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Replication of Epstein-Barr Virus oriLyt: Lack of a Dedicated Virally Encoded Origin-Binding Protein and Dependence on Zta in Cotransfection Assays

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Using a transient replication assay in which cosmid DNAs were cotransfected into Vero cells, we had previously demonstrated that oriLyt replication required six Epstein-Barr virus (EBV)-encoded replication genes. No oriLyt origin-binding protein was identified in this study, but oriLyt replication in the cotransfection assay was also dependent on the three lytic cycle transactivators Zta, Rta, and Mta and an activity encoded by the EBV *Sal*I F fragment. We have now used expression plasmids for the six known replication proteins to further examine the question of the requirement for an oriLyt origin-binding protein. The activity in *Sal*I-F was shown to be encoded by BKRF3. The predicted product of this open reading frame is an enzyme, uracyl DNA glycosylase, not an origin-binding protein, and is dispensable for replication in assays using expression plasmids. BBLF2, which is positionally related to the gene for the herpes simplex virus (HSV) UL9 origin-binding protein, was confirmed to be expressed as a spliced transcript with BBLF3 and not as an independent product. Examination of the requirement for the EBV transactivators revealed that Rta, while contributing to replication efficiency, was dispensable. Mta could be substituted by HSV IE63, and in complementation experiments with HSV replication genes, Mta was no longer required for replication of EBV oriLyt, suggesting that the contribution of Mta to replication may be indirect. Zta continued to be required for detectable oriLyt replication both with the EBV replication proteins and in the complementation assays with HSV replication proteins. We conclude that EBV does not encode an equivalent of HSV UL9 and that Zta is the sole virally encoded protein serving an essential origin-binding function.

Epstein-Barr virus (EBV) utilizes different origins of replication during lytic versus latent infection. The dependence of DNA replication on virally encoded proteins also differs in these two states. EBV establishes a latent infection in B cells. Latency replication occurs once per cell cycle (91), proceeds bidirectionally from oriP (32), and is dependent on cellular DNA replication proteins plus a single EBV-encoded protein, EBNA-1 (90). EBNA-1, which is unique to gamma one herpesviruses, binds to multiple sites within oriP, and binding leads to distortion of oriP sequences (30, 31, 42, 44, 69, 78). Lytic replication occurs predominantly in epithelial cells within the oropharynx (76). Multiple rounds of lytic DNA replication are initiated within a separate origin, oriLyt (36), and lytic replication has a greater dependence on EBV-encoded proteins. A cotransfection-replication assay modelled on that used to identify essential HSV replication proteins (7, 87) was previously used to demonstrate the functional requirement for six lytic replication proteins, BALF5 (DNA polymerase), BMRF1 (polymerase processivity factor), BALF2 (single-stranded DNA-binding protein), BSLF1 (primase), BBLF4 (helicase), and BBLF2/3 (helicase-primase-associated protein) (25). These replication proteins have either sequence or positional homology with known herpes simplex virus (HSV), varicella-zoster virus, and cytomegalovirus (CMV) replication proteins (10, 16, 55, 65). One activity that has not been identified in EBV is a lytic origin-binding protein that would be equivalent to the HSV UL9 protein.

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Three viral transactivators regulate lytic EBV gene expression. The key protein for activating the lytic cycle is Zta, encoded by the BZLF1 open reading frame (ORF) (13). Zta is a bZIP transcriptional activator with a unique non-leucine-based dimerization domain (9, 26, 27). Zta binds directly as a homodimer to both AP1 sites and to related sequences called Zta response elements (ZREs) (9, 23, 52, 80). Activation of transcription occurs through direct stabilizing contacts between the amino-terminal activation domain of Zta and TATA-binding protein (TBP) (50) and through interaction of Zta with TBP-associated factors to stimulate formation of a stable TFIIA-TFIID complex (51). Activation by Zta in the absence of DNA binding has also been observed with certain targets (28). The Rta transcriptional activator encoded by BRLF1 (38) also binds DNA as a dimer (34, 54) and contains a powerful negatively charged, VP16-like activation domain (39). Rta can contact both TBP and TFIIB (53). Zta and Rta in combination produce a synergistic response in targets containing binding sites for both factors (14, 68). The third transactivator, Mta (encoded by BSLF2/BMLF1), has a posttranscriptional mechanism of action (4, 45). Mta has homologs in the alpha and beta herpesviruses, and the mechanism of action of the HSV homolog, IE63, has been shown to include positive regulation at the level of poly(A) selection and negative regulation of spliced messages through redistribution of splicing small nuclear ribonucleoproteins out of coiled bodies (56, 67, 71).

The *cis*-acting sequences that form viral replication origins frequently overlap with transcriptional regulatory elements (18, 19). In some cases these elements are essential for replication, while in others they play an auxiliary role. Within the minimally defined EBV oriLyt are two transcriptional regions, the promoter and leader for the BHLF1 gene and an upstream

enhancer (36). The promoter contains four binding sites for the Zta transactivator and is strongly Zta responsive in transient expression assays (52). Deletion of the TATA box, ZRE1, ZRE2, and the CAAT box abolishes replication, while mutation of the ZRE1 and ZRE2 sites reduces replication efficiency fourfold (73). The enhancer contains binding sites for both Zta and Rta and responds synergistically to the presence of these transactivators (14). Mutation of the Zta binding sites in the enhancer in conjunction with mutation of the four promoter Zta binding sites ablates replication ability, providing evidence for an essential role for Zta in lytic replication (72). The enhancer also has considerable basal activity which can be abolished by mutation of a major late transcription factor (MLTF) binding site (70). Interestingly, the CMV oriLyt and a recently described cellular origin also contain MLTF binding sites (1, 33, 37). The presence and arrangement of transcriptional signals in EBV oriLyt is conserved in oriLyt of another gamma one herpesvirus, herpesvirus papio (70). A second *cis*-acting region that is required for oriLyt replication has been identified by deletional analysis (73). This region is reminiscent of pyrimidine-biased DNA that forms a structural feature in other origins (83), but its contribution to EBV oriLyt replication has yet to be fully elucidated.

