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Identification of Minimal oriP of Epstein-Barr Virus Required for DNA Replication[1]

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DNA replication from oriP of Epstein-Barr virus is mediated by the virus replication factor EBNA1 and cellular factors and occurs approximately once in each cell cycle. We have identified a minimal oriP element that is necessary and sufficient for DNA replication. We transfected plasmids containing several oriP fragments into HeLa cells expressing EBNA1 and analyzed their replication during four days after transfection using the methylation sensitive restriction endonuclease *DpnI*. All the oriP fragments containing the four EBNA1-binding sites known as the dyad symmetry sequence (DS) initiated DNA replication. The sequences flanking DS were not essential for DNA replication, but deletion of two or three EBNA1-binding sites in DS significantly reduced or totally abolished its replication activity. These results indicated that the four EBNA1-binding sites in DS constitute the minimal oriP element for DNA replication and suggest that DNA replication is initiated by recruitment of cellular replication factors onto or near the minimal oriP by EBNA1. We also found that the minimal oriP initiated DNA replication in mouse fibroblasts expressing EBNA1 but worked only at reduced efficiency, suggesting species specificity in DNA replication machineries.

Key words: DNA replication, Epstein-Barr virus, replication origin, transient replication assay.

OriP was identified in the Epstein-Barr virus (EBV) genome by its ability to replicate and maintain an oriP-containing plasmid in cells latently infected by EBV (*1*). Replication from oriP requires only EBNA1 as a viral protein (*2, 3*), which is a dimeric DNA-binding protein recognizing the 20-bp symmetric binding sequences (*4-8*). Unlike other viral replication factors such as large T antigen of SV40 and E1 protein of bovine papilloma virus, EBNA1 lacks DNA helicase activity (*9, 10*), and oriP replicates nearly once in each cell cycle (*11-13*). These indicate that oriP has some properties of cellular origin (for review, see Ref. *14*). EBNA1-binding sites are clustered in two regions of oriP, a family of repeats (FR) and a dyad symmetry sequence (DS) (*2, 15, 16*). FR contains 30 repeats of EBNA1-binding sites, while DS contains only 4 copies. These EBNA1 binding regions are separated by a 960-bp sequence. Both FR and DS are essential for the long-term replication and nuclear retention of oriP-containing plasmids (*15, 17*). Deletional mutation studies revealed that DS is important in the initiation of DNA replication (*17-19*), and FR activates the functions of DS (*15, 17*). More importantly, FR was shown to play a role in nuclear retention of oriP-containing plasmids (*15, 20*).

Replication of oriP-containing plasmids was analyzed in lymphocytes and other cell lines expressing only EBNA1 from the transfected gene, and replication was shown to initiate at or very near DS, proceed bidirectionally and complete at FR, which serves as a replication terminator (*18*). This and another study (*19*) suggested that the replication origin is present in a region containing DS. Expression of only EBNA1 as a viral protein in these cells resembles a distinct phenotype of EBV-infected cells (latency I), in which the expression of most viral genes is suppressed (*21-23*). A recent study revealed that a markedly different mode of EBV replication occurs in several EBV-infected Burkitt's lymphoma cell lines including Raji cells (*24*), which express all latent virus-encoded nuclear antigens, EBNA1 to 6, and latent membrane proteins, LMP1 and 2 (latency III phenotype). In Raji cells, the majority of initiation sites are dispersed in a broad region distant from oriP, and only a small portion of the episomal EBV genome initiates DNA replication from DS. This indicates that two types of replication occur in Raji cells. The major type does not occur in cells expressing only EBNA1, and it is unknown whether this replication is dependent on oriP and EBNA1.

An earlier study indicated that a 0.5-kb fragment containing DS but not FR can replicate efficiently in HeLa cells expressing EBNA1 (*19*). Another study showed that a 140-bp region containing DS can support replication of a plasmid at a low efficiency, although this replication was

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Abbreviations: EBV, Epstein-Barr virus; DS, a dyad symmetry sequence; FR, a family of repeats; PBS, phosphate-buffered saline.

analyzed in Raji cells and detailed deletional analysis was not done (*13*). A mutation study of oriP revealed that the number and spacing of EBNA1-binding sites in DS are important for the replication from oriP (*19*), but it has not been demonstrated that DS alone can initiate DNA replication. The regions flanking the EBNA1-binding sites contain TATA-box-like sequences and polyA adenylation signals (AATAAA), and it is possibile that these *cis*-acting elements may be important in DNA replication. It is not known what *cis*-acting elements in oriP are minimally required for DNA replication, although this knowledge is essential for studying mechanisms of initiation of DNA replication. In this study, we identified a minimal oriP that can initiate DNA replication in HeLa cells expressing EBNA1 by transient replication assays.

