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trans-Acting Requirements for Replication of Epstein-Barr Virus ori-Lyt

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Epstein-Barr virus (EBV) utilizes a completely different mode of DNA replication during the lytic cycle than that employed during latency. The latency origin of replication, ori-P, which functions in the replication of the latent episomal form of the EBV genome, requires only a single virally encoded protein, EBNA-1, for its activity. During the lytic cycle, a separate origin, ori-Lyt, is utilized. Relatively little is known about the *trans*-acting proteins involved in ori-Lyt replication. We established a cotransfection-replication assay to identify EBV genes whose products are required for replication of ori-Lyt. In this assay, a *Bam*HI-H plasmid containing ori-Lyt was replicated in Vero cells cotransfected with the *Bam*HI-H target, the three EBV lytic-cycle transactivators Zta, Rta, and Mta, and the EBV genome provided in the form of a set of six overlapping cosmid clones. By removing individual cosmids from the cotransfection mixture, we found that only three of the six cosmids were necessary for ori-Lyt replication. Subcloning of the essential cosmids led to the identification of six EBV genes that encode replication proteins. These genes and their functions (either known or predicted on the basis of sequence comparison with herpes simplex virus) are BALF5, the DNA polymerase; BALF2, the single-stranded DNA-binding protein homolog; BMRF1, the DNA polymerase processivity factor; BSLF1 and BBLF4, the primase and helicase homologs; and BBLF2/3, a potential homolog of the third component of the helicase-primase complex. In addition, ori-Lyt replication in this cotransfection assay was also dependent on one or more genes provided by the EBV *Sal*I-F fragment and on the three lytic-cycle transactivators Zta, Rta, and Mta.

Epstein-Barr virus (EBV), like all herpesviruses, has both a latent state and a lytic replicative cycle. In latently infected B cells, multiple copies of the viral genome are maintained predominantly as nucleosome-covered episomes that are replicated in synchrony with cell division (73). Latency replication proceeds from ori-P, which is composed of two domains (72). Region I, the family of repeats, contains 20 tandemly repeated binding sites for EBNA-1 (57) and functions as an EBNA-1-dependent enhancer whose activity is important for both ori-P replication and transcriptional activation of the *Bam*HI-C latency promoter (58, 63, 71). Region II, the dyad symmetry, contains two pairs of overlapping EBNA-1-binding sites (57) and is the site of initiation of latency replication (26). EBNA-1 is the only virally encoded protein required for replication of the episomal EBV genome, all other proteins, including the DNA polymerase, being provided by the host cell (74).

Lytic EBV replication occurs in mucosal epithelial cells of the oropharynx and genital tract (60) and can be activated in latently infected B cells in culture by treatment with phorbol esters (77), by superinfection with the P3HR-1 strain which carries defective rearranged viral genomes (53), or by introduction of the EBV Zta transactivator (13). Because of the limitations imposed by the lack of an EBV-infectable epithelial culture system, information on EBV lytic viral replication has been obtained predominantly in B-cell cultures undergoing reactivation. In this system, the transition from latency to a lytic replicative cycle is mediated by three viral regulatory proteins, the Zta (BZLF1, EB1, or ZEBRA) and Rta (BRLF1) transcriptional transactivators (9, 12, 13, 31, 34, 48) and Mta (BMLF1), which has a posttranscriptional mechanism of action (5, 39, 49). The concerted action of these three proteins results in activation of the complete cascade of early and late EBV gene expression.

Lytic DNA replication proceeds from a separate origin, ori-Lyt, and results in 100- to 1,000-fold amplification of the genome via concatemeric intermediates (18, 32, 59). In the prototype EBV genome, there are two copies of ori-Lyt, one in DS-L and one in DS-R. However, one copy is sufficient for lytic-cycle replication as exemplified by the EBV strain B95-8, which contains only DS-L. ori-Lyt covers 690 bp and can be divided into three essential domains. (i) The first domain is the promoter and leader of the BHLF1 gene whose transcript is the most abundantly synthesized of the lytic-cycle mRNAs (38). (In the DS-R origin, this promoter controls the related *Pst*I repeat gene.) The BHLF1 promoter contains four binding sites for the Zta transactivator and is strongly Zta responsive in transient expression assays (47, 48). (ii) The second domain is a central 225-bp region whose prominent features include two related AT-rich palindromes of 18 and 20 bp and an adjacent polypurine-polypyrimidine tract. Elements of this type destabilize helical structure and may serve as sites for the initiation or transmission of localized unwinding in origins of replication (42, 68). (iii) The third domain is a powerful enhancer element that responds to the Rta transactivator and contains two binding sites for Rta and one for Zta (14, 31, 48). Hammerschmidt and Sugden (32) found that origin function was retained when this enhancer was replaced with the enhancer from the human cytomegalovirus (HCMV) major immediate-early gene, suggesting that it was enhancer function per se that was provided by this region rather than a contribution involving specific protein interactions.

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The lytic origins of replication have been identified for a number of herpesviruses, including HCMV and simian cytomegalovirus (SCMV) (2, 33), varicella-zoster virus (VZV) (17), pseudorabies virus (43), equine herpesvirus 1 (4), Marek's disease virus (7), and herpes simplex virus (HSV) (51, 62, 67). Of these herpesviruses, replication of HSV has been the most extensively characterized. The HSV viral proteins involved in DNA replication were originally recognized through genetic studies (reviewed in reference 66). Subsequently, a complete set of seven essential genes was identified by Challberg et al. (8, 52, 70) using transient cotransfection replication assays.

We established a transient replication assay in Vero cells to determine exactly which EBV proteins are required to replicate an ori-Lyt-containing target. Utilizing this system, we identified six essential EBV replication genes. In addition to these genes, ori-Lyt replication in the transient cotransfection assay was also dependent on one or more genes provided by EBV *Sal*I-F and on the Zta, Rta, and Mta lytic-cycle transactivators.

MATERIALS AND METHODS

Cells and DNA transfections. Vero cells were maintained in Dulbecco modified Eagle medium plus 10% fetal calf serum. One day before transfection, 10^6 cells were plated in 100-mm dishes. Four hours before transfection, the medium was replaced with 10 ml of Dulbecco modified Eagle medium containing 10% fetal calf serum and antibiotics. DNA was transfected by using the calcium phosphate procedure originally described by Graham and van der Eb (30) as modified by Chen and Okayama (11). DNA (12.5 to 14.5 μg) was diluted with water to a total volume of 450 μl. To this was added 50 μl of 2.5 M $CaCl_2$ and 500 μl of 2× BES [*N*,*N*'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]-buffered saline (50 mM BES [pH 6.95], 280 mM NaCl, 1.5 mM Na_2HPO_4). This cocktail was incubated at room temperature for 20 min and then added dropwise to the cells. After incubation for 20 h at 35°C in 3.5% CO_2, the medium was removed, the cells were washed once with phosphate-buffered saline (PBS), and fresh medium containing antibiotics was added. The cells were harvested after a further 72-h incubation.