In previously published cotransfection-replication assays, the six EBV replication genes were provided as cosmid DNAs (25). In this setting, oriLyt replication was also dependent on the presence of the three EBV lytic transactivators, Zta, Rta, and Mta, and on the presence of an activity provided by the EBV *Sal*I F fragment. The apparent absence of an EBV-encoded oriLyt origin-binding protein equivalent to HSV UL9 was surprising. In the present study, we have used cotransfection assays with EBV replication genes expressed from heterologous promoters to eliminate the possibility that *Sal*I F encodes an origin binding activity and to further probe the contribution of the lytic transactivators to oriLyt replication.

MATERIALS AND METHODS

Plasmids. The *Bam*HI-H plasmid containing oriLyt that formed the target in the replication assays, the Zta, Rta, and Mta expression plasmids, and the expression plasmids for BMRF1, BALF5, BSLF1, BBLF2/3, BBLF4, and BALF2 have been described previously (25). Plasmids pEF66, pEF67, and pEF68 are derivatives of the EBV *Bam*HI-K-containing plasmid pGD5. The BKRF4 ORF in pEF66 carries a 210-bp deletion created by partial digestion with *Stu*I. An oligonucleotide encoding a triple terminator (CTAGTCTAGACTAG) was then ligated into the *Stu*I site. Cleavage of pGD5 with *Sal*I and *Sst*I followed by religation of the blunt-ended DNA resulted in the removal of the first 2,511 bp of BKRF1 (pEF67). A similar clone was constructed by using pEF66 as the parent. The resulting plasmid, pEF68, has both a deletion in BKRF1 and a triple terminator in BKRF4.

The BKRF3 expression plasmid, pEF72A, was generated by adding *Bam*HI linkers to a *Hin*cII fragment of pEF66 containing BKRF3 and cloning the fragment into the *Bam*HI site of the simian CMV expression vector pGH70. Plasmid pEF69 is a BBLF2/3 expression plasmid with a triple terminator introduced at the *Bgl*II site in the BBLF2 ORF, while pEF71 is a similar construction with a triple terminator at the *Sfi*I site in BBLF3. The expression vector for the synthetic cDNA version of BBLF2/3, pEF76A, was created by cleavage of the BBLF2/3 plasmid, pEF75A, partially with *Sfi*I and to completion with *Not*I and introduction of the *Sfi*I and *Not*I cleaved 191-bp cDNA PCR product that was generated from lytically induced Akata cells and shown by sequencing to represent the spliced message.

The complete set of expression plasmids for the HSV replication proteins was obtained from R. Heilbronn (43). The HSV oriS plasmid, pKR46, contains HSV type 1 (HSV-1) oriS as a 538-bp *Sau*3A fragment cloned into the pBR322 derivative pKP54. The HSV IE63 protein encoded by pJM200 is expressed from its own promoter and contains sequences from −250 to 580 bp downstream of the poly(A) motif. The derivative, pJM206, has an *Xba*I triple terminator inserted at the *Stu*I site and expresses a truncated IE63 protein (amino acids 1 to 406).

Isolation of RNA from Akata cells. EBV-positive Akata cells were lytically induced with anti-human immunoglobulin G (IgG) antibody (Cappel, Durham, N.C.) at a concentration of 0.1 mg/ml (79). Total RNA was isolated from 8×10^5 cells at 3 and 8 h after induction, using guanidine isothiocyanate. Control RNA was isolated in parallel from untreated Akata cells. RNA (1 mg) from each sample was reverse transcribed as instructed by the manufacturer, using random hexamers as primers and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The resulting cDNA samples were PCR amplified by using the primers 5′-CTACACGCGGCCGCTGAC (LGH566) and 5′-TGCGGGCCACGGTGGCCT (LGH567). The reaction mixtures were electrophoresed on an 8% polyacrylamide gel and stained with ethidium bromide to visualize the PCR products.

Cotransfection-replication assays. DNA transfection of Vero cells and oriLyt replication assays were performed as previously described (25).

RESULTS

The replication-augmenting gene provided by *Sal*I-F is BKRF3. We have previously described a cotransfection-replication assay in which the EBV genes required for oriLyt replication were provided in the form of cosmid DNAs (25). OriLyt replication in this assay was also dependent on the presence of the three lytic cycle transactivators Zta, Rta, and Mta and an EBV DNA fragment, *Sal*I-F. To identify the gene provided by *Sal*I-F, we substituted plasmid and cosmid constructions that contained *Sal*I-F sequences (Fig. 1A). In the replication assays shown in Fig. 1, the transfection mixture contained oriLyt-containing target plasmid *Bam*HI-H, the lytic transactivators, and the cosmids cMB14 and cM302-21. Cosmid cM301-99 was substituted by a BBLF4 expression plasmid, *Bam*HI-BG (containing BBLF2/3), plus the DNAs indicated above each lane.