MATERIALS AND METHODS

OriP-Containing Plasmids—To construct UBamC, the *Bam*HI C fragment of the EBV genome from strain B95-8 (from 3994 to 13215 in B95-8 coordinate) was inserted into the *Bam*HI site of pUC119. For UD1, the *Eco*RI-*Spe*I fragment of oriP was excised from the *Bam*HI C fragment and inserted between the *Eco*RI and *Xba*I sites of pUC119. To construct UD2, the *Spe*I-*Sac*II region was excised from the oriP-containing plasmid pREP4 (Invitrogen), in which the *Sac*II site was converted to *Xba*I, and inserted into the *Xba*I site of pUC119. The plasmid vector pAS (1.7 kb) was made by deleting two regions from *Eco*RI to *Afl*III and from *Hin*dIII to *Ssp*I of pUC18. For AD2, a fragment containing the *Spe*I-*Sac*II region of UD2 was excised by *Bam*HI and *Sal*I and inserted between the same sites of pAS. AD3, AD4, and AD5 were derivatives of AD2, which had deletions between *Eco*RI and *Eco*RV, *Eco*RV and *Hpa*I, or *Hpa*I and *Sac*II, respectively. For AD6, the *Hpa*I-*Sac*II region was deleted from AD3. AD7, AD8, AD9, AD10, and AD11 contained oriP fragments, 8995 to 9134, 8995 to 9077, 8995 to 9050, 9031 to 9050, or 9021 to 9134 in B95-8 coordinates, respectively. To construct these plasmids, these fragments were amplified by PCR and inserted into pAS plasmids using the *Bam*HI and *Sal*I sites embedded in PCR primers. AD7 and AD6 contained the same oriP fragment but differed in sequences linked to the fragment. KORI was constructed by ligating the *Eco*RI-*Eco*RV fragment of UBamC and the *Eco*RV-*Xba*I fragment of AD2, and inserting the product between the *Eco*RI and *Xba*I sites of pBluescript KS− (Stratagene). To construct KD11, the *Bam*HI-*Sal*I fragment containing minimal oriP was excised from AD11 and inserted at the same sites in pBluescript KS−. KD12, KD13, KD14, KD15, and KD16 were generated from KORI by external or internal deletions. Deleted regions were as follows: *Apa*I fragment for KD12, the region from *Hpa*I to *Sac*II for KD13, *Nco*I fragment for KD14, and the region from *Nco*I to *Eco*RV for KD15. The positions of these restriction sites in B95-8 were 7315 (*Eco*RI), 8029 (*Nco*I), 8622 (*Nco*I), 8668 (*Spe*I), 8992 (*Eco*RV), 9132 (*Hpa*I), 9245 (*Apa*I), 9464 (*Apa*I), and 9516 (*Sac*II).

Expression Plasmids—An expression plasmid for EBNA1, pHbEBNAneo, was constructed as follows. The complete coding region for EBNA1 was assembled from three fragments: the initiation codon to *Avr*II site, *Avr*II to *Sac*I site, and *Sac*I site to the termination codon. The *Avr*II-*Sac*I fragment was excised from p220.2, which was kindly provided by Dr. Sugden, and two other fragments were amplified by PCR. These fragments were ligated and inserted between the human β-actin promoter and the SV40 polyadenylation site of pHβAPr-1-neo (*25*).

Cells—For HeLa/EB1, HeLa cells were transfected with pHbEBNAneo and selected with G418 (Lifetech) at 400 μg/ml. G418-resistant cells were isolated, and expression of EBNA1 was analyzed by immunoblotting using a monoclonal antibody to EBNA1 (Oncogene Science). One of these clones, HeLa/EB1, expressed EBNA1 constantly during this study without addition of G418 into cultures. 3TEB1 cells were prepared similarly by transfection of pHbEBNAneo into mouse Balb/c3T3 fibroblasts.

