely that the other regions also function as origins at 48 h post-

Fig. 5. (A) Transcribed DNAs are lost at different rates in 293, C33A and 143B cells. RSVL was transfected into 293, C33A or 143B cells, and luciferase activity was measured at 24 h intervals from 48 h to 120 h post-transfection. The relative light units (RLU) observed at 48 h post-transfection were used to normalize the RLU observed at later time points. The decrease in luciferase activity observed over time in C33A and 143B cells represents an average of four experiments each, while the decrease observed in 293 cells represents a single experiment. Samples from 293 cells are represented by the closed squares, samples from C33A cells by the plus symbol, and samples from 143B cells by the open circles. (B) EBNA-1 facilitates the maintenance of transcribed *oriP* plasmids in proliferating cells. The *oriP*-containing luciferase reporter 1033 was transfected into 293, C33A and 143B cells along with 1553, an EBNA-1 expression plasmid. Luciferase activity was measured at 24 h intervals, and plotted as described for (A). Measurements in C33A and 143B cells represent the average of two experiments each, while the measurements in 293 cells represent a single experiment. Samples are distinguished by the same symbols as used in (A).

transfection. Two of these regions, FR and DS, contain sites that are bound by EBNA-1, and presumably bound by cellular proteins that bind EBNA-1 recognition sites (Wen *et al.*, 1990), while the third, Rep*, does not. Thus, while components of the cellular DNA synthetic machinery may bind EBNA-1 recognition sites, sequences that lack such sites also facilitate DNA synthesis.

Maintenance of newly synthesized *oriP* plasmids requires both EBNA-1 and FR; the absence of either results in rapid plasmid loss. We infer from this observation that human cells possess an active process by which inappropriately synthesized DNAs are destroyed or eliminated. This eradication also affects DNAs which are transcribed, but probably does not affect DNAs which have not homed to specific cellular compartments in which they act functionally. While newly synthesized *DpnI*-resistant plasmids were not detected by five generations after transfection, unreplicated plasmids have been shown to persist in mammalian cells for as many as 20 generations after transfection (Biamonti *et al.*, 1985). The process by which plasmid DNAs are eliminated from human cells occurs at differing rates in different cell lines. We note that the synthesis of extrachromosomal plasmids shortly after their introduction has been observed in 293 cells (Masukata *et al.*, 1993), a cell line in which the rate of loss of transcribed or newly synthesized DNA is ~50% per generation as contrasted to the 70 or 80% per generation observed in C33A and 143B cells, respectively. The observed loss of transfected plasmid DNA reflects at least two processes whose rates may vary between cell lines. The rate at which plasmid DNAs enter cellular compartments where they may be transcribed or synthesized, and the rate at which transcribed or newly synthesized plasmids are eliminated are each likely to depend upon the recipient cell. The cellular process that causes catastrophic loss of newly synthesized extrachromosomal plasmids explains the historical failure to identify mammalian ARS elements using plasmid assays (Biamonti *et al.*, 1985; DePamphilis, 1996). Far from being the *de facto* behavior of extrachro-

somal plasmids in human cells, stable replication not only requires an origin of DNA synthesis, but also requires an active plasmid maintenance function. Consistent with this interpretation, FR-containing plasmids with putative genomic origins are synthesized and maintained in EBNA-1-expressing cells (Krysan *et al.*, 1989; Masukata *et al.*, 1993). It is interesting to note that these chimeric plasmids did contain the FR and one half of Rep* which may have contributed to their support of DNA synthesis. We speculate that the cell's ability to eradicate newly synthesized exogenous DNAs that lack an equivalent of FR/EBNA-1 reflects a eukaryotic equivalent of the prokaryotic restriction/modification system evolved to rid the host of foreign, infectious DNA.

The mechanism by which EBNA-1 and FR prevent the catastrophic loss of newly synthesized *oriP* plasmids is unknown. The biochemical properties of EBNA-1 do, however, combine to provide a model for this maintenance function. EBNA-1 binds DNA site-specifically (Rawlins *et al.*, 1985; Ambinder *et al.*, 1990, 1991; Bochkarev *et al.*, 1996) and links bound DNAs through protein-protein associations mediated by its multiple 'linking' domains (Laine and Frappier, 1995; Mackey *et al.*, 1995; Mackey and Sugden, 1997). On binding site-specifically, both to FR and to hypothetical sites on host chromosomes, EBNA-1 molecules can link EBV or *oriP* plasmids to chromatin to ensure their retention in daughter nuclei at the end of mitosis. In fact, the catastrophic loss of plasmid DNAs synthesized in the absence of EBNA-1 may reflect their being relegated to the cytoplasm after mitosis, perhaps in a manner akin to the loss of double-minute chromosomes (Von Hoff *et al.*, 1992; Shimizu *et al.*, 1998). This model is consistent with the observations that EBNA-1 (Grogan *et al.*, 1983), EBV DNA (Delecluse *et al.*, 1993) and large *oriP* plasmids (Simpson *et al.*, 1996) associate with mitotic chromosomes, and that EBNA-1 enhances the retention of FR-containing DNAs in human cells (Middleton and Sugden, 1994). We note, however, that EBV DNA or *oriP* DNA does not consistently stain symmetrically on pairs