We first tried substituting the 8.0-kb *Sal*I-F plasmid with the smaller 5.0-kb *Bam*HI-K plasmid. Replication was positive (Fig. 1B, lane 2). The four intact ORFs located within *Bam*HI-K are BKRF1, which encodes the latency origin-binding protein EBNA-1; a late glycoprotein gene, BKRF2; and two early genes, BKRF3 and BKRF4 (24). Introduction of a deletion plus a stop codon into BKRF4 within the *Bam*HI-K plasmid had no effect on replication efficiency (lane 3), indicating that BKRF4 did not encode a replication function. EBNA-1 could also be eliminated as the relevant protein since substitution of a deleted construction lacking BKRF1 either in an otherwise wild-type *Bam*HI-K background (ΔE1; lane 4) or in conjunction with the BKRF4 stop codon (ΔE1/◇RF4; lane 5) did not negatively affect replication of oriLyt. On the other hand, replication did not occur when *Sal*I-F was substituted with cM302-23 (lane 3). The BKRF1 and BKRF2 ORFs are present in cM302-23, but BKRF3 is interrupted. Taken together, these experiments suggested that the requirement for *Sal*I-F represented a requirement for BKRF3 and replication was positive on inclusion of a BKRF3 expression plasmid. The protein encoded by BKRF3 has a high degree of homology to a family of uracyl DNA glycosylases (UDGs). As will be addressed in a later section, while BKRF3 was necessary when the replication genes were provided as cosmid DNAs, it did not continue to be a requirement in the presence of the cloned replication genes.

The BBLF2 and BBLF3 ORFs are transcribed as a spliced mRNA, and both exons are required for replication function. We had previously demonstrated that an expression plasmid containing the BBLF2 and BBLF3 ORFs provided an essential function in the cotransfection replication assay (25). BBLF2 and BBLF3 occupy the equivalent position in the EBV genome to UL8 of HSV. The BBLF2 ORF lacks a distinct polyadenylation signal, but there is a consensus signal downstream of BBLF3, and it has been stated in a review that these two ORFs are expressed as a spliced transcript (24). In contrast, UL8 consists of a single ORF and is not generated from a spliced transcript. The adjacent ORF in HSV is that encoding the

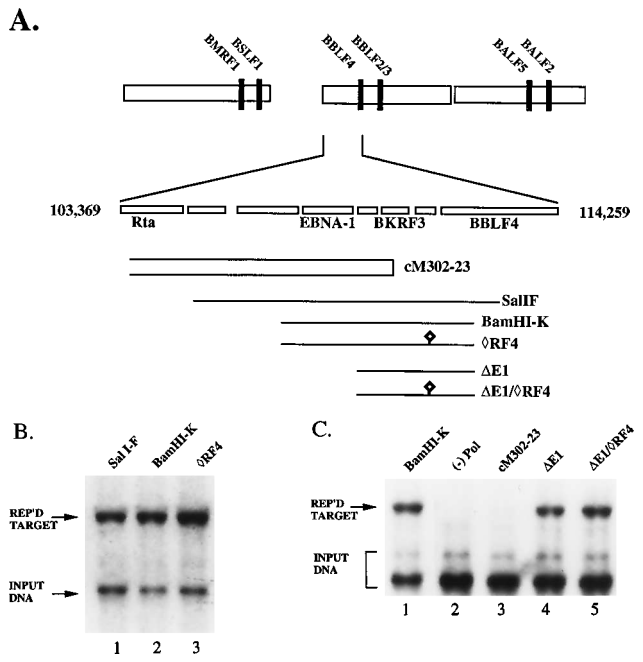

FIG. 1. BKRF3 is the gene within *Sal*I-F required for replication when the replication genes are provided as cosmid or plasmid DNAs. (A) The three cosmids carrying replication genes are diagrammed, and the locations of the individual genes are highlighted. An expanded region of cM301-99 is shown below, with the ORFs between 103,369 and 114,259 in the EBV genome (24) illustrated along with the regions contained within the individual plasmid and cosmid constructions. Introduced triple terminators are indicated (◇). (B and C) Southern blots of DNA isolated from transfected Vero cells were probed with a subfragment of *Bam*HI-H to detect *Dpn*I-resistant, replicated DNA. (B) Lane 1, the BMRF1, BSLF1, BALF5, and BALF2 genes were provided by cMB14 and cM302-21, while the third cosmid was replaced by plasmids *Bam*HI-BG (containing BBLF2/3) and *Sal*I-F plus a BBLF4 expression plasmid. Lane 2, *Sal*I-F was replaced by *Bam*HI-K. Lane 3, *Bam*HI-K was replaced by a derivative containing a triple terminator at 5′ end of BKRF4 (◇RF4). (C) Lane 1, positive control (plus *Bam*HI-K). Lane 2, cosmid cM302-21 was replaced with an expression plasmid for BALF2, but no source of BALF5 (polymerase) was provided. Lane 3, *Bam*HI-K was replaced by the cosmid clone cM302-23. Lane 4, *Bam*HI-K was replaced by ΔEBNA-1. Lane 5, *Bam*HI-K was replaced by ΔEBNA-1/◇RF4.

origin-binding protein, UL9. To eliminate the possibility that BBLF2 and BBLF3 represent coding sequences for two separate proteins, one of them the UL9 homolog, we examined the nature of the BBLF2/3 transcript by using a PCR protocol. EBV-positive Akata cells were induced for lytic cycle gene expression by treatment with anti-IgG antibody (79), and total RNA was isolated 3 or 8 h later. RNA isolated in parallel from noninduced cells provided the negative control. The isolated RNA was reverse transcribed by using random hexamers as primers, followed by PCR amplification of the cDNA across the putative splice junction by using primers that annealed to sequences outside of the boundaries of the predicted splice donor (AAGGTGAAT) and acceptor (ATCTTCCTC CAGGT) sites. The PCR product generated from a nonspliced message would be 320 bp, while that from a spliced message would be 191 bp (Fig. 2A). A PCR product of 191 bp was amplified from the induced cultures (Fig. 2B) and shown by PCR sequencing to represent the spliced BBLF2/3 cDNA. Thus, in Akata cells, the BBLF2/3 gene is indeed expressed early (3 h) after lytic cycle induction and is expressed as a spliced transcript.