Transient Replication Assay—Cells were plated onto 60-mm dishes at 50% confluence a day before transfection. OriP-containing plasmids (5 μg) were transfected into cells by the calcium phosphate method. Four hours after addition of calcium phosphate precipitates, glycerol shock was performed with 10% glycerol in PBS for 3 min. The next day the cells were replated onto three 100-mm dishes and cultured for 3 d. Cell number increased 10-fold during culture, which corresponded to about 3.2 cell cycles. To recover the plasmids from cultures, cells were scraped off, collected by centrifugation and washed once with PBS. Alkali-SDS methods were used for plasmid extraction and purification. Briefly, the cell pellets collected from 100-mm dishes were suspended in 100 μl of a solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, and incubated for 10 min at room temperature. Alkali-SDS solution (200 μl) containing 0.2 M NaOH and 1% SDS was added to these samples and incubated for a further 10 min. The samples were neutralized with acetate buffer (150 μl) containing 3 M potassium acetate and 2 M acetic acid, then 10 M LiCl (150 μl) was added. After separating denatured debris by centrifugation, 2-propanol (400 μl) was added to precipitate plasmids, followed by incubation at 4°C overnight. Propanol precipitates were collected and dissolved in 200 μl of TE. The plasmid samples were purified by extraction with phenol and chloroform, precipitated with ethanol and redissolved in 40 μl of TE. Due to incomplete separation, final samples contained some cellular DNA and RNA. The plasmid samples (5 to 10 μl) were digested with *Dpn*I (30 U, New England Biolabs) in 25 μl of the buffer supplied from the manufacturer. Complete digestion with *Dpn*I was checked by addition of *dam*-methylated plasmids into digestion mixtures. To linearize plasmids, samples were digested with an appropriate restriction enzyme (30U): *Xba*I for UBamC; *Eco*RI for UD1 and UD2; *Pst*I for AD2-11; *Xho*I for KORI; KD11-16 and KS−; *Hin*dIII for pSV2. In addition, RNaseA was also added at 10 μg/ml to digest RNA in samples. Digestion was carried at 37°C for 2 h. Preliminary experiments confirmed that addition of more enzyme or prolonged digestion did not affect the results. Digested products were separated in 0.7% agarose gels containing TBE buffer and transferred onto membranes for hybridization. α-^{32}P-labeled probes were prepared from vector plasmids for the experiments described in the legends to figures. Membranes were exposed to X-ray films for 3 to 5 h at room temperature and also analyzed with a Fuji BAS2000 image analyzer to estimate the ratio of *Dpn*I-resistant plasmids to total plasmids. Two independent transient assay experiments were performed for each

set of plasmids, and average ratios of *Dpn*I-resistant plasmids are shown in the figures.

Western Analysis—Total cellular proteins were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membranes. EBNA1 protein was detected by using monoclonal anti-EBNA1 antibody (Oncogene Science) and anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) and the ECM detection reagent (Amersham).

RESULTS

Transient Replication of a Plasmid Containing oriP in HeLa/EB1 Cells—To avoid the complexities of EBV replication which occur in EBV-infected lymphoma cell lines (*24*), we analyzed replication of oriP in cells expressing only EBNA1. HeLa/EB1 cells were prepared by transfecting an EBNA1 expression plasmid into HeLa cells. Immunoblotting analysis using an anti EBNA1 antibody confirmed expression of EBNA1 in HeLa/EB1 cells. To confirm that oriP replicates in HeLa/EB1 cells, we transfected the plasmid UBamC (pUC119 containing the 9-kb *Bam*HI C fragment of the B95-8 EBV genome including the complete 2.2-kb oriP) by the calcium phosphate method. These cells were cultured for 4 d after transfection, harvested. Cell number increased about 10-fold during this period, which corresponded to about 3.2 cell cycles. Plasmid DNA was then recovered from cells by the alkali-SDS method and its replication was analyzed by determining sensitivity to the restriction endonuclease *Dpn*I, which digests 5′-GATC-3′ sites only when both strands are methylated. Plasmids prepared from dam$^+$ *Escherichia coli* cells are methylated at these sites and thereby sensitive to *Dpn*I. If a plasmid replicates at least once in HeLa/EB1 cells (dam$^-$), the 5′-GATC-3′ sites become hemimethylated or unmethylated, and therefore the replicated plasmids are resistant to *Dpn*I. The plasmid samples were digested with another restriction enzyme for linearization. When the plasmid sample prepared from the UBamC-transfected cells was analyzed, *Dpn*I-resistant UBamC was detected at the position of the linear full-length plasmid (12 kb) (Fig. 1, lane 6). Complete digestion with *Dpn*I was checked by addition of *dam*-methylated plasmids into duplicated digestion mixtures. The ratio of *Dpn*I-resistant plasmids to the total recovered plasmids was more than 99%, indicating that UBamC replicated efficiently in HeLa/EB1 cells. When the same amount of a vector plasmid (pUC119, 3 kb) was transfected into HeLa/EB1 cells and analyzed similarly, *Dpn*I-resistant plasmids were not detected (lane 4), and short *Dpn*I-digests (<1 kb) were produced (not shown in the figure). This indicated that oriP-independent background replication did not occur above the level of detection in this transient assay (<1% of recovered plasmids). To confirm that replication of UBamC was dependent on EBNA1, we transfected the plasmid into parental HeLa cells which did not express EBNA1 and performed similar analyses. UBamC did not replicate in HeLa cells, and the unreplicated plasmids were lost rapidly from the cells (lanes 11 and 12). Comparison of the amount of recovered