DNA replication assay. Cell pellets were resuspended in 100 μl of PBS and then lysed in 2 ml of buffer containing 10 mM Tris-Cl (pH 8.0), 10 mM EDTA, 2% sodium dodecyl sulfate (SDS), and 100 μg of proteinase K per ml (8). After overnight incubation at 37°C, the samples were diluted to 4 ml with water. Sodium acetate (pH 5.2) was added to a final concentration of 0.3 M, and the samples were extracted with phenol-chloroform and chloroform and then subjected to ethanol precipitation. The DNA pellets were resuspended in 450 μl of water, treated with RNase, ethanol precipitated a second time, and finally resuspended in 200 μl of water. DNA (5.0 μg) was cut with 10 U of *Bam*HI or 10 U of *Bam*HI and 12 U of *Dpn*I (Boehringer Mannheim) in a reaction volume of 100 μl overnight at 37°C. To check the *Dpn*I activity, we incubated 4 μl of the *Dpn*I reaction digest with 500 ng of pUC19 DNA overnight at 37°C. Complete cutting of the pUC19 DNA was taken to indicate that the experimental DNA was also completely digested. The cellular DNA was then separated by electrophoresis on a 1% gel and transferred to a NYTRAN membrane following the method of Southern (61) as modified by the manufacturer (Schleicher & Schuell, Inc., Keene, N.H.). After the transfer, the NYTRAN membrane was neutralized in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) and baked under vacuum at 80°C for 1 h. The NYTRAN membrane was prehybridized for 2 h in buffer containing 1% SDS, 5 mg of nonfat dried milk per ml, 0.5 mg of heparin per ml, 0.2 mg of sonicated, denatured salmon sperm DNA per ml, 60 mg of polyethylene glycol 8000, 5× SSPE (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM Na_2EDTA), and 10% formamide (75). The membrane was then incubated overnight at 60°C with 2×10^6 to 5×10^6 cpm of EBV B95-8 *Bam*HI-H, M-ABA *Bam*HI-H (pPDL7), or pBR322 DNA probe per ml and radiolabeled by random priming (25) to a specific activity of 5×10^8 cpm/μg, after which the filters were washed twice in 0.1× SSC–0.1% SDS at 65°C for 45 min and exposed to X-ray film for 24 h at −80°C with an intensifying screen.

Plasmid constructions. The target *Bam*HI-H plasmid (pSL77) used in the transient replication assays has been described previously (49), as have the effector DNA plasmids, pPL17 (Zta), pMH48 (Rta), and pTS6 (Mta) (12, 34, 47). The variant ori-Lyt target, pPL2A, contains the *Bgl*II-C fragment from M-ABA (pM-B2-C [56]) in which the BHLF1 open reading frame has been disrupted by deletion of the *Not*I repeats. To express the B95-8 BMRF1 gene, a 3,026-bp *Bcl*I-*Eco*RI fragment from *Bam*HI-M was ligated into a *Bgl*II-*Eco*RI-cleaved SV2neo derivative, pGH52, which contains a *Hin*dIII-*Bgl*II-*Hin*dIII linker inserted at the *Hin*dIII site of SV2neo. A BSLF1 expression vector, pDH131, was generated by first ligating a 2,819-bp *Bgl*II-*Bam*HI fragment containing BSLF1 into the *Bam*HI site of pUC18 and then transferring the open reading frame as a *Hin*dIII-*Bam*HI fragment into *Hin*dIII-*Bam*HI-cleaved pSV2neo. To express the EBV M-ABA strain BBLF4 gene, 10-mer *Hin*dIII linkers were ligated onto a 3,003-bp *Stu*I fragment containing the entire BBLF4 open reading frame from the cosmid cM301-99 (3, 56), and this fragment was then cloned into the *Hin*dIII site of the pBR322 derivative, pGH59. This plasmid was cut with *Rsr*II, the overhang was filled in with the Klenow DNA polymerase, and the DNA was recut with *Eco*RI. The resulting fragment containing BBLF4 was then ligated into the *Sma*I-*Eco*RI-cleaved SCMV Colburn IE94 expression plasmid, pGH70, to generate pEF54A. To express the M-ABA strain BBLF2 and BBLF3 open reading frames, we inserted the 3,052-bp *Asp*718-*Sal*I fragment from the cosmid cM301-99 into *Asp*718-*Sal*I-cleaved pUC19, generating pEF58. This plasmid was cut to completion with *Sal*I and partially digested with *Bgl*II, and the resulting fragment was cloned into the *Bam*HI-*Sal*I-cleaved SCMV Colburn IE94 expression plasmid pGH177, generating EF59A.

To express the M-ABA strain BALF2 open reading frame, we moved a 3,912-bp *Bgl*II-*Eco*RI fragment that lacked the 5′ 1,005 bp of the BALF2 open reading frame from the cosmid cM966-20 (56) into the *Bgl*II-*Eco*RI-cleaved SCMV Colburn IE94 expression vector pGH179 to generate pEF55. The 5′ end of BALF2 was amplified by a polymerase chain reaction utilizing the following primers: at the 5′ end, 5′-CTAGGGATCCATGCAGGGTGCACAGACT-3′, and at the internal *Bgl*II site, 5′-GCAAAGATCTGCGTGGACAC-3′. The polymerase chain reaction mixtures contained 10 μl of 10× reaction buffer (Cetus), 10.0 μl of deoxynucleoside triphosphates (1.25 μM each dATP, dCTP, dGTP, and TTP), 5 μl of each primer at 20 μM, 10 ng of the appropriate plasmid DNA, and 0.5 μl of *Taq* polymerase (Cetus). The reaction mixtures were incubated in a thermocycler for 2 min at 94°C, 2 min at 52°C, and 3 min at 72°C for 40 cycles. Aliquots of the reaction mixtures were analyzed on an agarose gel for the presence of the desired 1,005-bp fragment, after which the DNA was digested with *Bam*HI and