To determine if each of the ORFs was required for activity of the CMV-BBLF2/3 expression plasmid in the replication assay, triple terminators were introduced into either BBLF2 or BBLF3. The stop codon in BBLF2 would allow translation of the first 217 amino acids of BBLF2, while that in BBLF3 would allow translation of all of BBLF2 plus the first 5 amino acids from BBLF3 (Fig. 3A). These mutant constructions were tested in a cotransfection-replication assay in which all of the other replication genes were provided as expression plasmids and the lytic cycle transactivators were also present. Replication of the oriLyt target was dependent on the presence of a functional BBLF2/3 product (Fig. 3B, lane 4). Introduction of a triple terminator into BBLF3 resulted in a product that was unable to support replication, indicating the essential nature of BBLF3 (lane 3). The construction containing the triple terminator in BBLF2 was severely impaired in its ability to support replication although not completely negative (lane 2). We believe that the limited replication obtained may reflect a low level of translation reinitiation at an internal, in-frame methionine immediately downstream of the triple terminator.

To provide additional evidence that the BBLF2/3 product was expressed from a spliced transcript, a synthetic cDNA was generated. The 320-bp region between the *Not*I and *Sfi*I restriction sites of the genomic version of BBLF2 and BBLF3 was replaced with the 191-bp PCR product amplified from induced Akata cells. (The *Not*I and *Sfi*I sites formed the outer boundaries of the PCR primers used to generate this product.) An expression plasmid containing the synthetic cDNA was able to support oriLyt replication (Fig. 3C), further strengthening the suggestion that the BBLF2 and BBLF3 ORFs are spliced to generate the functional product.

Requirement for the lytic cycle transactivators. In the initial transient replication assays, expression plasmids encoding the three EBV transactivators Zta, Rta, and Mta were cotransfected with the overlapping cosmids to ensure efficient expression of the early EBV replication genes from the cosmid DNAs. However, Zta and Rta binding sites also occur within the promoter and enhancer sequences that form part of the minimally defined oriLyt (Fig. 4A), and a mutational analysis has indicated a requirement for the Zta binding sites (72). The generation of expression plasmids for the complete set of replication genes allowed us to reassess the contribution of the transactivators in the cotransfection assay. We found that even when the replication genes were expressed from the strong heterologous CMV or simian virus 40 (SV40) promoter, the presence of the EBV transactivators altered the efficiency of oriLyt replication (Fig. 4B). Replication was least affected by the absence of Rta (Fig. 4B, lane 4). In cotransfections lacking Rta, the replication efficiency ranged from 20 to 60% of that obtained in the presence of all three transactivators. (Quantitation was performed by densitometer scanning of the replicated DNA band, with the input DNA band serving as a control for transfection efficiency.) Omission of either Zta or Mta resulted in a dramatic decrease in oriLyt replication (lanes 3 and 5).

The apparent requirement for Mta was surprising since Mta is a transactivator with a posttranscriptional mechanism of action. The Mta gene is conserved throughout the alpha, beta, and gamma herpesviruses. We asked whether the HSV homolog, IE63, was functionally equivalent to Mta. OriLyt replication was detected in the presence of an intact IE63 gene although at level lower than that observed in the presence of Mta (Fig. 5, lanes 1 and 2). An IE63 derivative that contains a terminator at codon 406 and does not function as a transactivator in transient expression assays (40, 57) was unable to substitute for Mta in the replication assay (lane 3). Thus, IE63 transactivator function was a necessary component of the IE63 contribution to replication.

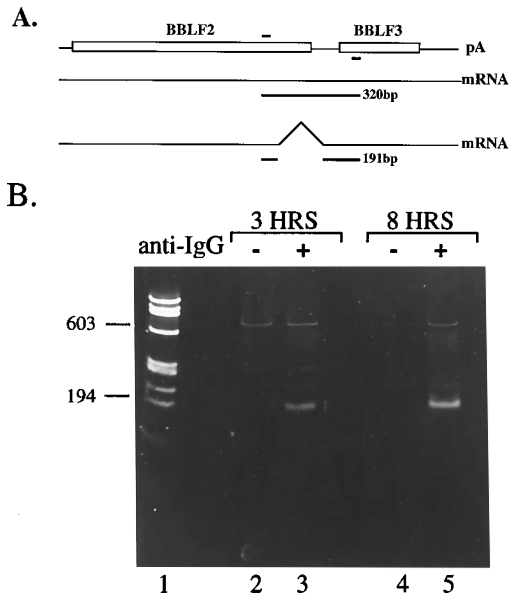

FIG. 2. A spliced BBLF2 and BBLF3 transcript is detected in induced Akata cells. (A) The BBLF2 and BBLF3 ORFs are diagrammed. The locations of the PCR primers (−) and the sizes of potential PCR products from spliced and unspliced cDNA templates are shown. (B) Ethidium bromide-stained 8% polyacrylamide gel showing the separated PCR products. Lane 1, ϕX size markers. Lanes 2 and 3, PCR products from RNA isolated 3 h after mock induction (−) or anti-Ig induction (+) of Akata cells. Lanes 4 and 5, PCR products from RNA isolated 8 h after mock induction (−) or anti-Ig induction (+).

