Fusions between Epstein-Barr Viral Nuclear Antigen-1 of Epstein-Barr Virus and the Large T-antigen of Simian Virus 40 Replicate Their Cognate Origins*

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Epstein-Barr viral nuclear antigen-1 (EBNA-1) is required for the stable replication of plasmids that contain oriP, the origin of DNA synthesis used during the latent phase of the Epstein-Barr virus life cycle. EBNA-1 acts post-synthetically through unknown mechanisms to facilitate the continued synthesis of oriP plasmids in ensuing S phases. In contrast to viral replicons such as that of SV40, DNA synthesis of oriP is restricted to a single round during each cell cycle. Large T-antigen of SV40 is a DNA helicase and activates the synthesis of SV40 DNA by recruiting cellular proteins required for DNA synthesis to the origin of SV40. Using fusion proteins of EBNA-1 and large T-antigen, we tested whether tethering large T-antigen to oriP is sufficient to initiate multiple rounds of DNA synthesis from oriP during each cell cycle. We report here that, although these fusion proteins retain the biological activities of both EBNA-1 and large T-antigen, their constituent proteins do not confer the properties of one on the other. Thus, it is not possible to subvert the cellular controls that restrict DNA synthesis from oriP to a single round per cell cycle. These results also provide insights into architectural constraints at oriP and at the SV40 ori.

The Epstein-Barr virus (EBV)1 genome is maintained as a plasmid within latently infected cells. Synthesis and maintenance of this plasmid requires a viral cis-acting sequence, oriP, and a single viral protein, the Epstein-Barr viral nuclear antigen 1 (EBNA-1) (1–3). In latently infected cells, the EBV genome is synthesized only once every cell cycle (4), as are oriP plasmids in cells that express EBNA-1 (5).

There are 24 binding sites for EBNA-1 within oriP (6). These sites are organized into two clusters, referred to as the family of repeats (FR) and the dyad symmetry element (7, 8). FR contains 20 binding sites for EBNA-1, while the dyad symmetry element has 4 binding sites for EBNA-1, 2 of which are present within an extended palindrome (1, 8). Unlike the origin-binding proteins of some viruses, EBNA-1 does not possess enzymatic activities such as a DNA helicase activity that could contribute directly to synthesis of oriP DNA (9, 10). Results from a deletion analysis indicate that EBNA-1 is unlikely to function as an enzyme, as small overlapping deletions in regions other than the domain required for DNA binding do not disrupt functions of EBNA-1 (11). We have recently demonstrated that EBNA-1 is not required for the synthesis of oriP plasmids (12). However, in the absence of EBNA-1, newly synthesized oriP plasmids are lost rapidly from proliferating cells. Thus, EBNA-1 bound to oriP functions post-synthetically to ensure plasmid maintenance and segregation in dividing cells. EBNA-1 bound at FR has another function of significant biological consequence to EBV. When bound at FR, EBNA-1 activates two EBV promoters, the BamHI-C promoter and the LMP1 promoter, the activation of which is critical to the establishment of viral latency (13–15). In addition to sequence-specific DNA binding, EBNA-1 molecules bound to cognate binding sites interact with each other, thereby “linking” the two binding sites (10, 16, 17). Deleted derivatives of EBNA-1 that lack domains required for DNA linking are unable to support the stable replication of oriP plasmids, and fail to activate the EBNA-1-responsive BamHI-C promoter (18).2 These results indicate that EBNA-1’s ability to link DNA is likely to be important to EBV.

Replication of the oriP replicon differs substantially from the replication of other viral replicons such as those of simian virus 40 (SV40) or bovine papilloma virus (BPV) (20, 21). The biochemical contributions toward DNA synthesis by the origin binding proteins of these viruses, large T-antigen and E1, are largely understood (20, 22–24). In contrast to oriP, the replicons of SV40 and BPV are not restricted by cellular controls to a single round of DNA synthesis during each cell cycle (20, 21). Large T-antigen of SV40 and E1 of BPV possess an ATP-hydrolysis-dependent DNA helicase activity in addition to binding specifically to their cognate origins (22, 25, 26). These proteins also interact directly with cellular proteins involved in initiation of DNA synthesis such as DNA polymerase α/primase and recruit them to the viral origin (27–30), suggesting one possible mechanism by which the SV40 and BPV replicons bypass the cellular mechanisms that restrict chromosomal and oriP DNA synthesis to a single round per cell cycle.