Fig. 6. Quantitation of plasmids bearing FR and a trimer of Rep*, FR+Rep* (A and C), or *oriP* (B and D), synthesized in 143B cells 48 h (A and B) and 96 h (C and D) post-transfection. The presence or absence of a co-transfected EBNA-1 expression plasmid is indicated above each set of quantitative PCRs. PCRs were performed using Hirt extract from 2×10^5 transfected cells. The gels are labeled as in Figure 1.

of sister chromatids. Therefore, while association with mitotic chromosomes may contribute toward maintenance of *oriP* plasmids, it is unlikely to be the mechanism by which *oriP* plasmids are segregated equally in proliferating cells that express EBNA-1. Studies designed to elucidate the function of EBNA-1 and FR will probably also illuminate the mechanism by which other non-centromeric eukaryotic plasmids, such as the 2μ plasmid of yeast (Wu *et al.*, 1987; Veit and Fangman, 1988), or plasmids bearing the origin of BPV-1 (Piirsoo *et al.*, 1996; Lehman and Botchan, 1998) are maintained.

Another facet of *oriP* not considered in this model is

Fig. 7. Quantitation of DS-containing plasmids synthesized in C33A cells 48 h (A) and 96 h (B) post-transfection. The presence or absence of a co-transfected EBNA-1 expression plasmid is indicated above each set of quantitative PCRs. PCRs on samples harvested 48 h post-transfection were performed using Hirt extract from 5×10^4 transfected cells. Processed Hirt extracts from 1×10^5 transfected cells were used in quantitative PCRs on samples harvested 96 h post-transfection. (C) Quantitation of FR-containing plasmids synthesized in 143B cells 48 and 96 h post-transfection in the absence of EBNA-1. PCRs were performed using Hirt extract from 2×10^5 transfected cells. The gels are labeled as in Figure 1.

that DS has binding sites for EBNA-1 (Baer *et al.*, 1984; Hearing *et al.*, 1992; Hsieh *et al.*, 1993). Not all DNAs which may mediate initiation of DNA synthesis and can be maintained by FR and EBNA-1 have binding sites for EBNA-1 (Kirchmaier and Sugden, 1998). FR is a barrier to replication fork migration (Gahn and Schildkraut, 1989), and the binding sites for EBNA-1 in DS may allow *oriP* to remain tethered to chromosomal sites via EBNA-1's linking while fork migration displaces EBNA-1 at FR. It is also possible that EBNA-1 bound to DS may augment synthesis of *oriP* in a manner akin to the function of the transcription factors ABF1 or GAL4 at ARS1 (Marahrens and Stillman, 1992), consistent with the observation that individual EBNA-1-binding sites within DS can be mutated without loss of origin function (Harrison *et al.*, 1994). It has been shown that the position of ARS1 on a plasmid relative to the positioned nucleosomes on that plasmid significantly affects DNA synthesis from ARS1 (Simpson, 1990). In this light, we speculate that EBNA-1 bound at DS may facilitate the appropriate positioning of nucleosomes

around DS to ensure efficient recognition of *oriP* as an origin in sequential cell cycles.

oriP is a striking origin in that it contains multiple regions that independently facilitate DNA synthesis. Yet it is compact, and contains a second element that ensures its preservation and re-replication in sequential cell cycles.