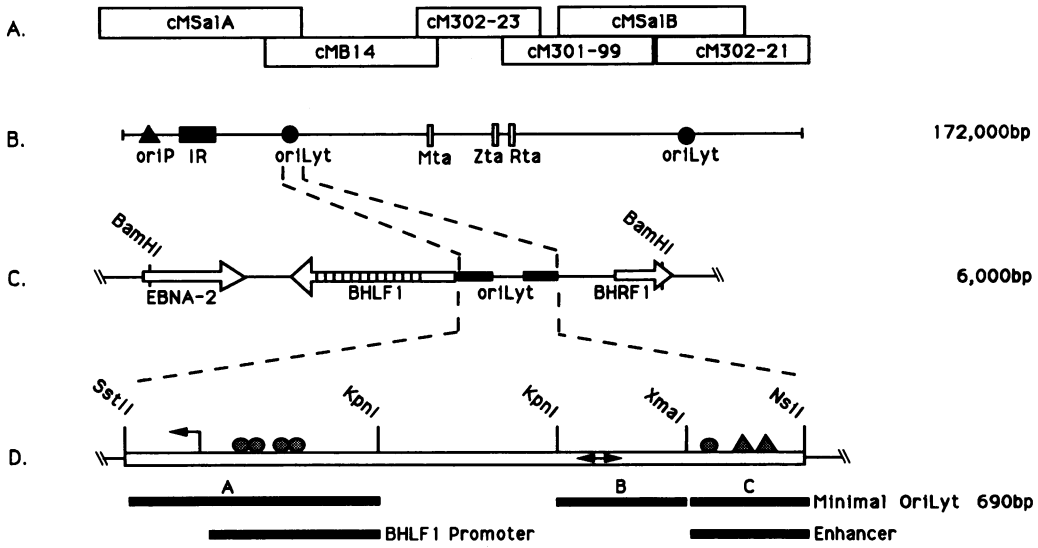


FIG. 1. Diagrammatic representation of the EBV genome showing the map locations of the genes of interest and the structure of ori-Lyt. (A) Six overlapping cosmids from the EBV strain M-ABA that were utilized to provide the entire EBV genome. (B) EBV genome. The locations of the *Bam*HI-W internal repeat (striped rectangle) and the origins of replication, ori-P (triangle) and ori-Lyt (circles), as well as the locations of the three lytic-cycle transactivators, Zta, Rta, and Mta (open rectangles), are shown. (C) 6-kb *Bam*HI-H fragment from EBV that served as the target in the replication assays. *Bam*HI-H contains 3′ sequences of the EBNA-2 gene, the entire BHLF1 open reading frame, ori-Lyt, and 5′ sequences of the BHRF1 gene. (D) Minimal ori-Lyt, which is contained within a 690-bp *Sst*II-*Nsi*I fragment, is composed of three domains. Domain A contains the promoter and leader region of the BHLF1 gene and four Zta-binding sites (ZREs, shaded circles). Domain B contains two AT-rich palindromes, one 18 bp and the other 20 bp, designated by the double-headed arrow. Domain C is composed of an enhancer element, which contains one ZRE (shaded circle) and two binding sites for the Rta transactivator (shaded triangles). The central *Kpn*I fragment is not required for ori-Lyt function (32).

*Bgl*II and cloned into pEF55 at the *Bgl*II site. The correct orientation regenerated the intact BALF2 open reading frame (pEF56A). The B95-8 DNA polymerase expression plasmid was a gift from Don Coen (Harvard University) and contains the DNA polymerase open reading frame BALF5 under the control of the simian virus 40 early promoter. The *Sal*I-F subclone (pGD4) contains the *Sal*I-F fragment from B95-8 cloned into pBR322 at the *Sal*I site. The *Bam*HI-BG clone (pDH33) contains the *Bam*HI-B and *Bam*HI-G fragments from P3HR-1 cloned into pBR322 at the *Bam*HI site. The plasmid pJMH4 (*Bam*HIΔBΔG) is an M-ABA subclone containing the 3′ 6,107 bp of *Bam*HI-B and the 5′ 3,003 bp of *Bam*HI-G cloned into the pHC79 vector.

RESULTS

Establishment of an ori-Lyt replication assay. To identify viral proteins required to replicate ori-Lyt, we established a transient cotransfection assay similar to that described by Challberg (8) for HSV type 1 (HSV-1). In that assay, large restriction fragments of the HSV-1 genome were cotransfected into Vero cells with a plasmid containing the HSV-1 origin of replication, ori-S, and *Dpn*I sensitivity was used to discriminate between input and replicated DNA (55). The *Dpn*I assay is based on the differential ability of *Dpn*I to cleave the input bacterially synthesized DNA which is methylated on the A residue of the *Dpn*I cleavage site GATC and the lack of cleavage when this methylation is lost after replication in eukaryotic cells. In the experiments described here, Vero cells (an African green monkey kidney cell line) were also used, and the exogenous EBV genome was provided by a set of six overlapping cosmids from the EBV strain M-ABA (56) (Fig. 1). The replication origin was provided by a plasmid carrying the EBV *Bam*HI-H fragment which encompasses the DS-L ori-Lyt. The transfection mixture also contained expression plasmids for the lytic-cycle transactivators, Zta, Rta, and Mta, to ensure adequate expression of the EBV early genes from the transfected cosmid clones. To determine the optimal assay conditions, we transfected Vero cells with the complete set of cosmids, the *Bam*HI-H target, and increasing amounts (0 to 2.5 μg) of the expression plasmids encoding the transactivators. The isolated cellular DNA was cleaved with restriction enzymes, and the DNA fragments were separated by gel electrophoresis and transferred onto a nylon membrane. The input, transfected DNA was visualized by hybridization with a radiolabeled pBR322 probe. The *Bam*HI-cleaved DNA is shown in Fig. 2A, and *Bam*HI-plus-*Dpn*I-cleaved DNA is shown in Fig. 2B. At a concentration of 2.5 μg of each expression plasmid, maximal replication of the target occurred (lane 4), as indicated by the presence of the *Dpn*I-resistant ori-Lyt band (arrowed). If no transactivators were included, no detectable replication occurred (lane 1). Below 2.5 μg of the transactivators, either no detectable replication occurred (lane 2) or minimal replication of the target occurred (lane 3). Replication was origin specific. Of all the input DNAs, only the *Bam*HI-H target (and to a lesser extent the cosmids that contain DS-R and DS-L) showed *Dpn*I resistance.