We made fusions between EBNA-1 and the large T-antigen of SV40 to examine whether localizing a protein competent to assemble an unregulated DNA synthetic complex to oriP is sufficient to trigger multiple rounds of DNA synthesis from oriP. Biological characterization of these fusion proteins reveals that they retain the ability to recognize and replicate their cognate origins but do not confer the DNA synthetic phenotype of one on the other. Fusions that contain intact EBNA-1 activate transcription and replication similarly to unfused EBNA-1, indicating that EBNA-1’s post-synthetic contribution to oriP replication is not hampered by the extraneous domain fused to EBNA-1. A fusion protein, which contains

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The abbreviations used are: EBV, Epstein-Barr virus; EBNA-1, Epstein-Barr viral nuclear antigen 1; SV40, simian virus 40; FR, family of repeats; IE, immediate early; CMV, cytomegalovirus; aa, amino acids; ORF, open reading frame; bp, base pairs; PCR, polymerase chain reaction; BPV, bovine papilloma virus; RPA, replication protein A.

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Characterization of Fusions between EBNA-1 and T-antigen

T-antigen was used as the primary antibody, purified rabbit anti-hamster IgG (Cortex) was used as secondary antibody prior to detection with 32P-labeled donkey anti-rabbit IgG.

Preparation of Nuclear Extracts—293 cells were transfected with vectors expressing EBNA-1, large T-antigen, or a fusion protein 48 h prior to preparation of nuclear extracts (18). Plasmids 1602 and 1033 cells were harvested in 50 ml of cold phosphate-buffered saline, pelleted, and washed twice with 5 ml of cold phosphate-buffered saline. The washed pellets were resuspended in a buffer containing 20 mM HEPES pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM aminothiol, and 0.05% N-lauroyl sarcosine (Calbiochem) and 0.05% N-lauroyl sarcosine (Calbiochem) and 0.05% N-lauroyl sarcosine (Calbiochem). Nuclei were pelleted by centrifugation at 4 °C for 10 min at 2500 rpm. Nuclei were resuspended at 106 cell/ml in a buffer of the same composition as the wash buffer, except the NaCl concentration was raised to 0.42 M. The resuspended nuclei were rocked gently at 4 °C for 30 min. After transfer to cold Oakridge tubes, nuclei were centrifuged for 20 min at 20,000 rpm in a Beckman Ti50 rotor. The supernatant was frozen as aliquots at −70 °C.