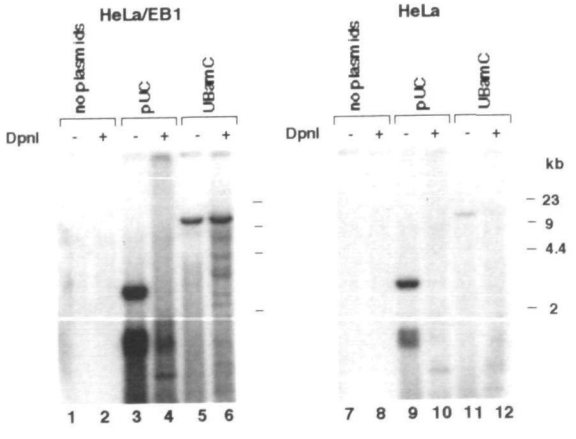

Fig. 1. **Replication of an oriP-containing plasmid in HeLa/EB1 cells.** Replication of plasmids was analyzed by transient replication assays in HeLa/EB1 and HeLa cells. The results of Southern hybridization analysis using a plasmid vector as a probe are shown here. UBamC (12 kb), plasmid containing the *Bam*HI C fragment of the EBV genome, which included the 2.2-kb oriP (Fig. 2); pUC, plasmid vector pUC119 (3 kb); no plasmids, mock-transfected control. *Dpn*I-digests (<1 kb) are not shown in the figure. HeLa/EB1 cells were prepared by transfecting an EBNA1 expression plasmid into HeLa cells. All plasmid samples were linearized with *Eco*RI. Plasmids which replicated at least once in cells were resistant to *Dpn*I digestion and detected at the position of the full-length linear plasmid.

