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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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 replication: 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...
oriP: cellular synthesis with viral maintenance

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 2×10⁴ 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

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>cDNA library</th>
<th>No. of transformants screened</th>
<th>cDNAs recovered (DDJB/EMBL/GenBank NID)</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>oriP-HIS3</td>
<td>EBV-immortalized B-lymphocytes</td>
<td>6×10⁶</td>
<td>gC1q-R (g472955)</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EBNA-1 DNA-binding domain (g59074)</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p75 nuclear phosphoprotein (g402147)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CpG island DNA</td>
<td>1</td>
</tr>
<tr>
<td>DS-HIS3</td>
<td>EBV-immortalized B-lymphocytes</td>
<td>5.1×10⁶</td>
<td>gC1q-R (g472955)</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p75 nuclear phosphoprotein (g402147)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rchl a-karyopherin (g791184)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Histone H1 (g184071)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human homolog of lipocalin (g211502)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nucleolar GTP-binding protein (g179284)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ORF similar to yeast SCD6 (g939713)</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CpG island DNA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D38491 human ORF of unknown function (g559327)</td>
<td>1</td>
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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 1.53×10⁷ 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-1a

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<th>Hours post-transfection</th>
<th>(+) EBNA-1</th>
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<tr>
<td></td>
<td>oriP</td>
<td>Backbone</td>
</tr>
<tr>
<td>48 h⁵</td>
<td>6.8 ± 1.5</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>72 h⁵</td>
<td>5.7 ± 0.8</td>
<td>&lt;0.6</td>
</tr>
<tr>
<td>96 h⁵</td>
<td>9.6 ± 1.1</td>
<td>&lt;0.7</td>
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aNumbers reflect the number of DpnI-resistant plasmid molecules present per transfected cell.

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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
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 co-migrated 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 (Wysokinski and Yates, 1989). The presence of oriP increases replication of plasmid DNAs substantially relative to that of the prokaryotic backbone.

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
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

### Table III. Activity of DpnI on methylated, hemi-methylated and unmethylated DNAs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Input digested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’gtcGATCgtcactGATCgtaacGATCtccgatGATCCTAGcagtgaCTAGtcaggtCTAGcattgcCTAGaggctaCTAGgcgc 5’</td>
<td>97</td>
</tr>
<tr>
<td>5’gtcGATCgtcactGATCgtaacGATCtccgatGATCCTAGcagtgaCTAGtcaggtCTAGcattgcCTAGaggctaCTAGgcgc 5’</td>
<td>95b, 97c</td>
</tr>
<tr>
<td>5’gtcGATCgtcactGATCgtaacGATCtccgatGATCCTAGcagtgaCTAGtcaggtCTAGcattgcCTAGaggctaCTAGgcgc 5’</td>
<td>3</td>
</tr>
</tbody>
</table>

*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 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 $10^7–2\times10^7$ 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.
measuring the rates of loss of luciferase activity in different cells transfected with replicating and non-replicating plasmids that express EBV. 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-
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 may be transcribed or synthesized, and the rate at which plasmid DNAs enter cellular compartments influences the rate at which plasmid DNAs are destroyed or eliminated. This eradication also affects DNAs which are transcribed or newly synthesized DNAs are destroyed or eliminated. This eradication also affects DNAs which are transcribed or newly synthesized DNAs are destroyed or eliminated.

We note, however, that EBV DNA or oriP-containing 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 plasmids 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 of chromosomes.
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 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 facilitate the appropriate positioning of nucleosomes 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 2μ plasmid pAS1-Tet (DDBJ/EMBL/GenBank accession No. g1236518) in these strains. An EBV-immortalized B-lymphocyte library (Dufre 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-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 transferred back into the same strain to confirm the positive phenotype, after which they were transformed into *Escherichia coli* DH5α 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 CoEl 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 Mlu 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 neoB 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, 1878, and 1938 were propagated in *E.coli* MC1061/F3 using conditions described previously (Aiyar et al., 1996). Plasmid 1380, that is distinguished from 1878 and 1920 by a second length polymorphism in its neoB 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-BamH-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 (Knudson and Yee, 1987).

Measurement of DNA synthesis by quantitative PCR

Plasmids 1920 and 1878 (10 μg of each) were transfected together with 1553 or pEQ176 into 4 × 10^4 C33A cells, or electroporated into 10^7 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 μg/ml) and proteinase K (200 μg/ml), and then extracted sequentially with phenol and phenol/chloroform:isoamyl alcohol (25:24:1). Precipitated samples were resuspended at 1×10^6 cell equivalents/μl in 1× KGB and digested with 1 U of *DpnI* per 2.5×10^5–5×10^6 cell equivalents for a minimum of 48 h to digest selectively the dimethylated transfected DNAs (Lacks, 1980). *DpnI*-digested samples were digested with *MluI* to linearize the plasmid 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 μl 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% agarose gels electrophoresed in 0.5× TBE. Gels were fixed by soaking for 20 min in 7.5% trichloracetic 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′-gctGATCgctacGATGAccteGATGtacgGATCtcggatGATC-GATCtcggatGATC; B: 5′-gctGATCgctacGATGAccteGATGtacgGATCtcggatGATC; C: 5′-cgccGATCgctacGATGAccteGATGtacgGATCtcggatGATC; and D: 5′-cgccGATCgctacGATGAccteGATGtacgGATCtcggatGATC) were synthesized such that two contained 5′-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 μg of 994, which contains 29 sites for *DpnI*, and Hirt extract from the equivalent of 10^7 mock-transfected cells, in a final volume of 100 μl of 1× 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 6×10^6 293 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 hypotonic 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 μl 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 spectrophotometry. Fractions were digested with *Bst*HI to linearize the plasmids, and then separated along with standards on 0.8% agarose gels electrophoresed in 0.5× 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 compared 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 143B 293 cells with *MluI* to linearize the plasmid. Amplified DNAs were digested with *MluI* to linearize the plasmid and 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 compared with the added standards.
Luciferase assays were performed using extracts from 4 x 10⁵ 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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References


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