Protein-DNA Co-immunoprecipitations—Plasmid 304 (10), which contains 10 binding sites from the FR, was digested with ApaLI and EcoRI, and used as probe in co-immunoprecipitation assays to detect site-specific DNA binding by EBNA-1. Plasmid 1602, digested with ApaLI and BstXI, was used as probe in co-immunoprecipitation assays to detect site-specific DNA binding by large T-antigen. A probe was created with treated with shrimp alkaline phosphatase (Amersham Pharmacia Bio-tech), before 5′ end-labeling with [γ-32P]ATP using T4 polynucleotide kinase. Unincorporated label was removed by gel filtration chromatography on Sephadex G-25 spin columns (Boehringer Mannheim). The DNA concentration of the probe was adjusted to 2.5 ng/μl (304) or 5 ng/μl (1602); 2.5 ng of probe or 5 ng of probe 2.5 μg of sheared salmon sperm DNA, and 10 μg of poly(dI/dC) were added to 80 μl of a solution containing 13.75 mM HEPES, pH 7.9, 8.75 mM MgCl2, 2.5 mM dithiothreitol, and 0.05% Triton X-100. To this, 20 μl of nuclear extract were added along with the appropriate antibodies. For immunoprecipitations to detect site-specific binding by EBNA-1, 1 μl of a 1.5 dilution of affinity-purified rabbit anti-EBNA-1 IgG was added. For immunoprecipitations to detect site-specific binding by large T-antigen, 1 μl of 1.5 dilution of hamster anti-large T-antigen antisera was added, along with 1 μl of a 1:2 dilution of the affinity-purified rabbit anti-hamster IgG. The immunoprecipitations were incubated at room temperature for 20 min before 15 μl of a 20% solution of protein A-Sepharose beads were added, followed by an incubation for 30 min at room temperature. Pellets were recovered by centrifugation at 6000 rpm for 1 min in a table top microfuge with 300 μl of wash buffer. The supernatant was extracted with 50 μl of sheared salmon sperm DNA, and 0.05 μM poly(dI/dC). After the washes, the pellets were resuspended in 100 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) containing 1% SDS and incubated at 65 °C for 5 min. They were extracted with an equal volume of phenol. The aqueous phase was extracted sequentially with equal volumes of phenol:chloroform:isoamyl alcohol (24:1), and chloroform:isoamyl alcohol (24:1). Twenty μg of glycerone were added to the aqueous phase, and the final salt concentration was adjusted to 0.3 M NaCl. DNA was recovered by precipitation with two volumes of 100% ethanol, and separated on a 1% agarose gel electrophoresed in 0.5 Tris-borate EDTA (31). The gel was fixed in 7.5% trichloroacetic acid, and dried on DE-81 paper using a Bio-Rad gel dryer for 1 h at 70 °C. Samples were quantified using a PhosphorImager (Molecular Dynamics).

Measurement of Transcriptional Activation or Repression—The plasmids ori-P-BamHI-C luciferase, and pSVL were used as reporters to test the properties of the expressed proteins in the activation or repression of transcription. Transfections that used the ori-P-BamHI-C-luciferase reporter were performed in 293 cells or 143B cells, while those that utilized pSVL were performed in 293 cells. Cells were harvested 48 h after transfection, fractionated, and lysed at a concentration of 2 × 106 cells/ml in lysis buffer from the Promega luciferase assay system. Luciferase assays were performed using extracts from 4 × 106 cells as per the instructions of the manufacturer. Luminescence was measured using a Bio-Tek plate reader (Analytical Luminescence Laboratory) as described previously (18).

Measurement of DNA Replication—A total of 10 μg of the oriP reporter, 994, or the SV40 ori reporter, 1602, was electroporated together with 10 μg of the replication control plasmid 1381, and 10 μg of the effector plasmid into 106 143B cells. OriP replication was assayed 96 h after electroporation, while SV40 ori replication was assayed 48 h after electroporation. The 143B cells were grown in Dulbecco's modified Eagle's medium/glucose supplemented with 10% calf serum, 2% 104 cells were transfected with 2 μg of the reporter plasmid and 20 μg of the effector plasmid under test. Cells were harvested 48 h after transfection, fractionated, and lysed at a concentration of 2 × 106 cells/ml in lysis buffer from the Promega luciferase assay system. Luciferase assays were performed using extracts from 4 × 106 cells as per the instructions of the manufacturer. Luminescence was measured using a Bio-Tek plate reader (Analytical Luminescence Laboratory) as described previously (18).
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RESULTS

Expression of Fusions between EBNA-1 and SV40 Large T-antigen—DNA synthesis from oriP is known to be restricted to a single round during the S phase of the cell cycle in human cells (4, 5). Replication of the SV40 replicon has been biochemically characterized, and the contributions of large T-antigen to the replication of SV40 DNA have been studied in depth (24, 25, 44, 45). Large T-antigen interacts site-specifically with the origin of SV40 (20, 22). Large T-antigen possesses a DNA helicase activity, and interacts with cellular proteins that participate directly in DNA synthesis (28, 44, 46). In contrast to oriP, the SV40 replicon is not restricted to a single round of DNA replication during each cell cycle (5, 20). We made fusions between large T-antigen and EBNA-1 to test whether tethering large T-antigen to oriP is sufficient to trigger multiple rounds of DNA synthesis during each cell cycle from oriP.