Replication genes are located on three separate cosmids. Having established the assay conditions, we next determined whether any of the cosmids were dispensable. The assay was repeated with one of the cosmids being omitted from each transfection mix (Fig. 3B). A *Dpn*I-resistant band was observed in the absence of cMSalB, cM302-23, or cMSalA, indicating that these three cosmids were not re-

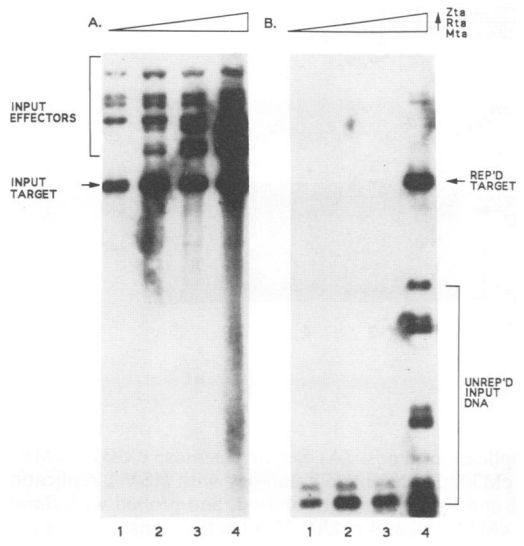


FIG. 2. Six overlapping cosmids support replication of the *Bam*HI-H target in the presence of the lytic-cycle transactivators. (A) Southern blot of transfected cell DNA cut with *Bam*HI and probed with radiolabeled pBR322 DNA. The top four bands of input DNA represent cosmids, and the central three bands represent the Zta, Rta, and Mta transactivators. Lane 1, no transactivators added; lanes 2 to 4, increasing amounts (0.1, 0.5, and 2.5 μg, respectively) of the transactivator expression plasmids. (B) As in panel A, except that the DNA was digested with *Bam*HI plus *Dpn*I. Replicated *Dpn*I-resistant target is readily detected with 2.5 μg of the Zta, Rta, and Mta expression constructs (lane 4).

quired for replication. On the other hand, if cM302-21, cM301-99, or cMB14 was removed, replication was not detected. Thus, in the presence of the lytic-cycle transactivators, only three of the cosmids, cMB14, cM301-99, and cM302-21, were required for replication of ori-Lyt.

To establish that the three essential cosmids, cMB14, cM301-99, and cM302-21, were sufficient for replication of

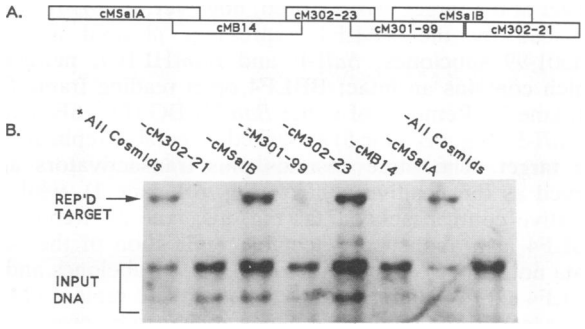


FIG. 3. Three cosmids, cMB14, cM301-99, and cM302-21, are each required for replication of the target. (A) Set of overlapping cosmids. (B) Southern blot of transfected cell DNA digested with *Bam*HI plus *Dpn*I and probed with pBR322 to detect *Dpn*I-resistant, replicated DNA. The positive control contained all six cosmids transfected with 2.5 μg of the expression constructs for Zta, Rta, and Mta. The target was resistant to cleavage with *Dpn*I. Replication of the target was negative when the cosmid cM302-21, cM301-99, or cMB14 was omitted. Replication of the target was positive in the absence of the cosmid cMSalB, cM302-23, or cMSalA. In the negative control, all cosmids were omitted.

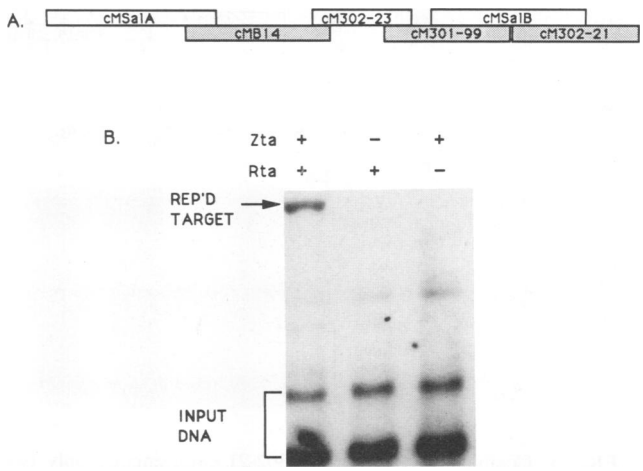


FIG. 4. Three essential cosmids, cMB14, cM301-99, and cM302-21, are sufficient for replication of the target, but only in the presence of the Zta and Rta expression plasmids. (A) Set of overlapping cosmids with the three essential cosmids highlighted. (B) Transfected cell DNA cut with *Bam*HI and *Dpn*I, Southern blotted, and probed with *Bam*HI-H to detect *Dpn*I-resistant, replicated DNA. The requirement for Zta and Rta was examined in cells cotransfected with cMB14, cM301-99, cM302-21, and Mta.

ori-Lyt, we cotransfected them with expression plasmids for Zta, Rta, and Mta. Efficient replication of the target occurred in the presence of these three cosmids (Fig. 4), demonstrating that cMB14, cM301-99, and cM302-21 not only were required for replication, but were sufficient. Addition of both the Zta and Rta transactivators remained a requirement for replication. If either was left out of the transfection mix, detectable replication of *Bam*HI-H did not occur. Thus, to obtain replication of ori-Lyt, three of the cosmids were required as well as two transcriptional activators, Zta and Rta. Zta and Rta could be functioning directly to transactivate the ori-Lyt promoter and enhancer or indirectly to increase expression from the cosmid-encoded genes needed for replication. The requirement for Mta could not be assessed in these experiments because Mta is encoded within one of the essential cosmids, cMB14.

ori-Lyt replication requires the EBV genes BALF5, BALF2, BMRF1, and BSLF1. The seven genes of HSV-1 whose products are essential and sufficient to replicate an HSV-1 origin-containing target plasmid are the DNA polymerase, UL30; the single-stranded DNA-binding protein, UL29; the tripartite helicase-primase complex containing UL5, UL8,

TABLE 1. Putative EBV homologs of the essential HSV replication proteins

HSV gene	EBV ORF[a]	Identity (%)	Function
UL30	BALF5	33	DNA polymerase (POL)
UL42	BMRF1	—[b]	Polymerase processivity factor (PPF)
UL29	BALF2	25	Single-stranded DNA-binding protein (SSB)
UL5	BBLF4	34	Helicase (HEL) } Complex
UL52	BSLF1	23	Primase (PRI) } Complex
UL8	BBLF2/3	—	Primase-associated factor (PAF)
UL9	?		Origin-binding protein (OBP)

[a] ORF, open reading frame.
[b] —, equivalent genomic location only.