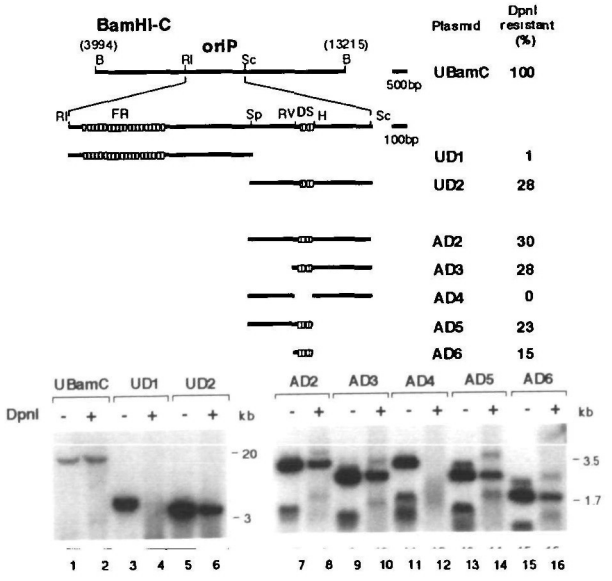

Fig. 2. **Replication of deletion mutants of oriP.** Deletion mutants of oriP were prepared as shown in the upper panel. These fragments were inserted into a vector plasmid and analyzed by transient replication assays in HeLa/EB1 cells. Southern hybridization results are shown in the lower panels. *Dpn*I-digests (<1 kb) are not shown in the figure. All samples were also digested with a restriction enzyme to linearize plasmids. Replicated plasmids were resistant to *Dpn*I digestion and detected at positions of linear plasmids. The ratios of *Dpn*I-resistant plasmids to recovered plasmids are shown as averages of two independent experiments. Restriction sites used for plasmid constructions are indicated in the map: B, *Bam*HI; RI, *Eco*RI; Sc, *Sac*II; Sp, *Spe*I; RV, *Eco*RV; H, *Hpa*I. Numbers in parenthesis indicate the *Bam*HI sites of the *Bam*HI C fragment in B98-5. EBNA1-binding sites (20 bp) are indicated by open boxes; FR, the family of repeats; DS, the dyad symmetry sequence. Plasmid vectors used to construct these plasmids were pUC119 (3 kb) for UBamC, UD1, UD2, and pAS (1.7 kb) for AD2-AD6.

plasmids indicated that HeLa/EB1 cells retained plasmids more efficiently than HeLa cells. A similar observation was reported previously (*26*). The hybridized DNA found at a position shorter than the linear plasmid (lanes 3 and 9) were the plasmids denatured during the alkali-SDS purification. Denatured plasmids were also observed in the experiments described in Figs. 2, 3, and 4.

Identification of Minimal oriP That Can Initiate DNA Replication—To find minimal *cis*-acting elements for DNA replication within the oriP region, we examined replication of plasmids containing fragments of oriP. The entire oriP region was first divided into two regions, UD1 containing a region including FR, and UD2 containing the rest where DS is located (Fig. 2). When UD1 and UD2 were transfected into HeLa/EB1 cells for transient replication assays, UD2 replicated efficiently (lane 6), but replication of UD1 was barely detected (lane 4). Ratios of *Dpn*I-resistant plasmids to total recovered plasmids were estimated as 28% for UD2 and 1% for UD1. The short *Dpn*I-digests (<1 kb) are not shown in the figure. To analyze the region containing DS in detail, we inserted the oriP fragment into a small vector, pAS (1.7 kb), and obtained AD2. We considered that use of smaller plasmids could help in detecting lower replication activity. When AD2 was analyzed in a similar transient replication assay, AD2 replicated as efficiently as UD2 (lane 8). To narrow down the region containing the origin, we made several deletion mutants of AD2. AD3 and AD5 had a deletion in the region flanking either end of DS, but these plasmids still retained much of the original replication activity (lanes 10 and 14). AD4 had an internal deletion of a small fragment including DS, and this deletion completely abolished replication activity of the plasmid (lane 12). AD6, which contained only this small region, replicated efficiently (lane 16). The ratio of *Dpn*I-resistant plasmids to total plasmids recovered was 15% for AD6, which corresponded to half of AD2. From these results, the replication origin was localized to a small region (140 bp) from *Eco*RV to *Hpa*I, which contained four EBNA1-binding sites (DS, 95 bp) and two flanking sequences of DS (36 and 9 bp).

To determine the minimal oriP within the *Eco*RV-*Hpa*I region, we amplified several fragments by PCR and analyzed their origin activity (Fig. 3). AD7 had the same *Eco*RV-*Hpa*I fragment but differed in the linked vector sequence. AD7 replicated as efficiently as AD2 (lanes 2). In this experiment, some plasmids were detected above the linear DNA positions in both *Dpn*I-digested and undigested samples (lanes 1, 2, 9, and 10): these were plasmids denatured during alkali-SDS preparation. AD8 had one pair of EBNA1-binding sites and the 36-bp flanking sequence, and this plasmid replicated at a very low efficiency (lanes 4), corresponding to less than one-tenth of AD7. The plasmids containing a single EBNA1-binding site (AD9 and AD10) did not replicate (lanes 6 and 8). In contrast, a plasmid (AD11) containing only the four EBNA1-binding sites (DS) replicated efficiently (lane 10). As replication