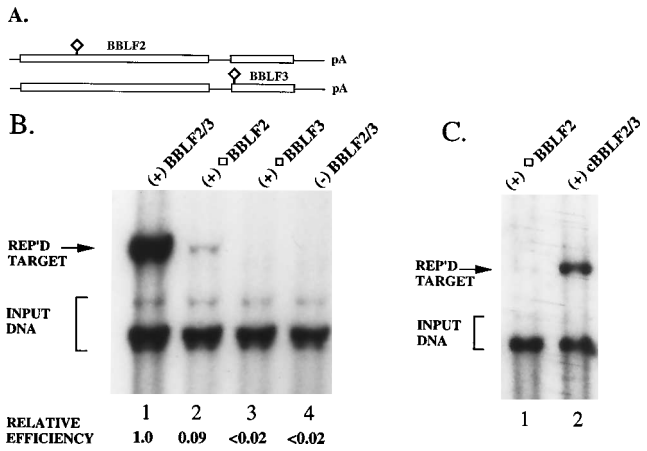

FIG. 3. Both the BBLF2 and BBLF3 ORFs are required for efficient replication of oriLyt. (A) The BBLF2 and BBLF3 ORFs are diagrammed. Location of triple terminators introduced into each ORF (◇) are shown. (B and C) Southern blots of *Bam*HI- and *Dpn*I-digested DNA probed with a subfragment of *Bam*HI-H. (B) Lane 1, Vero cells were transfected with the *Bam*HI-H target and expression plasmids encoding the replication genes and Zta, Rta, and Mta. Lane 2, BBLF2/3 was replaced by a derivative containing a triple terminator in the BBLF2 ORF. Lane 3, BBLF2/3 was replaced by a derivative containing a triple terminator in the BBLF3 ORF. Lane 4, BBLF2/3 was omitted. The resulting autoradiogram was scanned by densitometry, and the ratio of the *Dpn*I-resistant band to the first *Dpn*I-sensitive band was determined. This value for the positive control (lane 1) was set at 1. (C) Lane 1, BBLF2/3 was replaced by a derivative containing a triple terminator in BBLF2. Lane 2, BBLF2/3 was replaced by a synthetic cDNA version of BBLF2/3.

Subtraction assays with cloned genes identify six dedicated EBV replication proteins that are essential for oriLyt replication. Now that a complete set of cloned replication genes was available, it was also possible to definitively examine the requirement for each gene by removing individual expression plasmids from the transfection mixture. In the presence of all seven genes plus the lytic cycle transactivators, efficient replication of the oriLyt target occurred (Fig. 6, lane 1). Omission of any one of the six genes BMRF1, BSLF1, BBLF4, BBLF2/3, BALF5, and BALF2 abolished replication, reinforcing the essential nature of their protein products (lanes 2, 3, and 5 to 8). In contrast, in the context of cloned genes, omission of BKRF3 resulted in only a twofold decrease in oriLyt replication (lane 4). Possible reasons for the differential requirement for BKRF3 in experiments in which the EBV genome was provided in the form of cosmid DNAs, as opposed to assays using cloned genes, will be discussed. However, the data in Fig. 6 clearly indicate that BKRF3 is not an essential replication gene.

Complementation between HSV and EBV replication genes. The six essential EBV replication genes have homologs in HSV (55). The striking differences between replication of HSV oriLyt and EBV oriLyt are (i) the requirement in HSV for a virally encoded origin-binding protein, UL9, and the lack of an EBV equivalent of this protein and (ii) the central role of the lytic transactivators in replication of EBV oriLyt compared with their more ancillary contribution to HSV lytic DNA replication (85). To gain further insight into EBV oriLyt replication, we examined whether the EBV genes could be substituted by their HSV homologs and whether the EBV lytic transactivators contributed in the presence of HSV replication genes.

A previously described set of HSV replication genes cloned into human CMV expression plasmids (43) was shown to replicate HSV oriS in a cotransfection-replication assay (Fig. 7, lane 1). When the genes for the HSV DNA polymerase and the polymerase processivity factor (UL30 and UL42) were substituted for their EBV counterparts, BALF5 and BMRF1, no replication of EBV oriLyt was observed (lane 3), and a similar result was obtained when the HSV proteins of the helicase-primase complex (UL5, UL8, and UL52) were substituted for