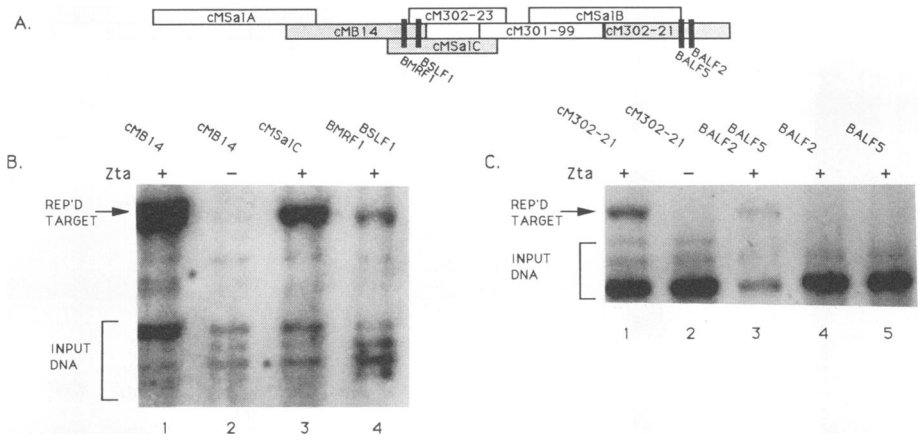


FIG. 5. Cosmids cMB14 and cM302-21 each encode only two essential replication genes. (A) Set of cosmids; cMB14, cMSalC, and cM302-21 are highlighted. The locations of the genes on cMB14, cMSalC, and cM302-21 that have homology with HSV-1 replication genes are also shown. In panels B and C, transfected cell DNA was cut with *Bam*HI and *Dpn*I, Southern blotted, and probed with *Bam*HI-H to detect *Dpn*I-resistant, replicated DNA. (B) Lane 1, the three cosmids, cMB14, cM301-99, and cM302-21, plus the transactivator expression plasmids. Lane 2, no Zta added. Lane 3, the cosmid cMB14 was replaced by the cosmid cMSalC. Lane 4, the cosmid cMB14 was replaced by expression plasmids encoding BMRF1, the polymerase processivity factor, and BSLF1, the primase homolog. (C) Lane 1, the three cosmids, cMB14, cM301-99, and cM302-21, plus the transactivator expression plasmids. Lane 2, no Zta added. Lane 3, the cosmid cM302-21 was replaced by expression plasmids encoding BALF5, the DNA polymerase, and BALF2, the single-stranded DNA-binding protein homolog. Lane 4, minus BALF5. Lane 5, minus BALF2.

and UL52; the DNA polymerase processivity factor, UL42; and the origin-binding protein, UL9. Table 1 shows the seven HSV-1 replication genes, their potential EBV homologs, and the percent homology which they share (52).

Upon inspection of the map location of those EBV genes that had potential homology with the HSV-1 replication genes, it became apparent that they were all located within the three cosmids that were essential for replication in our assay. To determine whether these genes indeed encoded functional replication proteins, the EBV DNA polymerase, BALF5, and its processivity factor, BMRF1, as well as BSLF1, BBLF4, and BALF2 were placed under the control of the strong simian virus 40 early or CMV major immediate-early promoters in eukaryotic expression vectors and tested in substitution experiments. First, the cosmid cMB14 was replaced with another cosmid, cMSalC, which has five open reading frames in common with cMB14. This cosmid was able to substitute functionally for cMB14 (Fig. 5B, lane 3), indicating that the replication genes provided by cMB14 were located within the region that overlaps with cMSalC. The common sequences contain BMRF1 and BSLF1 plus BMLF1 (Mta), BMRF2 (a late gene), and BORF2 and BaRF1, which encode the large and small subunits of ribonucleotide reductase (3, 24). The cosmid cMB14 was next replaced by expression plasmids for the two genes, BSLF1 and BMRF1, that were potential homologs for HSV replication genes. Replication was positive in the presence of BSLF1 and BMRF1, although the replication signal was reduced compared with that obtained with cMB14 or cMSalC (Fig. 5B, lane 4). Transfection of the three essential cosmids in the presence of the lytic-cycle transactivators served as the positive control (Fig. 5B, lane 1). Removal of Zta from the transfection mix abolished detectable replication of the target and served as the negative control (Fig. 5B, lane 2). In a parallel experiment, the cosmid cM302-21 was successfully replaced by expression plasmids for the two predicted replication genes that mapped within cM302-21, the DNA polymerase, BALF5, and BALF2, the putative single-stranded DNA-binding protein (Fig. 5C, lane 3). If either BALF5 (Fig. 5C, lane 4) or BALF2 (Fig. 5C, lane 5) was omitted, replication of the target did not occur. Therefore, the cosmid cMB14 encodes only two essential replication proteins, BSLF1, the primase homolog, and BMRF1, the processivity factor. Similarly, the cosmid cM302-21 also encodes only two replication proteins, the DNA polymerase, BALF5, and BALF2, the single-stranded DNA-binding protein homolog.

ori-Lyt replication also requires BBLF4, BBLF2/3, and *Sal*I-F. A diagram of the remaining essential cosmid, cM301-99, showing the map locations of relevant open reading frames is presented in Fig. 6A. The only gene encoded by cM301-99 which has recognized sequence homology to a replication protein of HSV-1 is BBLF4. However, when this cosmid was replaced by an expression plasmid for BBLF4, no detectable replication occurred (data not shown). Replication of the target was restored, however, if cM301-99 was replaced by the BBLF4 expression plasmid and two cM301-99 subclones, *Sal*I-F and *Bam*HI-BG, neither of which contains an intact BBLF4 open reading frame (Fig. 6B, lane 3). Removal of either *Bam*HI-BG (Fig. 6B, lane 4) or *Sal*I-F (Fig. 6B, lane 5) abolished detectable replication of the target. The three cosmids plus transactivators again served as the positive control (Fig. 6B, lane 1), while the negative control lacked Zta (Fig. 6B, lane 2). Removal of BBLF4 also abolished detectable replication of the target (data not shown). Because two cM301-99 subclones and the BBLF4 expression plasmid were required to replace cM301-99, at least three replication genes appear to be provided by this cosmid.