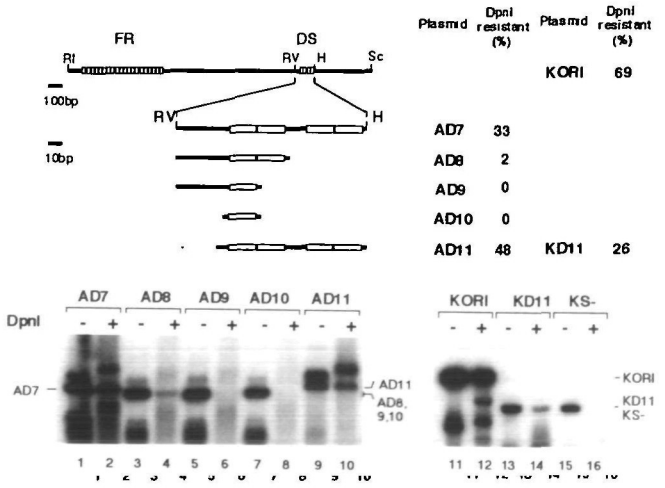

Fig. 3. **Identification of minimal oriP.** Origin activities of short fragments within the *Eco*RV-*Hpa*I region were analyzed by transient replication assays in HeLa/EB1 cells. The analyzed fragments and the average ratio of *Dpn*I-resistant plasmids in recovered plasmids are shown in the upper panel. Results of Southern hybridization analyses are shown in the lower panels. Replicated plasmids were resistant to *Dpn*I digestion and detected at positions of linear plasmids, which are indicated by plasmid names. *Dpn*I-digests (<1 kb) are not shown in the figure. All plasmids were linearized by a restriction enzyme. AD7 and AD6 (Fig. 2) had the identical *Eco*RV-*Hpa*I fragment but differed in vector sequences linked to the oriP fragment. Restriction sites were: RI, *Eco*RI; Sc, *Sac*II; RV, *Eco*RV; H, *Hpa*I. EBNA1 binding sites (20 bp) are indicated by open boxes; FR, the family of repeats; DS, the dyad symmetry sequence. Plasmid vectors used to construct these plasmids were pAS (1.7 kb) for AD7-AD11, and pBluescript KS− (KS−, 3 kb) for KORI and KD11.

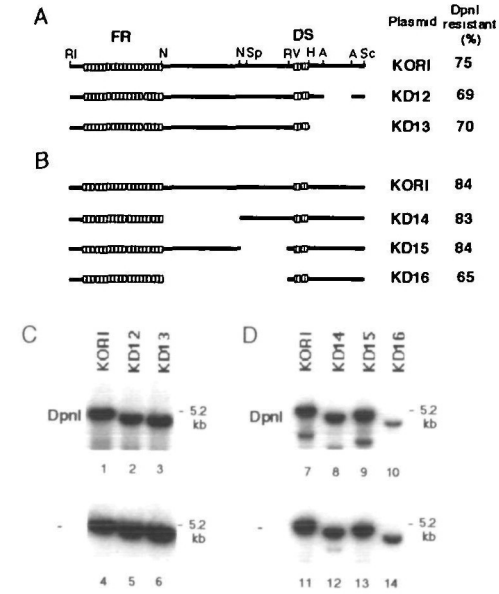

Fig. 4. **Deletions of the sequences flanking EBNA1-binding sites did not affect replication of minimal oriP.** Deletion mutants of KORI were analyzed by transient replication assays. (A) Deletions in the *Hpa*I-*Sac*II region. (B) Deletion in the region between FR and minimal oriP (DS). (C) and (D) show Southern hybridization results of these deletion mutants. Results of the *Dpn*I-resistant plasmids (*Dpn*I: lanes 1-3 and 7-10) and the recovered plasmids (-: lanes 4-6, 11-14) are shown laterally. Replicated plasmids were resistant to *Dpn*I digestion and detected at positions of linear plasmids, which are indicated by plasmid names. *Dpn*I-digests (<1 kb) are not shown in the figure. All plasmids were linearized with a restriction enzyme. Restriction sites used for plasmid construction are indicated in the map: RI, *Eco*RI; N, *Nco*I; Sp, *Spe*I; RV, *Eco*RV; H, *Hpa*I; A, *Apa*I; Sc, *Sac*II. EBNA1-binding sites (20 bp) are indicated by open boxes; FR, family of repeats; DS, dyad symmetry sequence.