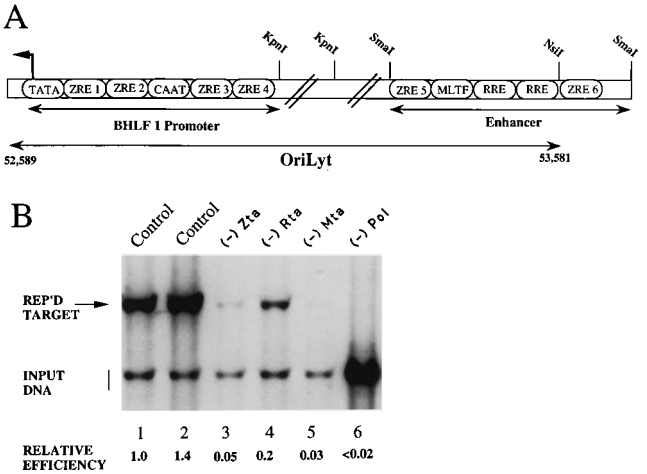

FIG. 4. Contribution of the Zta, Rta, and Mta transactivators to oriLyt replication. (A) Organization of oriLyt. The diagram shows the relative positioning of binding sites within the promoter and enhancer elements for the cellular transcription factors TFIID (TATA), CTF (CAAT), and MLTF and for the EBV Zta (ZRE) and Rta (Rta response element [RRE]) proteins. (B) Southern blot of *Bam*HI- and *Dpn*I-digested DNA isolated from transfected Vero cells. Lane 1, cells were cotransfected with the *Bam*HI-H target and expression plasmids for the seven replication genes and for Zta, Rta, and Mta. Lane 2, a different Rta expression plasmid (pDH201A) was used. Lane 3, Zta was omitted. Lane 4, Rta was omitted. Lane 5, Mta was omitted. Lane 6, DNA polymerase (BALF5) was omitted.

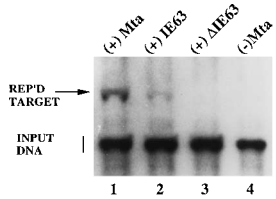

FIG. 5. The Mta transactivator can be substituted by the HSV-1 IE63 protein. A Southern blot of transfected cell DNA cut with *Bam*HI and *Dpn*I and probed with a subfragment of *Bam*HI-H is shown. Lane 1, Vero cells were cotransfected with the *Bam*HI-H target and expression plasmids encoding the replication genes and the three transactivators. Lane 2, Mta was replaced by HSV-1 IE63. Lane 3, Mta was replaced by a carboxy-terminal truncated HSV-1 IE63 protein. Lane 4, Mta was omitted.

EBV BBLF4, BBLF2/3, and BSLF1 (lane 4). However, the complete set of HSV proteins (minus UL9) was capable of replicating the EBV oriLyt in the presence of the Zta and Rta transactivators (lane 7). Replication of EBV oriLyt was below detectable levels in the absence of either the Zta and Rta transactivators or the absence of Zta (lanes 5 and 6).

This experiment indicates that there is functional equivalence between the HSV replication complex comprising UL30, UL42, UL5, UL8, UL52, and UL28 and the complex formed by the EBV BALF5, BMRF1, BBLF4, BSLF1, BBLF2/3, and BALF2 proteins but that individual proteins within the complex are not readily interchangeable. The data also indicate that the Mta transactivator is not absolutely essential for EBV oriLyt replication. Mta was not included in the assays using the HSV expression plasmids, and replication of oriLyt was observed in its absence (Fig. 7, lane 7). On the other hand, the importance of the Zta transactivator for efficient oriLyt replication was reemphasized by the inability to obtain detectable levels of oriLyt replication in the presence of the HSV replication proteins but the absence of Zta.

DISCUSSION

Replication origins consist of a core region that determines the position at which replication begins and auxiliary components that usually contain binding sites for transcription factors and contribute to replication efficiency, timing, and in some

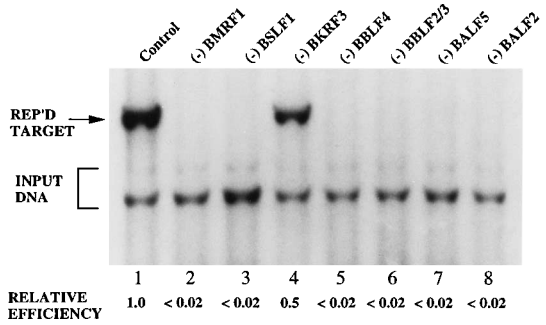

FIG. 6. Six replication genes are essential for oriLyt replication in the transient assay. A Southern blot of DNA isolated from transfected Vero cells and probed with a subfragment of *Bam*HI-H to detect *Dpn*I-resistant, replicated DNA is shown. Lane 1, cells were cotransfected with the *Bam*HI-H target, expression plasmids encoding BMRF1, BSLF1, BKRF3, BBLF4, BBLF2/3, BALF5, and BALF2, as well as expression plasmids encoding Zta, Rta, and Mta. One gene was omitted from each of the subsequent transfections. Lane 2, minus BMRF1; lane 3, minus BSLF1; lane 4, minus BKRF3; lane 5, minus BBLF4; lane 6, minus BBLF2/3; lane 7, minus BALF5; lane 8, minus BALF2. BKRF3 was the only nonessential gene in this assay.

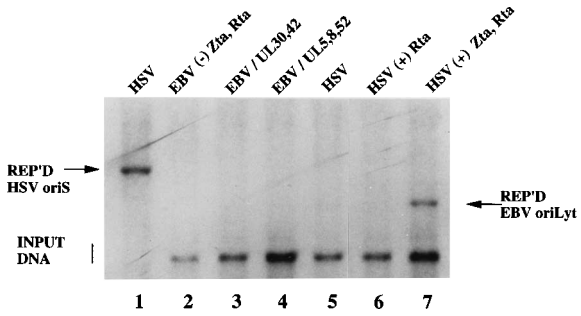

FIG. 7. HSV-1 replication genes can support EBV oriLyt replication. A Southern blot of transfected cell DNA cut with *Bam*HI and *Dpn*I and probed with a mixture of HSV oriS and EBV oriLyt DNAs is shown. Lane 1, Vero cells were cotransfected with expression plasmids for the HSV replication genes plus an HSV-1 oriS target plasmid. Lanes 2 to 7, cells were transfected with an EBV oriLyt target plus the following. Lane 2, expression plasmids for the EBV replication genes. Lane 3, expression plasmids for Zta, Rta, and Mta plus a subset of the EBV replication genes (BMRF1, the polymerase processivity factor, and BALF5, the DNA polymerase) were replaced by their HSV analogs, UL42 and UL30. Lane 4, as in lane 3 except that the EBV genes replaced were BBLF4, BBLF2/3, and BSLF1, which were replaced by their HSV analogs, UL5, UL8, and UL52. Lane 5, Vero cells were cotransfected with expression plasmids for the HSV replication genes (minus UL9). Lane 6, HSV replication genes (minus UL9) plus Rta. Lane 7, HSV replication genes (minus UL9) plus Zta and Rta.