Materials and methods

Yeast one-hybrid screen

We constructed two strains of *Saccharomyces cerevisiae*, Y1526 and Y1617, which contained integrated DS-HIS3 and *oriP*-HIS3 reporter constructions, respectively. A functional derivative of EBNA-1 which lacks all but 15 residues of the internal glycine-glycine-alanine repeat (Yates *et al.*, 1985) was expressed from the 2 μ plasmid pAS1-Tet (DDBJ/EMBL/GenBank accession No. g1236518) in these strains. An EBV-immortalized B-lymphocyte library (Durfee *et al.*, 1993) and a commercially available human K562 erythroleukemia cDNA library (Clontech) in pACT or pACT2 were transformed into these strains using protocols described elsewhere (Sandberg *et al.*, 1997). Transformants were selected for growth in the absence of histidine. The stringency of this selection was raised by growing the transformed yeast in the presence of 50 mM 3-amino-1,2,4-triazole (3AT), which is an inhibitor of imidazole glycerol phosphate dehydratase, the enzyme encoded by the *HIS3* gene. Plasmid DNAs recovered from positive colonies were transformed back into the same strain to confirm the positive phenotype, after which they were transformed into *Escherichia coli* DH5 α from where they were isolated for sequence analysis.

DNA constructions

Plasmid 1553 was used to express EBNA-1 (Aiyar and Sugden, 1998). pEQ176, used to express β -galactosidase from the CMV-IE promoter, has been described previously (Schleiss *et al.*, 1991). pcDNA3 was obtained from Invitrogen. DNA synthesis was monitored using plasmids 994 (Kirchmaier and Sugden, 1995), 1676 (*oriP* without Rep*), 1677 (contains FR and sequences between FR and DS until the *EcoRV* recognition site), 1925 (FR and three copies of Rep*) (Kirchmaier and Sugden, 1998) and 1920. Plasmid 1920 was derived by cloning *oriP* and the TK-*neo* cassette from 994 into a prokaryotic backbone that contains a derivative of the ColE1 origin and a *supF* marker for selection in *E. coli*. The DS-containing plasmid 1938 used in our studies was constructed by deleting FR from 1920. Plasmid 1938 also contains sequences between FR and DS from the *MluI* recognition site to the *EcoRV* recognition site. Plasmid 1878, which does not contain *oriP* and which can be distinguished from 1920 by a length polymorphism in the *neo*^R gene, was used as the backbone control plasmid. Plasmid 1878 was constructed by cloning the TK-*neo* cassette from 1381 (Kirchmaier and Sugden, 1997) into the same backbone as 1920. Plasmids 1920, 1938 and 1878 were propagated in *E. coli* MC1061/P3 using conditions described previously (Aiyar *et al.*, 1996). Plasmid 1380, that is distinguished from 1878 and 1920 by a second length polymorphism in its *neo*^R gene, was used as competitor during quantitative PCRs (Kirchmaier and Sugden, 1997). Plasmids RSVL (RSV LTR-luciferase) (de Wet *et al.*, 1987) and 1033 (*oriP*-BamHI-C-luciferase) (Kirchmaier and Sugden, 1997) were used as transcription reporters. Transfection efficiencies were measured using pEQ176 or pEGFP.

Cell culture and transfections

The human cell lines 293 (Graham *et al.*, 1977), C33A (Yee *et al.*, 1985) and 143B (Bacchetti and Graham, 1977) were used in this study. The 293 cells were grown in DMEM/HG supplemented with 10% fetal calf serum and antibiotics. C33A and 143B cells were grown in DMEM/HG supplemented with 10% calf serum and antibiotics. Plasmids were introduced into cells by the calcium phosphate method (Sambrook *et al.*, 1989) or by electroporation (Knutson and Yee, 1987).

Measurement of DNA synthesis by quantitative PCR

Plasmids 1920 and 1878 (10 μ g of each) were transfected together with 1553 or pEQ176 into 4 $\times$ 10⁶ C33A cells, or electroporated into 10⁷ 143B cells. Plasmid DNAs were harvested from cells at the indicated times using the Hirt extraction procedure (Hirt, 1967). Supernatants were digested with RNase A (100 μ g/ml) and proteinase K (200 μ g/ml), and then extracted sequentially with phenol and phenol:chloroform:isoamyl alcohol (25:24:1). Precipitated samples were resuspended at