To determine the gene or genes provided by *Bam*HI-BG, we used subclones of this DNA fragment. The clone *Bam*HIΔBΔG(pJMH4) was able to substitute for *Bam*HI-BG in the replication assay (Fig. 6C, lane 4). Both *Bam*HI-BG and *Bam*HIΔBΔG contain the two open reading frames BBLF2 and BBLF3 (Fig. 6A), which are potential UL9 and UL8 homologs based on their genome locations (52). The linkage with UL8 was further strengthened by the recognition that BBLF2 has a stretch of 55 amino acids which are

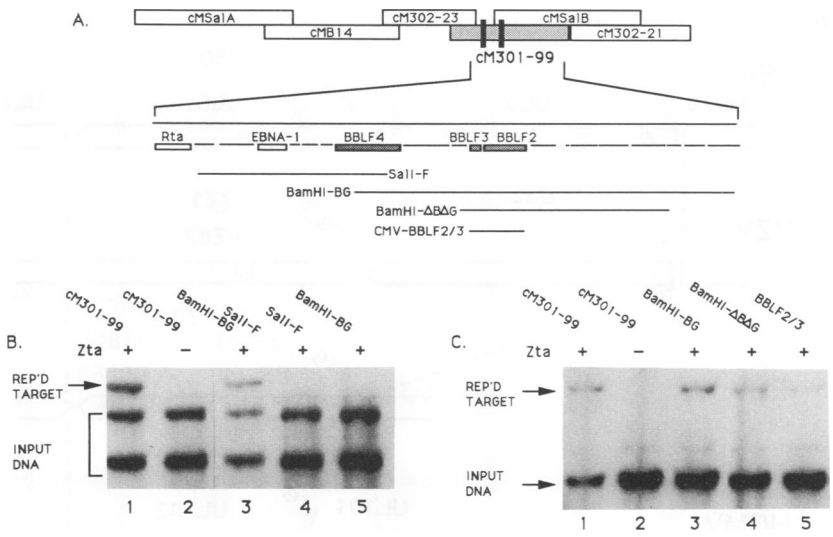


FIG. 6. Cosmid cM301-99 encodes at least three replication genes. (A) Set of overlapping cosmids; cM301-99 is highlighted, and the locations of the genes on cM301-99 known to be required for replication are designated. In the expanded segment of cM301-99, the structure of subclones of this region is shown, along with the location of relevant open reading frames. In panels B and C, transfected cell DNA was cut with *Bam*HI and *Dpn*I, Southern blotted, and probed with *Bam*HI-H (B) or with a subfragment of *Bam*HI-H (C) to detect *Dpn*I-resistant, replicated DNA. (B) Lane 1, the three essential cosmids plus the transactivator expression plasmids. Lane 2, no Zta added. Lane 3, the cosmid cM301-99 was replaced with the BBLF4 expression plasmid and the two subclones, *Sal*I-F and *Bam*HI-BG plus the transactivator expression plasmids. Lane 4, no *Bam*HI-BG added. Lane 5, no *Sal*I-F added. (C) Lane 1, the three essential cosmids transfected with the transactivator expression plasmids. Lane 2, no Zta added. Lane 3, cells were transfected with cMB14, cM302-21, the BBLF4 expression plasmid, the two subclones, *Sal*I-F and *Bam*HI-BG, as well as the transactivator expression plasmids. Lane 4, *Bam*HI-BG was replaced by *Bam*HIΔBΔG. Lane 5, *Bam*HI-BG was replaced by a CMV-driven expression plasmid encoding BBLF2 and BBLF3.

similar to UL8 of HSV-1. This region of similarity is also found in the VZV gene 52 and the HCMV gene UL102 (Fig. 7). Because of this sequence and positional homology, we cloned these two open reading frames into an expression vector. This BBLF2/3 expression construct, when cotransfected with *Sal*I-F and the BBLF4 expression plasmid, successfully substituted for *Bam*HIΔBΔG (Fig. 6C, lane 5). We conclude that the cosmid cM301-99 encodes at least three replication proteins. One of these is the helicase homolog encoded by the open reading frame BBLF4. The open reading frames BBLF2 and BBLF3 are thought to be spliced into one message in EBV (24) and hence would encode only one protein, potentially functioning as the UL8 homolog. The other gene (or genes) required for replication of ori-Lyt is located on the subclone *Sal*I-F.

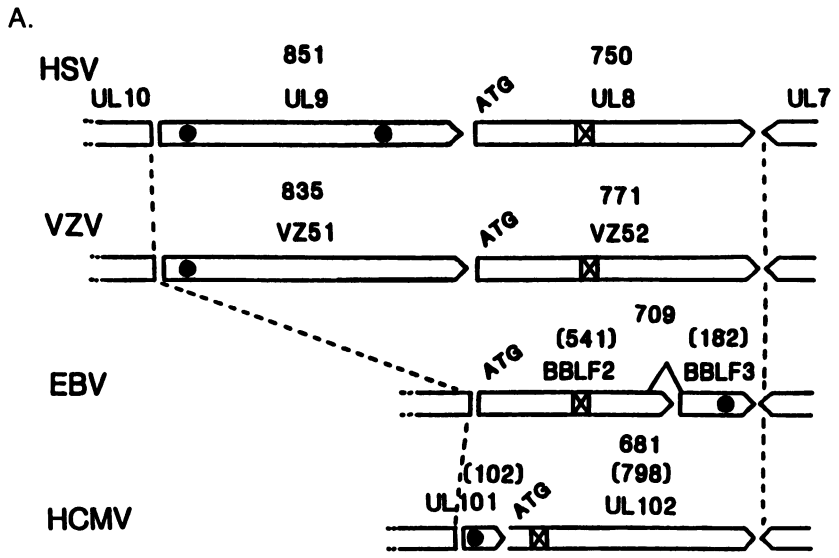
The cotransfection assays identified six genes encoded by EBV whose products were individually essential for transient replication of an ori-Lyt-containing target in Vero cells. We also demonstrated that the combination of these six genes plus *Sal*I-F and the Zta, Rta, and Mta transcriptional transactivators were sufficient to support replication of ori-Lyt (Fig. 8, lane 4). In an earlier experiment (Fig. 4), we demonstrated that Rta and Zta were both required for replication. To address the question of the requirement for the Mta transactivator, we used the set of six cloned genes plus *Sal*I-F. As shown in Fig. 8 (lane 5), replication of ori-Lyt was significantly reduced but not eliminated in the absence of added Mta, even though both Zta and Rta were present. Therefore, all three transactivators appear to be necessary for maximal replication.