cases cell type specificity (18, 35). In viral systems that have been studied to date, the core region is bound by a virally specified origin-binding protein. For example, this function is performed in SV40 by T antigen (8), in papillomavirus by the E1 protein (60), and in HSV by UL9 (22, 63). Binding of these proteins leads to distortion of the origin DNA and localized melting, and in addition T antigen, E1, and UL9 have intrinsic helicase activities that produce an initial unwinding of origin sequences prior to entrance of the remainder of the replication machinery (3, 17, 84, 89). EBV encodes a latency origin-binding protein, EBNA-1, that distorts origin DNA sequences and facilitates looping between the two regions of oriP (30, 42, 44, 78), but EBNA-1 does not have helicase activity. In a previous study to determine the virally encoded proteins required for EBV oriLyt replication, six genes were identified but the question of an oriLyt origin binding protein equivalent to HSV UL9 was left unresolved (25). The lytic cycle transactivator, Zta, binds to six sites in oriLyt. Mutation of these sites abolishes replication, providing evidence for the essential contribution of Zta to replication (72). A primary goal of the present study was to determine whether EBV encodes an origin-binding protein in addition to Zta or whether Zta alone serves this function. Functions that were required in the earlier cotransfection-replication assays using cosmid DNAs and which we have investigated further are an activity provided by *Sal*I-F, BBLF2/3, and the lytic cycle transactivators.

The requirement for *Sal*I-F was mapped to the BKRF3 ORF. The protein product of BKRF3 has a high degree of amino acid homology to (UDG) enzymes from *Escherichia coli* (81), *Saccharomyces cerevisiae* (66), humans (64), and HSV (86). UDG participates in the removal uracil from DNA and the repair of the resulting apyrimidinic site. Uracil can be incorporated into DNA as the result of misincorporation of dUMP directly during DNA synthesis or by the deamination of cytosine, which can result in GC-to-AT transition mutations. The UDG gene is dispensable for growth of HSV in tissue culture (61) and was not one of the essential replication genes identified by Wu et al. in their cotransfection-replication assays (87). However, the UDG of the poxvirus Shope fibroma virus is essential for virus viability (77), and a vaccinia virus temper-

ature-sensitive mutant in this gene is severely impaired in DNA replication (59). Synthesis of UDG activity is cell cycle regulated (62), and there is evidence for physical association of the enzyme with replicating DNA (46), suggesting that there may be some linkage between repair and replication. It has also been suggested that the presence of uracil in the target DNA sequences of origin-binding proteins may reduce binding affinity and hence replication efficiency (29).

Why would the EBV UDG be required when cosmid clones were used but have only a small impact on oriLyt replication when expression plasmids were providing the essential *trans*-acting replication proteins in the cotransfection assay? If one makes the assumption that the UDG was acting to remove misincorporated uracil from newly synthesized DNA, then it can be hypothesized that templates which had undergone one round of replication without repair might not be effective substrates for subsequent rounds of replication, whereas in the presence of UDG, multiple rounds of replication from these same templates could occur. As will be discussed, efficient oriLyt replication in the transient assay was dependent on the Zta and Rta lytic cycle transactivators and on Zta in particular. Both Zta and Rta bind sequences within oriLyt. If the transactivators were present in limiting quantities, then those templates already activated by Zta and Rta would be the favored replication templates. Under conditions in which the cosmids were used, the entire EBV genome was present to compete for Zta and Rta binding, potentially limiting the number of oriLyt templates which could then be bound to and activated by the transactivators. When the expression plasmids were used reinitiation of a small number of templates would no longer be essential because in the absence of competing binding sites, there would now be abundant Zta and Rta to activate templates for replication.

In general, genes involved in nucleotide metabolism may play an ancillary role in DNA replication. Herpesviruses encode a number of these enzymes, including one or both subunits of the ribonucleotide reductase, thymidine kinase, dUTPase, and possibly a dCMP deaminase. The requirement for the virally encoded enzymes can depend on the cell type and growth conditions (reviewed in reference 82).

HSV-1-infected cells contain novel helicase and primase activities that reside within a three-subunit complex (15). The three subunits were shown by immunoblot analysis to be encoded by the HSV genes UL5, UL8, and UL52, which are three of the seven essential genes identified in the transient replication assay (87). Subsequently, the helicase, DNA-dependent ATPase, and primase activities were purified from triply infected Sf9 insect cells and from cells doubly infected with UL5 and UL52 (6, 20). Although the UL8 protein is associated with UL5 and UL52 in HSV-infected cells, the exact function of the UL8 protein in the complex has been elusive. In cells expressing only UL5, UL8, or UL52 (or UL5 plus UL52), the location of each protein was primarily cytoplasmic. However, when all three proteins were coexpressed, they were localized in the nucleus to structures resembling the prereplicative sites associated with the UL29 single-stranded DNA-binding protein of HSV (5). This may indicate that UL8 is required for formation of a UL5-UL8-UL52 complex which is competent to enter the nucleus. Using an in vitro system which measures DNA synthesis of M13 closed circles, Sherman et al. (75) proposed a role for UL8 in primer elongation. In essence, in the absence of the UL8 protein, primers synthesized by the helicase-primase complex dissociated from the DNA template prior to elongation by the DNA polymerase because of their short length (20 bases).