Schematic representations of EBNA-1, large T-antigen, and the fusion proteins used in this study are shown in Fig. 1. The derivative of EBNA-1 used in this study lacks all but 15 residues of the internal Gly-Gly-Ala repeats, and is referred to as “intact EBNA-1” in this report. Intact EBNA-1 and wild-type EBNA-1 support replication of oriP plasmids, and activate transcription from EBNA-1-responsive promoters similarly (3, 47). In two of the fusion proteins described here, intact EBNA-1 was fused to either the amino terminus or the carboxyl terminus of large T-antigen. These two proteins are referred to as EBNA-1-TAg and TAg-EBNA-1, respectively. A third fusion protein, N450 derivative of EBNA-1 has been demonstrated to be a cleavage event within large T-antigen that removes or disrupts major epitopes in that protein. Quantitative immunoblot analysis performed on extracts from 293 and murine (101) cells indicates that the five effector proteins used in this study are expressed similarly (within a factor of 10; data not shown). Their different levels of expression correlate with different levels of their activities. Next, we examined the ability of these proteins to bind DNA in a site-specific manner and characterized their biological activities.

DNA Binding by Fusion Proteins—Both EBNA-1 and large T-antigen are site-specific DNA-binding proteins (6, 22, 26). We tested the fusion proteins for their ability to bind cognate binding sites for EBNA-1 and large T-antigen using DNA co-immunoprecipitation assays. We used this assay to avoid the confusion of EBNA-1’s linking DNAs to which it binds such that they do not enter polyacrylamide gels (17).

Whether EBNA-1 binds DNA site-specifically was tested using the plasmid 304, which contains 10 binding sites from FR. 304 was digested with ApaLI and EcoRI, and the restriction fragments were 5’-end-labeled. Co-immunoprecipitations were performed by the addition of rabbit anti-EBNA-1 antisera along with protein A-Sepharose beads, using nuclear extracts from 2.7 × 10⁶ 293 cells. The results of this experiment are shown in Fig. 2A, with controls shown in Fig. 2B. Specific immunoprecipitation of an 896-bp fragment that contains the EBNA-1-binding sites was observed, demonstrating that all three of the fusion proteins specifically bind EBNA-1-binding sites. In contrast, this fragment was not co-immunoprecipitated when nuclear extracts from 293 cells expressing large T-antigen were used in conjunction with rabbit anti-EBNA-1 antisera. In three independent experiments, the FR-containing fragment was preferentially retained in co-immunoprecipitations from 10-fold to 60-fold better than other restriction fragments from the same plasmid.

The ability of the fusion proteins to bind binding sites for large T-antigen was tested using plasmid 1602, which contains the origin of SV40. 1602 was digested with ApaLI and Bsu36I, and the restriction fragments were 5’-end-labeled. Co-immunoprecipitations were performed by the addition of hamster anti-large T-antigen antisera, along with rabbit anti-hamster IgG and protein A-Sepharose beads using nuclear extracts from 2.7 × 10⁶ 293 cells. The result of this assay, which demonstrates that all three of the fusion proteins specifically co-immunoprecipitated a 1695-bp fragment containing the three large T-antigen-binding sites within the origin of SV40, is shown in Fig. 2C, which was not co-immunoprecipitated when extracts from EBNA-1 expressing cells were used (Fig. 2D). The SV40 ori-containing fragment was preferentially retained from 3-fold to 40-fold over other restriction digest fragments from the same plasmid.

As the fusion proteins synthesized in vivo bind DNA in a site-specific manner in vitro, we conclude that both the EBNA-1 and large T-antigen domains of the fusion proteins are likely to be folded correctly.