Finally, our standard ori-Lyt target was the *Bam*HI-H fragment, which contains an intact BHLF1 gene in addition to ori-Lyt (Fig. 1). Since a plasmid with this open reading frame was present in all the replication assays shown, the potential existed for the BHLF1 gene product to be contributing to ori-Lyt replication. To address this question, we substituted a modified *Bam*HI-H target (pPL2A) carrying a 1,375-bp deletion in the BHLF1 open reading frame for *Bam*HI-H. This modified ori-Lyt target was still replication competent when cotransfected with the six cloned replication genes, plus *Sal*I-F and the three transactivators, indicating that BHLF1 is not essential (Fig. 8, lane 1).

DISCUSSION

The identity of the virally encoded *trans*-acting proteins involved in HSV DNA replication was established through a combination of genetic studies and the use of transient cotransfection-replication assays. Several of the HSV replication genes have potential homologs in EBV based on sequence similarities. However, with the exception of the EBV DNA polymerase (BALF5) and the associated polymerase processivity factor (BMRF1), the functional equivalence of these genes has not previously been examined, nor has the full complement of genes required for lytic EBV DNA replication been determined. We established a cotransfection-replication assay utilizing Vero cells and overlapping cosmid clones of the EBV genome to examine the requirements for replication of an ori-Lyt-containing target plasmid. Our studies identified six EBV genes, BALF5, BMRF1, BALF2, BBLF4, BSLF1, and BBLF2/3, along with the viral lytic-cycle transactivators, Zta, Rta, and Mta, and an unidentified gene in *Sal*I-F, as being essential for ori-Lyt replication in the transient assay.

Two of the essential genes, the DNA polymerase and the polymerase processivity factor, were already known to serve a replication function. The first evidence for an EBV-encoded polymerase came from studies demonstrating that



B.

HSV-1	270	PALVALFRGWHTTVHAAPGALAPVFAFLGPEFAVRGGPV-PYFAVLGFPGWPTFTVP	324
VZV	278	LSILATYRLWYAAAFGKPGVVRPIFAYLGPELNPKGEDRD-YFCTVGFPGWTTLRTQ	332
EBV	261	GGLYAFLRSIYASCYANHRGTKPIFYLLDPELCPGGSDFQPYVP--GFPFLPIHYVG	315
CMV	183	LLLQSAWTHLYDVLFRGFAGQPPLRDYLGPDLFETGAARSFFFP--GFPPVPVYAVH	237

FIG. 7. BBLF2/3 shares genomic location and limited amino acid sequence similarity with UL8 of HSV, gene 51 of VZV, and UL102 of HCMV. (A) Relationship of the BBLF2 and BBLF3 open reading frames to the open reading frames in the equivalent regions of the HSV, VZV, and HCMV genomes (11, 19, 53). Potential ATP-binding motifs are indicated (●), as is a region of amino acid similarity (☒). (B) Comparison of the sequences denoted in panel A by the symbol ☒. Identical residues are indicated by asterisks (*), and equivalent residues are indicated by vertical lines (|).

viral lytic-cycle replication was selectively inhibited by drugs such as phosphonoacetic acid and acyclovir (16, 45). Sequencing of the EBV genome revealed an open reading frame (BALF5) whose protein product shares 33% sequence identity with the HSV DNA polymerase (3, 52) and like the HSV polymerase has several regions that are highly conserved in prokaryotic and eukaryotic viral DNA polymerases and in mammalian DNA polymerase alpha (64). More recently, BALF5 has been synthesized in vitro and shown to exhibit polymerase activity (50). The BMRF1 gene is the positional equivalent of the HSV UL42 polymerase processivity factor (20, 27, 29, 36). Although the EBV protein has no significant sequence similarity to UL42, it is a functional homolog. The BMRF1 protein copurifies with the EBV polymerase and has been shown to stimulate polymerase activity in vitro (40, 46).

The four other EBV genes identified as being required for ori-Lyt replication in the cotransfection-replication assay are BALF2, BBLF4, BSLF1, and BBLF2/3. The BALF2 gene product has 25% overall identity with the HSV single-stranded DNA-binding protein (UL29). BALF2 also contains a series of motifs that are highly conserved in the HSV, VZV, SCMV, and HCMV single-stranded DNA-binding proteins (1, 10, 65). The HSV and EBV proteins are virtually identical in size at 1,196 and 1,128 amino acids, respectively. The BBLF4 and BSLF1 proteins have significant sequence identity with the HSV UL5 and UL52 genes. UL5 and UL52 form a tripartite complex with UL8 in HSV-infected cells and in insect cells coinfected with recombinant baculoviruses expressing these genes (15, 21). This complex has both helicase and primase activities. Recently, helicase and pri-

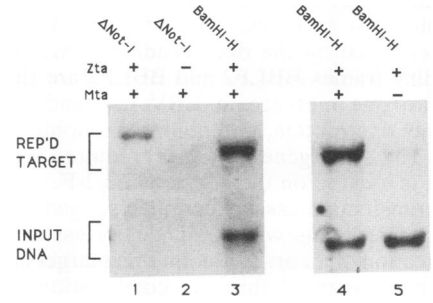


FIG. 8. Six cloned replication genes plus *Sal*I-F and the lytic-cycle transactivators support replication of the target; the BHLF1 gene product is not required for replication. The ori-Lyt-containing target was transfected with expression constructions for the six cloned genes: BMRF1, the polymerase processivity factor; BSLF1, the primase homolog; BBLF4, the helicase homolog; BBLF2/3, the potential UL8 homolog; BALF5, the DNA polymerase; and BALF2, the single-stranded DNA-binding protein homolog. *Sal*I-F was also added, as well as expression constructions for the three transactivators. A Southern blot of transfected cell DNA digested with *Bam*HI and *Dpn*I was probed with *Bam*HI-H to detect *Dpn*I-resistant, replicated DNA. Lane 1, an ori-Lyt target (ΔNotI) carrying a deletion within the BHLF1 open reading frame. Lane 2, no Zta added. Lane 3, the standard *Bam*HI-H target. Lane 4, the standard *Bam*HI-H target in the presence of Zta, Rta, and Mta. Lane 5, no Mta added.