BBLF2/3 was believed to encode the functional equivalent of the HSV UL8 protein on the basis of positional similarity of the two genes in their respective genomes, a small stretch of amino acid similarity with UL8 of HSV and gene 51 of varicella-zoster virus within a 55-amino-acid domain in BBLF2 (25), and the similarity in size of the predicted BBLF2/3 product (709 amino acids) and the UL8 protein (750 amino acids). However, HSV UL8, which lies adjacent to UL9, is encoded by a single ORF, not two, and it was theoretically possible that BBLF2 and BBLF3 were independently expressed ORFs encoding UL9 and UL8 homologs that were much smaller in size than their HSV counterparts. Additional evidence is now available to eliminate this latter possibility. First, BBLF2/3 was shown to be expressed as a spliced RNA; second, the cDNA version of BBLF2/3 was shown to support oriLyt replication; and third, oriLyt replication was observed in a complementation experiment in which the HSV replication proteins (minus UL9) substituted for the EBV proteins. These results are consistent with BBLF2/3 encoding a single protein and with that protein being a UL8 equivalent rather than a UL9 equivalent.

The six EBV replication genes, BALF5 (DNA polymerase), BMRF1 (polymerase processivity factor), BALF2 (single-stranded DNA-binding protein), BSLF1 (primase), BBLF4 (helicase), and BBLF2/3 (helicase-primase associated protein), were each essential for oriLyt replication: removal of any one of them from the transfection mixture abolished replication ability. Further, the functional homology between these genes and their HSV counterparts was demonstrated by the ability to substitute UL30, UL42, UL29, UL5, UL8, and UL52 and obtain replication of EBV oriLyt.

Efficient oriLyt replication continued to be significantly dependent on the presence of the lytic transactivators Zta, Rta, and Mta. The role of Mta is likely to be predominantly indirect. Little is known about the mechanism of action of Mta aside from the fact that it acts posttranscriptionally. However, its HSV homolog IE63, which can partially substitute for Mta in the replication assay, has a positive regulatory effect mediated at the level of poly(A) selection (56, 71). Although the EBV replication genes were cloned into expression vectors, all except the BALF5 polymerase retained their natural 3′ processing signals, including the poly(A) sites. It seems likely that a major contribution of Mta to the replication assay lay in efficient expression of the EBV replication proteins. Reinforcing this conclusion is the observation that replication of oriLyt by the HSV replication proteins was not dependent on the presence of Mta. It is clear, therefore, that Mta is not absolutely essential for oriLyt replication in a transient assay.

Both Zta and Rta bind to sequences within oriLyt. Rta augmented oriLyt replication efficiency but was not essential. Zta continued to be required for efficient oriLyt replication in the presence of both the EBV replication proteins and the HSV replication proteins. This result complements the observation by Schepers et al. (72) that mutation of all the Zta binding sites in oriLyt destroys replication ability. That the contribution of transcription factors to replication can be independent of their transcriptional activity was initially demonstrated directly in the SV40 in vitro system, in which replication was shown to be insensitive to α-amanitin (47). Postulated roles for transcription factors include facilitation of the assembly of the replication complex and removal of chromatin structure which would interfere with binding of the replication proteins (20, 35). NF1 (CTF) interacts with adenovirus DNA polymerase to stabilize the DNA-bound polymerase-preterminal protein complex, and this function does not require the NF1 activation domain (8, 11, 58). Similarly, the bovine papillomavirus transactivator E2 stabilizes binding of the E1 origin-binding protein (88). Stimulation of the assembly of the

initial replication complex through interaction with the cellular replication protein A is also mediated by certain transcription factors. SV40 T antigen interacts directly with replication protein A (21), as do the activation domains of the papillomavirus E2, HSV VP16, cellular p53, and yeast Gal4 proteins (41, 48). In vitro repression of SV40 DNA replication by nucleosomes is alleviated by prebinding of Gal4-VP16 to DNA (12). E1-mediated replication of bovine papillomavirus in vitro is also repressed by nucleosomal assembly. E2 counteracts this repression, and other transcription factors with acidic activation domains, namely, Gal4-VP16 and p53(1-73), can substitute for E2 (49).

Zta, when bound to the BHLF1 promoter and the oriLyt enhancer, may activate replication by displacing histones present on the oriLyt sequences (74). During EBV latency in B cells, the DNA is assembled as chromatin, and Zta is able to activate early EBV promoters that are quiescent during latency and certainly nucleosome associated prior to Zta expression. The activation domains of both Zta and Rta are required for their replication function (24a, 72), and Zta in particular, but also Rta, may additionally contribute to oriLyt replication by a mechanism involving protein-protein contacts and stabilization of an oriLyt-replication complex.

The lack of a dedicated virally encoded origin-binding protein and the dependence of oriLyt replication on transcription factors, primarily Zta, suggest that oriLyt may more closely resemble a cellular replication origin in its organization than is the case for the alpha herpesvirus origins. Interestingly, the beta herpesvirus CMV oriLyt also consists of multiple and repeated transcription factor binding sites, and as yet no CMV-encoded origin-binding protein has been identified (1, 2, 37, 65).

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