Transcriptional Activation of the EBNA-1-responsive EBV BamHI-C Promoter—EBNA-1 is known to activate transcriptionally the BamHI-C promoter of EBV (15, 18). We examined the ability of the fusion proteins to activate transcription from a luciferase reporter under the control of the oriP-BamHI-C promoter in 293 cells. The results of this analysis are in Table I. EBNA-1, EBNA-1-TAg, and TAg-EBNA-1 transactivated the oriP-BamHI-C luciferase reporter to similar extents (15.6-, 8.2-, and 7.5-fold, respectively). The differences in activation by these proteins reflects their order of expression, EBNA-1 being expressed at 2–4-fold higher levels than the fusion proteins. The third fusion protein, N450-TAg, did not activate transcription from this reporter. This result was expected because the N450 derivative of EBNA-1 has been demonstrated to be
FIG. 1. A, schematic representation of EBNA-1. Wild-type EBNA-1 from the prototypic B95–8 strain of EBV is 641 amino acids (aa) long. EBNA-1's DNA-binding and dimerization domain lies between aa 461 and 608 (50, 51). The three DNA-linking domains (17) and a nuclear localization sequence identified between aa 379 and 386 of EBNA-1 (50) are indicated. The EBNA-1 protein used in this study contains only 15 residues of the Gly-Gly-Ala repeat present between aa 90 and 328 of wild-type EBNA-1 protein. This protein, which is 417 aa long, activates transcription from EBNA-1-dependent reporters and supports long term replication of oriP plasmids, similarly to wild-type EBNA-1 (46).

B, schematic representation of SV40 large T-antigen. Large T-antigen is 708 aa long. The carboxyl-terminal half of large T-antigen contains the domain of T-antigen responsible for enzymatic ATP hydrolysis. The DNA-binding domain lies between aa 131 and 259. The ATP hydrolysis-dependent DNA helicase domain encompasses the domains required for site-specific DNA binding and ATP hydrolysis. DNA polymerase α/primase.
severely impaired in its ability to trans-activate the oriP-BamHI-C promoter (18). We also observed transactivation of the oriP-BamHI-C luciferase reporter by large T-antigen. We think that this low level of transactivation (3-fold over background) is likely to be nonspecific, as large T-antigen has been shown to activate promiscuously several promoters that do not contain binding sites for large T-antigen, perhaps by interacting with transcriptional repressors (32). These results indicate that the EBNA-1-TAg and TAg-EBNA-1 fusion proteins retain the ability to bind cognate EBNA-1-binding sites in vivo, and activate transcription while bound at these sites, similarly to these functions of unfused EBNA-1.

**NΔ450-TAg Inhibits Transcriptional Activation by Wild-type EBNA-1**—The NΔ450 derivative of EBNA-1 is a potent dominant-negative inhibitor of wild-type EBNA-1’s capacity to activate transcription from the oriP-BamHI-C promoter. The ability of the EBNA-1-TAg and TAg-EBNA-1 fusions to repress transcriptional activation was determined in transfection experiments in 293 cells. The data, measured as relative light units, are expressed as fold transactivation relative to vector alone, which was set to 1.

**Table I**

| Effector         | Transactivation* | fold |
|------------------|------------------|------|
| Vector           | 1.0              |      |
| EBNA-1           | 15.6 ± 3.7       |      |
| EBNA-1-TAg       | 8.2 ± 4.6        |      |
| TAg-EBNA-1       | 7.5 ± 2.9        |      |
| NΔ450-TAg        | 1.1 ± 1.7        |      |
| Large T-antigen  | 3.0 ± 2.4        |      |

* Data represent an average ± standard deviation of the results from three sets of transfections into 293 cells. 4 × 10⁵ cells on two 100-mm dishes were transfected with 20 μg of effector and 2 μg of reporter. Cells from each pair of plates were harvested as a pool 48 h after transfection, and luciferase activity was measured as described under “Experimental Procedures.” The data, measured as relative light units, are expressed as fold transactivation relative to vector alone, which was set to 1.
Characterization of Fusions between EBNA-1 and T-antigen