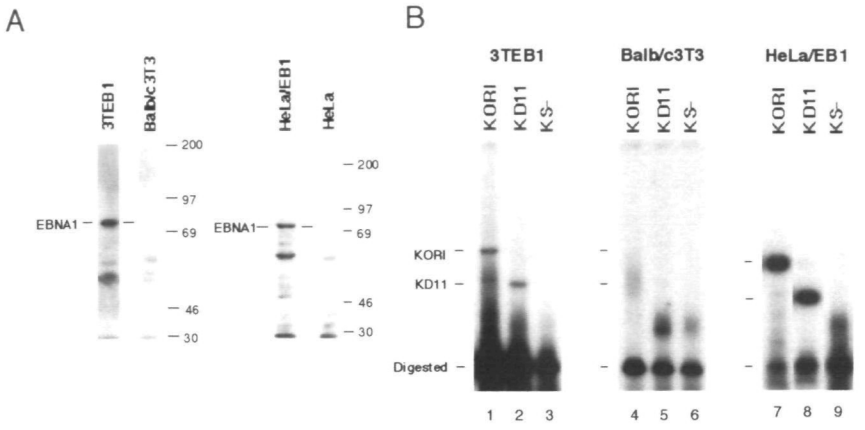

Fig. 5. **Replication of oriP in mouse fibroblasts.** KORI (complete oriP) and KD11 (minimal oriP) replication were analyzed by transient replication assays in either 3TEB1, Balb/c3T3, or HeLa/EB1 cells. (A) Expression of EBNA1 protein. 3TEB1 cells were the Balb/c3T3 cells transfected with an EBNA1 expression plasmid. Proteins of the same number of cells (1×10^4) were loaded on each lane. (B) Transient replication analysis. Results of Southern hybridization analyses of *Dpn*I-digested samples are shown. Positions of linear KORI and KD11 plasmids, and a 1-kb fragment from *Dpn*I digests (digested) are indicated on the left. The hybridization probe was the 1-kb *Dpn*I fragment of pBluescript KS− vector. Replicated (*Dpn*I-resistant) and unreplicated plasmids (*Dpn*I-sensitive) were detected at the full-length positions or at 1 kb, respectively.

activity differed the most between AD11 (48%) and AD8 (2%), we concluded that a 100-bp region containing the EBNA1-binding sequence (DS) is the minimal oriP region that can effectively initiate DNA replication.

To exclude the possibility that vector sequences linked to minimal oriP might provide some *cis*-acting elements required for DNA replication, we constructed another plasmid, KD11, in which minimal oriP was linked to different vector sequences from those in AD11. Although replication activity of minimal oriP in the KS− vector (KD11) was lower than that in the pAS vector (AD11), we confirmed that KD11 replicated in HeLa/EB1 cells (lane 14). As a control, replication of a plasmid containing the entire oriP (KORI) was also analyzed (lane 12). KORI replicated efficiently and the ratio of *Dpn*I-resistant plasmids was 69%, which was about 3-fold higher than KD11 (26%).

We next searched for *cis*-acting elements that might activate or suppress the minimal oriP only when FR was present in the same plasmid. We constructed several deletion mutants of KORI and analyzed their replication in HeLa/EB1 cells. KD12 and KD13 had a deletion in the *Hpa*I-*Sac*II region flanking the minimal oriP (Fig. 4A). These deletions did not affect replication (Fig. 4C, lanes 2 and 3). KD14, KD15, and KD16 had a deletion in the intervening sequence between FR and minimal oriP (Fig. 4B). Although these mutations shortened the distance between two EBNA1-binding regions, the deletions neither enhanced nor suppressed the replication activity of minimal oriP (Fig. 4D, lanes 8–10). We therefore found no auxiliary *cis*-acting elements for DNA replication in these flanking sequences of the EBNA1-binding sites.

DNA Replication from Minimal oriP in Mouse Fibroblasts—Plasmids containing both oriP and the EBNA1 gene cannot be maintained in rodent cells (*3*). We examined transient replication of KORI and KD11 in mouse fibroblasts to know whether oriP can initiate DNA replication in rodent cells as well as human cells. We prepared mouse fibroblasts expressing EBNA1 (3TEB1) by transfecting the EBNA1-expression plasmid into Balb/c3T3 cells. Western analysis confirmed that 3TEB1 expressed EBNA1 at a level similar to that in HeLa/EB1 cells (Fig. 5A). When KORI and KD11 were transfected into 3TEB1 cells for transient replication assays, we detected replication of KORI and KD11 (Fig. 5B, lanes 1 and 2). No such replication was detected in the parental Balb/c3T3 cells (lanes 4 and 5) and no background replication was observed in 3TEB1 cells (lane 3). These observations indicated that replication of KORI and KD11 depended on oriP and the expression of EBNA1. However, the ratio of *Dpn*I-resistant plasmids was only 2% for both plasmids, which was significantly lower than those observed in HeLa/EB1 cells: 69% for KORI, 26% for KD11 (lanes 7 and 8). Apparently there is a species specificity in DNA replication from minimal oriP. As there is no evidence that EBNA1 is involved in the elongation process of DNA replication, we suggest that this species specificity is due to the process which initiates DNA replication from minimal oriP.