1 $\times$ 10³ cell equivalents/ μ l in 1 $\times$ KGB and digested with 1 U of *DpnI* per 2.5 $\times$ 10³–5 $\times$ 10³ cell equivalents for a minimum of 48 h to digest selectively the *dam*-methylated transfected DNAs (Lacks, 1980). *DpnI*-digested samples were digested with *MluI* to linearize the plasmids prior to quantitative competitive PCR analysis performed as previously described (Kirchmaier and Sugden, 1997). In brief, a constant amount of each sample was mixed with varying amounts of competitor DNA and amplified. The amplified products from sample DNAs and competitor DNA were distinguished by length polymorphisms. Primers used for quantitative, competitive PCR were 5' end-labeled by T4 polynucleotide kinase using [γ -³²P]ATP to enable detection and quantitation of products. PCRs were performed in a final volume of 50 μ l using the following conditions for 20–25 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 60 s. Amplified products were separated on 1.1% agarose gels electrophoresed in 0.5 $\times$ TBE. Gels were fixed by soaking for 20 min in 7.5% trichloroacetic acid (TCA), and dried on DE-81 paper (Whatman) using a Bio-Rad gel dryer for 1 h at 70°C. Detected signals were quantified by PhosphorImage analysis (Molecular Dynamics). The number of amplified DNA molecules detected was normalized to the percentage of cells transfected, which was measured by counting β -galactosidase-positive cells (pEQ176) or cells expressing green fluorescent protein (GFP; pEGFP). The transfection efficiency of 143B cells ranged from 10 to 15%, that for C33A cells from 12 to 20% and that for 293 cells from 24 to 60%.

Activity of *DpnI* on methylated, unmethylated and hemi-methylated DNAs

Four oligodeoxynucleotides (A: 5' gtcGATCgtcactGATCagtcGATCgtacgGATCtcgcatGATC; B: 5' gtcGATCgtcactGATCagtcGATCgtacgGATCtcgcatGATC; C: 5' cgcgGATCcatcggaGATCcggtacGATCtggtacGATCagtcGATC; and D: 5' cgcgGATCcatcggaGATCcggtacGATCtggtacGATCagtcGATC) were synthesized such that two contained N⁶-methylated deoxyadenosines, indicated as A, in the context of *DpnI* recognition sites. The oligodeoxynucleotides were 5' end-labeled with [γ -³²P]ATP by T4 polynucleotide kinase, and annealed to form methylated (B*/D), hemi-methylated (B*/C and B/C*) or unmethylated (A/C*) double-stranded substrates. The asterisk indicates the presence of a 5' end label. Two picomoles of each substrate were added to 1 μ g of 994, which contains 29 sites for *DpnI*, and Hirt extract from the equivalent of 10⁵ mock-transfected cells, in a final volume of 100 μ l of 1 $\times$ KGB. The reactions were digested with 40 U of *DpnI* for 48 h, at which point aliquots were electrophoresed independently on 10% denaturing and 10% native polyacrylamide gels. Digestion was assayed by cleavage of the full-length duplex and quantified by phosphorimage analysis.

Equilibrium density analysis of cells labeled with BrdU

Plasmids 1920 and 1878, along with either 1553 or pcDNA3, were transfected into 6 $\times$ 10⁶ 293 cells. Cells were labeled with 0.1 mM BrdU and 0.2 mM deoxycytidine for 60 h from the time of transfection (Meuth and Green, 1974; Yates and Guan, 1991). At this time, total nucleic acid was harvested by lysing cells in 5 mM EDTA, 1% sarkosyl, followed by proteinase K digestion. CsCl was added to a final concentration of 1.74 g/ml and samples were spun at 37 500 r.p.m. for 96 h in a Beckman Type 70.1Ti rotor. After centrifugation, the gradients were fractionated into 300 μ l fractions, whose density was measured using a refractometer or by weight. Each fraction was precipitated twice and resuspended in TE. The total DNA present in each fraction was measured by UV or fluorescence spectroscopy. Fractions were digested with *Bss*HII to linearize the plasmids, and then separated along with standards on 0.8% agarose gels electrophoresed in 0.5 $\times$ TBE. Electrophoresed samples were transferred to Hybond (Amersham) nylon membranes, which were then hybridized to randomly primed, labeled DNAs synthesized using 1878 as template (Sambrook *et al.*, 1989). The signals were detected and quantified by phosphorimage analysis and comparison with the added standards.

Measurement of transcription

The plasmid 1033 and RSVL were used to measure the loss of transcription reporter plasmids in proliferating cells. Transfections were performed in 143B, C33A and 293 cells. A total of 4 $\times$ 10⁶ 293 cells, 4 $\times$ 10⁶ C33A cells or 2 $\times$ 10⁶ 143B cells were transfected with 1 μ g of the reporter plasmid and 10 μ g of either 1553 or the backbone effector. Cells were split 24 h post-transfection such that they would not have reached confluence when harvested 48, 72 and 96 h post-transfection. Harvested cells were counted and lysed at a concentration of 2 $\times$ 10⁴ cells/ml in lysis buffer from the Promega luciferase assay system

(Promega). Luciferase assays were performed using extracts from 4×10^5 cells as per the instructions of the manufacturer. Luminescence was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) as described previously (Kirchmaier and Sugden, 1997).

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