Table II

| Effector                  | Transactivation* |
|---------------------------|------------------|
| Vector                    | 1.0              |
| EBNA-1 + vector           | 24.0 ± 15        |
| Large T-antigen + vector  | 4.5 ± 1.3        |
| NA450 + vector            | 2.8              |
| NA450-TAg + vector        | 3.7 ± 0.6        |
| EBNA-1 + NA450            | 1.1 ± 0.8        |
| EBNA-1 + NA450-TAg        | 2.6 ± 1.8        |

* Data represent an average ± the standard deviation of the results from two sets of electroporations into 143B cells. 2 × 10^7 cells were electroporated with 20 μg of effector and 2 μg of reporter in two electroporations that were pooled and plated on a 150-mm dish. Cells were harvested 48 h after transfection, and luciferase activity was measured as described under "Experimental Procedures." The data, measured as relative light units, are expressed as -fold transactivation relative to vector alone, which was set to 1.

Table III

| Effector                  | Transactivation* |
|---------------------------|------------------|
| Vector                    | 1.0              |
| Large T-antigen           | 0.5 ± 0.12       |
| EBNA-1                    | 3.5 ± 0.8        |
| EBNA-1-TAg                | 1.3 ± 0.08       |
| TAg-EBNA-1                | 0.6 ± 0.5        |
| NA450-TAg                 | 0.4 ± 0.3        |

* Data represent an average ± the standard deviation of the results from three sets of transfections into murine (101)1 cells. 1.6 × 10^6 cells on two 100-mm dishes were transfected with 20 μg of effector and 2 μg of reporter. Cells from each pair of plates were harvested as a pool 48 h after transfection, and luciferase activity was measured as described under "Experimental Procedures." The data, measured as relative light units, are expressed as -fold transactivation relative to vector alone, which was set to 1.

Fig. 3. A, replication of plasmids containing SV40 ori reporter measured using quantitative, competitive PCR 48 h after electroporation. 10 μg each of the SV40 ori reporter, the origin-minus control, and the effector plasmid indicated above each set of PCRs were electroporated into 10^7 143B cells. The positions of the amplified fragments from the competitor DNA, the SV40 ori replication reporter, and the origin-minus control are indicated. The amount of competitor DNA used in each PCR is indicated below each lane. PCRs performed with cells transfected with the EBNA-1-TAg and TAg-EBNA-1 effector plasmids contained DpnI-digested Hirt extract equivalent to 10^7 cells. PCRs performed with cells transfected with the large T-antigen and NA450-TAg effector plasmids contained DpnI-digested Hirt extract equivalent to 10^6 cells. B, replication of plasmids containing oriP reporter measured using quantitative, competitive PCR 96 h after electroporation. 10 μg each of the oriP reporter, the origin-minus control, and the effector plasmid indicated above each set of PCRs were electroporated into 10^7 143B cells. The positions of the amplified fragments from the competitor DNA, the oriP replication reporter, and the origin-minus control are indicated. The amount of competitor DNA used in each PCR is indicated below each lane. Each PCR contained DpnI-digested Hirt extract equivalent to 10^6 cells.
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TABLE IV
Replication of oriP and SV40 ori reporters in 143B cells

| Effector          | oriP reporter<sup>a</sup> | SV40 ori reporter<sup>a</sup> |
|-------------------|---------------------------|-------------------------------|
| EBNA-1            | 14 ± 4.2<sup>b</sup>     | <0.64<sup>c</sup>             |
| TAg               | <0.64<sup>d</sup>        | ~370<sup>e</sup>              |
| EBNA-1-TAg        | 12 ± 3.4<sup>d</sup>     | <0.64<sup>c</sup>             |
| NΔ450-TAg         | <0.64<sup>c</sup>        | ~270<sup>e</sup>              |
| TAg-EBNA-1        | 9 ± 3.7<sup>d</sup>      | <0.64<sup>e</sup>             |