DISCUSSION

In this study, we demonstrated that the four EBNA1 binding sites in DS constitute the minimal oriP that can initiate DNA replication. Sequences flanking the minimal oriP are not essential and apparently do not have any enhancing or supressing functions in DNA replication (Figs. 3 and 4). We also demonstrated that minimal oriP can initiate DNA replication in mouse fibroblasts expressing EBNA1, but the efficiency is lower than that observed in human cells, the natural host of Epstein-Barr virus (Fig. 5).

Identification of the minimal origin element will provide insight into how replication initiates from the minimal oriP. The minimal oriP contains only EBNA1-binding sites, and no other *cis*-acting elements are required for DNA replication. An important conclusion from our study is that DNA replication from the minimal oriP is initiated by recruitment of cellular replication factors onto or near the minimal oriP by EBNA1. *In vivo* footprinting analysis (*27*) revealed that EBNA1 binds to the minimal oriP constitutively throughout the cell cycle. Hsieh *et al.* (*27*) suggested that when all four sites are occupied by EBNA1, no other DNA-binding factors can interact with the minimal oriP, because the recognition sequence of EBNA1 is 20 bp and the minimal oriP is 100 bp. Therefore, EBNA1 is the only factor that binds to the minimal oriP in a sequence-specific manner. It is still unknown, however, what functions EBNA1 has in DNA replication. No known enzymatic activities related to DNA replication are attributed to

EBNA1 (*9, 10*). EBNA1 may have a function similar to the origin recognition complex (ORC) in DNA replication of cellular origin, a launching pad for the replication machinery (*28-30*). It is, therefore, important to determine which factors are associated with EBNA1 at the site of the replication origin, but no EBNA1-associated replication factors have yet been identified.

Some mammalian origins as well as viral origins contain the DNA unwinding element (DUE), sequences which are unwound easily. DUE is often found near origin recognition elements (ORE) (*31, 32*). SV40 large T antigen unwinds DNA at DUE and initiates bidirectional replication. Interestingly, a study predicts that the EBNA1-binding sequences in the minimal oriP (DS) are potential DUEs (*33*). Since the minimal oriP sequence contains sufficient *cis*-acting elements to initiate DNA replication, DNA replication may be initiated within the minimal oriP region. Studies using chemical modification of DNA as probes indicated that EBNA1 distorts the DNA of binding sites (*27, 34, 35*) but does not unwind the minimal oriP. Therefore, cellular replication factors may assist EBNA1 to unwind DNA at the minimal oriP. Alternatively, DUE may not be essential for replication from the oriP, and a cellular replicative helicase may be loaded by EBNA1 and unwind a region near the minimal oriP.

Although FR lacks the ability to initiate DNA replication, presumably because spacing between EBNA1-binding sites is different in FR and DS (*17, 19, 36*), it can activate the functions of DS (*15, 17*). The results of our transient replication assays (Figs. 3 and 4) confirmed that FR enhances DNA replication from the minimal oriP. This replication enhancement may be due to binding to FR by EBNA1 with higher affinity than to DS (*4, 9, 37*), and the EBNA1 complex bound to FR may stabilize the EBNA1 complex bound to DS by the interaction between the two complexes (*10, 38, 39*). Our study showed, however, that low-level EBNA1-dependent replication from the minimal oriP is not enhanced by FR in mouse fibroblasts (Fig. 5). This indicates that some cellular factor is involved in the replication enhancement. A mutation study of DS indicated that only one pair of EBNA1-binding sites is sufficient for long-term maintenance of oriP-containing plasmids when FR is present in the plasmids (*19*). This can be explained by enhancement by FR of very low origin activity of a pair of EBNA1-binding sites, as observed in our experiments (Fig. 3, lane 4). As there was a large difference in origin activiy between one pair (AD8) and two pairs of EBNA1 (AD11), the four EBNA1 binding sites appear to act as a functional minimal oriP.

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