mase activities were demonstrated in a bipartite complex of UL5 and UL52 (6, 22). Analysis of the UL5 amino acid sequence has revealed six motifs, including an ATP-binding motif, that are present in other families of helicases (28, 37, 44), and hence UL5 and BBLF4 are predicted to function as helicases. Indeed, individual mutations in each of the six conserved motifs of UL5 abolish its ability to complement a replication-deficient null mutant in a transient replication assay (76). UL52 is the putative primase of HSV, although an association with UL8 and UL5 may be required for complete activity. BSLF1 is the candidate primase of EBV. Furthermore, we believe that BBLF2/3 is the homolog of HSV UL8, the third member of the helicase-primase complex. BBLF3 and BBLF2 are the positional equivalents of HSV UL8 and UL9. However, in EBV, these two open reading frames are believed to be spliced into a single transcript and hence would presumably encode only one protein. Previous reports noted no significant similarity between BBLF2/3 and UL8 or UL9. However, a visual alignment (Fig. 7) reveals a 55-amino-acid region of BBLF2 that is conserved in HSV UL8, VZV 52, and HCMV UL102. For this reason, we believe that BBLF2/3 is likely to be the homolog of HSV UL8. The spliced BBLF2/3 transcript would encode 709 amino acids compared with 750 for HSV UL8.

At least one other gene encoded by EBV *Sal*I-F is required for ori-Lyt replication in the cotransfection assay. Interestingly, *Sal*I-F encodes the latency origin-binding protein EBNA-1. However, EBNA-1 is thought not to be the required gene product because *Sal*I-F cannot be replaced by other subclones that contain the EBNA-1 open reading frame (unpublished data). One of the replication proteins that has not been identified is an ori-Lyt origin-binding protein equivalent to the HSV origin-binding protein, UL9 (23, 41, 54). Since ori-Lyt is unrelated in sequence to HSV ori-S and ori-L, it is not surprising that comparative analyses have not revealed a homolog for UL9. It is possible that the gene for the EBV origin-binding protein is located in *Sal*I-F or that one of the proteins already shown to be required in the cotransfection assay is multifunctional and also provides origin-binding activity. Another alternative is that the origin-binding protein is not virally encoded and that a cellular factor serves in this capacity.

The EBV-encoded proteins identified in this study as being required for ori-Lyt replication are homologs of HSV proteins that participate directly in DNA replication. Herpesviruses also encode a number of enzymes, alkaline nuclease, ribonucleotide reductase, thymidine kinase, dUTPase, and uracil DNA glycosylase, that are involved in nucleotide metabolism and play an ancillary role in DNA synthesis (reviewed in reference 66). Genetic studies with HSV indicate that under certain conditions such as growth at high temperature, in growth-arrested cells, or in the animal host, HSV DNA replication may become dependent on the virally encoded alkaline nuclease, thymidine kinase, and ribonucleotide reductase. In our cotransfection-replication assays, the cosmid cMB14 could be replaced by a cosmid cMSalC which has five open reading frames in common with cMB14. The common genes are BMRF1 (the polymerase processivity factor), BSLF1 (the putative primase homolog), BMLF1 (the Mta transactivator), BMRF2, BORF2, and BaRF1. When these cosmids were replaced by expression vectors for BMRF1 and BSLF1, replication of ori-Lyt did occur but at a reduced level. This observation raises the possibility that cMB14 and cMSalC were providing an additional nonessential, replication-related function. Mta can be discounted since it was being provided exogenously by an expression vector, and BMRF2 is a late gene and therefore not a likely candidate for a replication-related function. The remaining common open reading frames, BORF2 and BaRF1, encode the large and small subunits of ribonucleotide reductase. One interpretation of the data is that the EBV-encoded ribonucleotide reductase is providing an auxiliary function that increases replication efficiency in these assays. The replication signal was also reduced when the *Bam*HI-H ori-Lyt target was replaced with a modified target carrying a deletion in the BHLF1 gene. In this case, the apparent reduction in replication efficiency may simply represent decreased hybridization of the probe to the target DNA which no longer contains the *Not*I repeats.

The minimal ori-Lyt as defined by Hammerschmidt and Sugden (32) consists of three essential subdomains (Fig. 1). Two of these, the BHLF1 promoter and the upstream enhancer, also function in transcriptional regulation of the flanking BHLF1 and BHRF1 genes. Characterized replication origins are commonly associated with transcriptional elements (reviewed in reference 19). These elements may be integrally linked to replication functions or may serve an auxiliary role by contributing to replication efficiency. For example, the cloned minimal HSV-1 origin, ori-S, can function in transient replication assays. However, if the flanking sequences containing the divergent promoters for the immediate-early IE175 and IE68 genes are also included, replication of the target is substantially increased (69). The EBV latency origin of replication (ori-P) consists of two domains, an EBNA-1-dependent enhancer and a region of dyad symmetry that is the site of initiation of replication (26, 72). With ori-P, replication is strictly dependent on the presence of the enhancer domain. The recently identified CMV origin of replication contains multiple binding sites for transcription factors (2, 33), although their requirement for replication has yet to be addressed by mutational analyses.

The BHLF1 promoter that constitutes one of the essential domains of EBV ori-Lyt contains four binding sites for Zta and is efficiently activated by Zta in cotransfection assays. The ori-Lyt enhancer contains two binding sites for a second viral transcriptional activator, Rta, and one binding site for Zta (31, 48). The enhancer is strongly activated by Rta and responds synergistically to the combination of Rta and Zta (14). In the cotransfection-replication assays, ori-Lyt replication was dependent on the presence of Zta, Rta, and Mta. In the assays using the cosmid clones, the transactivators would have been required for efficient expression of the replication genes encoded within the cosmids. Even in the final assays described here, at least one of the genes necessary for replication was provided on *Sal*I-F, and again it is likely that the transactivators would be needed for expression from this plasmid. In HSV, the immediate-early transactivators were required for replication of ori-S in the transient replication assay when viral DNA fragments were used to provide the replication functions. In contrast, when each of the seven replication genes was expressed from the strong constitutive HCMV promoter, the requirement for these transcription factors in the transient replication assay was alleviated (35). In EBV, however, the inclusion of the promoter and enhancer elements within the defined limits of the minimal ori-Lyt makes it highly probable that Zta and Rta contribute to replication directly through transcriptional activation, through DNA binding, or through interactions with components of the replication complex. The role of these transactivators in ori-Lyt replication can be addressed

more directly when the full complement of EBV replication genes has been identified.

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