<sup>a</sup> Ten micrometers of effector along with 10 mg of each of replication reporter and origin-minus replication control plasmid was electroporated into two electroporations into 2 × 10<sup>4</sup> 143B cells. Electroporations were pooled and plated into a single 150-mm dish. Hirt extracts were performed 96 h after transfection when the oriP replication reporter was tested, and 48 h after transfection when the SV40 ori reporter was tested. Recovered DNAs were digested with DpnI for a minimum of 48 h, following which competitive PCRs were performed on 1 × 10<sup>6</sup> cell eq of sample per PCR. The numbers reported represent molecules of replicated reporter DNA present per cell. To calculate this number a standard curve of signal versus concentration of competitor DNA was plotted for each set of PCRs. The phosphorimagery signal from the replication reporter was determined at each competitor concentration, and the average of these signals was used to calculate the number of molecules of replication reporter from the standard curve of competitor DNA.

<sup>b</sup> Data represent an average with the standard deviation of measurements on three independent pairs of electroporations. The numbers represent molecules of replicated plasmid detected per transfected cell. The transfection efficiency in these experiments was 14%.

<sup>c</sup> 0.64 molecules/cell is the lowest amount the competitor DNA visualized after PCR shown in Fig. 3. In other experiments, the lowest amount of competitor DNA visualized after PCR was 0.8 molecules/cell or 0.45 molecules/cell, respectively.

<sup>d</sup> In two other experiments, the number of replicated molecules per transfected cell was estimated to be >200 molecules/cell, which was the greatest amount of competitor DNA visualized in those experiments after PCR. The number of molecules of competitor DNA listed was calculated from the competitive PCR shown in Fig. 3, where 1 × 10<sup>6</sup> cell eq were used in each PCR.

<sup>e</sup> In one other experiment, the number of replicated molecules per transfected cell was estimated to be >200 molecules/cell, which was the greatest amount of competitor DNA visualized in that experiment after PCR. The number of molecules of competitor DNA listed was calculated from the competitive PCR shown in Fig. 3, where 1 × 10<sup>6</sup> cell eq equivalents were used in each PCR.

Replication Activities of Fusion Proteins—Large T-antigen and the fusion proteins were tested for their ability to support replication of a plasmid containing the SV40 origin in 143B cells. The SV40 ori reporter, together with an origin-minus replication reporter and the appropriate effector plasmid, were electroporated into 143B cells. Hirt extractions were performed on transfected cells 48 h after electroporation (42). This time period was found to be optimal to detect replication of the SV40 ori reporter in these cells. Hirt extracts were digested exhaustively with the restriction endonuclease DpnI to digest selectively the transfected DNA, which is dam-methylated (43). Replication of the SV40 ori reporter was assessed by measuring the level of DpnI-resistant, replicated plasmid DNA using quantitative, competitive PCRs as described under “Experimental Procedures.” Representative results of quantitative, competitive PCR analysis are shown in Fig. 3A, and summarized in Table IV. Forty-eight hours after transfection, approximately 370 molecules of replicated reporter were detected per transfected cell when large T-antigen was provided as effector. Neither the EBNA-1-TAg nor the TAg-EBNA-1 fusion proteins supported the replication of the SV40 ori reporter either 48 h after transfection (Fig. 3A, Table IV) or 96 h after transfection (data not shown). The NΔ450-TAg fusion protein supported replication of the SV40 ori reporter to an extent similar to that for large T-antigen (approximately 270 molecules/transfected cell). This result indicates that NΔ450-TAg, which has the ability to bind EBNA-1 DNA-binding sites (Fig. 2A, Table II), is competent to assemble a replication complex at the SV40 ori in a manner indistinguishable from large T-antigen.

The fusion proteins were next tested for their ability to support replication of an oriP plasmid 96 h after their introduction into 143B cells. By this time, cellularly synthesized oriP plasmids are lost from proliferating 143B cells in the absence of EBNA-1 (12, 18). 143B cells were electroporated with the oriP replication reporter, origin-minus control plasmid, and effector plasmid. Hirt extracts were prepared 96 h later and were digested with DpnI to digest the input bacterially methylated DNA (42, 43). DpnI-digested Hirt extracts were used in quantitative, competitive PCR analyses as described under “Experimental Procedures,” to determine the number of replicated oriP reporter molecules present per transfected cell. Representative results are shown in Fig. 3B, and summarized in Table IV. Ninety-six hours after electroporation, 14 molecules of replicated oriP reporter were detected per transfected cell when EBNA-1 was provided as effector. Similar levels of replicated oriP reporter were detected when EBNA-1-TAg and TAg-EBNA-1 fusion proteins were provided as effectors (12 and 9 molecules/transfected cell, respectively). No replication was detected when the NΔ450-TAg effector was used, although this protein is capable of binding oriP as shown by the results in Fig. 2A and Table III, and supports DNA synthesis from the SV40 ori (Fig. 3A, Table IV). NΔ450-TAg behaves like the NΔ450 derivative of EBNA-1, which has been shown to support oriP replication to less than 1% the levels of wild-type EBNA-1 at 96 h after electroporation (18).

Our results with the EBNA-1-TAg and TAg-EBNA-1 fusion proteins demonstrate that the contributions of EBNA-1 to the post-synthetic stabilization of oriP plasmids, and to transcription from these plasmids, are not impaired by large extraneous protein domains. The results also demonstrate that tethering a helicase competent to assemble an active DNA synthetic complex to oriP is not sufficient to activate DNA synthesis from oriP.

DISCUSSION
EBNA-1 is required for the stable replication of oriP plasmids in human cells (3, 36). Deletion derivatives of EBNA-1 have been extensively characterized, and the molecular details of the interactions between EBNA-1 and oriP DNA have been elucidated through biochemical and structural studies (54–56). While the DNA-binding domain of EBNA-1 has been characterized recently to interact with human RPA in vivo (57), we have failed to detect this interaction in vitro (12). Additionally, the DNA-binding domain of EBNA-1, the region of EBNA-1 to which RPA binds, inhibits the replication of oriP plasmids (18), and RPA also interacts with proteins that do not participate in DNA synthesis such as GAL4 and EBNA-2 (58, 59). Thus, the mechanisms by which EBNA-1 facilitates stable replication of oriP plasmids remain enigmatic. Unlike other viral origin-binding proteins, such as E1 of BPV, or the large T-antigen of SV40, EBNA-1 has not been found to have enzymatic activities, such as a DNA unwinding activity, that can contribute directly to the synthesis of DNA (9, 10), consistent with our recent appreciation that EBNA-1 is not required for DNA synthesis from oriP (12). We have several features of oriP replication that distinguish it from the replication of other viral plasmid replicons such as those of SV40 and BPV (1, 20, 21). One central difference between the replication of oriP and the replication of these other replicons is that oriP-containing plasmids are synthesized only once every S phase (5). It is a striking parallel that viral replicons that encode their own helicases all lack this control exhibited by oriP and EBNA-1.
Characterization of Fusions between EBNA-1 and T-antigen

Calos and co-workers conducted studies using chimeric plasmids that contain both oriP and the origin of SV40. Their results indicate that transient expression of large T-antigen within cells that stably maintain the oriP/SV40 ori chimeric plasmid increased the copy number of the chimeric plasmid from 10-fold to 50-fold (60). This result indicates that, while DNA synthesis from oriP is restricted to a single round per S phase, oriP present in cis does not impose cellular regulation on runaway DNA synthesis from an SV40 ori present on the same plasmid.

We wished to examine whether the cellular control of DNA synthesis from oriP could be bypassed by localizing to oriP a protein competent to assemble a DNA synthetic complex that is not restricted by the cell, and therefore created fusions between EBNA-1 and the large T-antigen of SV40. We chose to fuse the large T-antigen of SV40 to EBNA-1, as it facilitates unfettered synthesis from these plasmids. Similar fusions of EBNA-1 with the ligand-binding domain of steroid receptors or GFP may prove useful to study the mechanisms by which EBNA-1 facilitates the stable replication of oriP plasmids.

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