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# A Replication Function Associated with the Activation Domain of the Epstein-Barr Virus Zta Transactivator

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**The Zta transactivator is crucial for both Epstein-Barr virus (EBV) lytic gene expression and lytic DNA replication. We have used a cotransfection-replication assay to examine the effect of mutations in the Zta activation domain (amino acids [aa] 1 to 167) on Zta replication activity. Deletion of Zta aa 25 to 86, which are critical for transcriptional activation of ori-Lyt, or aa 93 to 141 did not adversely affect replication of an ori-Lyt-containing target plasmid. However, removal of aa 2 to 25 (Δ2-25) abolished replication activity. Within this subdomain, deletion of aa 2 to 10 (Δ2-10) or mutation of codons 18 and 19 (m18/19) or 22 and 26 (m22/26) did not affect replication competency, while deletion of codons 13 to 19 (Δ13-19) or mutation at codons 12 and 13 (m12/13) impaired Zta replication function. Each of the replication-negative Zta variants was capable of transactivating expression from both BHLF1 promoter-chloramphenicol acetyltransferase constructions and the BMRF1 promoter on endogenous EBV genomes in Raji cells with efficiency comparable to that of the wild-type polypeptide. Thus, a replication contribution of Zta was functionally separable from its transactivation activity and was supplied by the N-terminal region encompassing aa 11 to 25. Replication by a subset of the impaired Zta mutants was partially rescued upon the addition of Rta to the replication assay. The contribution of Rta mapped to domain II of the Rta activation domain and was specific for this region. A chimeric Rta–EBNA-2 transactivation domain fusion, which retains the DNA-binding and transactivation properties associated with wild-type Rta, failed to rescue replication-deficient Zta. Our data suggest that Rta may act as an ancillary replication factor in EBV ori-Lyt DNA synthesis by stabilizing Zta-replisome interactions.**

Maintenance of both a latent state and a lytic replicative cycle by the human gammaherpesvirus Epstein-Barr virus (EBV) requires distinct genetic elements, ori-P and ori-Lyt, respectively (25, 30, 71). In latently infected, immortalized B lymphocytes, multiple copies of the viral genome are maintained as extrachromosomal episomes. The latent state is characterized by a lack of virus production, limited viral gene expression, and replication of the episome in a manner synchronous with cell division (72). The absolute dependence on a multitude of host cell proteins yet only a single virus-encoded polypeptide, EBNA-1 (56, 71), for amplification establishes an intimate association of replication of the episomal EBV genome with the latently infected cell. Lytic replication differs from the latent amplification state in that multiple rounds of replication are initiated within ori-Lyt, and the replication process has a greater dependence on EBV-encoded proteins. Moreover, lytic DNA replication results in the generation of high-molecular-weight concatemeric molecules which are subsequently cleaved into unit-length genomes and packaged into virions (16, 59, 73). The lytic replication cycle, which occurs in the oropharynx (65), can be induced in latently infected B cells by treatment with phorbol esters, by cross-linking of surface immunoglobulin, or by introduction of the EBV BZLF1-encoded transactivator, Zta (13, 66, 74). The first genes expressed on induction are those for the immediate-early Zta (BZLF1) and Rta (BRLF1) transactivators (23, 53). The initial transcription of these genes is further up-regulated by Zta, which binds to sequences in both promoters (22, 45, 64). Zta and Rta then activate expression of the third transactivator, Mta (BMLF1) (12, 27, 34, 42). The concerted action of these three viral regulatory proteins culminates in the sequential activation of early gene expression followed by the lytic cascade of replication and late gene expression (20, 23, 60, 64, 66).

The EBV genome possesses two copies of ori-Lyt, although one is sufficient for lytic-cycle replication. The 695-bp origin consists of two essential or core elements and one auxiliary domain (30, 61). The first core element encompasses the promoter and leader region of the BHLF1 gene, which contains four binding sites for Zta (Zta response elements [ZREs]) and is Zta responsive in transient expression assays (40, 44). Deletion of the TATA box, ZRE1, ZRE2, and the CAAT box abolishes replication, while mutation of the ZRE1 and ZRE2 sites reduces replication efficiency (61). The second essential domain is delineated by a central 225-bp region including two adenine-thymidine (AT)-rich palindromes and an adjacent polypurine-polypyrimidine tract (28, 57). Such elements are vital to facilitate the localized unwinding and helical destabilization essential for initiation of replication (38, 63). The third domain, a nonessential auxiliary element, is a powerful en-

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hancer region which contains DNA-binding sites for Rta and Zta and responds synergistically to the presence of these transactivators (14, 27). Hammerschmidt and Sugden (30) functionally replaced this domain with the human cytomegalovirus (HCMV) major immediate-early gene enhancer, thus implicating the enhancer function of the third domain, and not a specific sequence element, as indispensable for lytic replication.

Previously, our laboratory used a transient replication assay in Vero cells to determine which EBV-encoded proteins are required to replicate an ori-Lyt target (19, 20). This method has also proven invaluable for identifying the replication genes for herpes simplex virus type 1 (HSV-1) (5, 69) and human cytomegalovirus (HCMV) (54). In this assay, six essential core EBV replication genes were identified for ori-Lyt DNA replication as well as the Zta and Mta lytic-cycle transactivators (20). Rta, encoded by BRLF1, is a transcriptional activator that binds DNA as a dimer (26, 31, 48) and acts in combination with Zta to produce a synergistic response in targets containing binding sites for both factors (14, 55). Rta augments DNA replication but is not absolutely required (20). Mta, encoded by BSLF2/BMLF1 (41), primarily serves a posttranscriptional function (3, 12, 35) and contributes to replication in an indirect manner (20). In contrast, an absolute dependence was demonstrated for Zta, the single virus-encoded protein which serves an essential origin-binding function (20). Moreover, mutation of all ZRE sites in the target origin abrogates replication function, further implicating Zta as an obligatory participant in lytic replication (60).

Zta is a bZIP transcriptional activator with a basic DNA recognition domain that is closely related to that of Jun, Fos, and other bZIP proteins (18, 44, 68). However, unlike the rest of this family, the Zta dimerization domain does not consist of a heptad repeat of leucines (7, 21). One consequence of this structural difference is that Zta does not undergo heterodimerization interactions with other family members. Zta binds as a homodimer to AP-1 sites and to the related ZREs (4, 7, 18, 44, 68), and its binding to DNA is modified by phosphorylation (37). Activation of transcription occurs primarily through direct interaction between the amino-terminal activation domain of Zta and TATA-binding protein (45) and TATA-binding-protein-associated factors (43) to stimulate formation of a stable TFIID-TFIIA complex (11, 43). However, the mechanism by which Zta functions to fulfill its essential role in the replication process is not currently understood. Chimeric Zta polypeptides in which the Zta activation domain, extending from amino acids (aa) 1 to 168, was replaced by the potent transcriptional activation domain from the HSV-1 VP16 protein failed to support ori-Lyt replication (60). This finding implies that a property intrinsic to the Zta activation domain, other than the transcriptional activation features common to VP16, may be essential for replication.

This report represents an initial examination of the effects of Zta variants carrying deletions or mutations within the activation domain on ori-Lyt replication in a transient cotransfection-replication assay. We have identified a region within the N terminus of Zta that genetically separates transcriptional activity from replication function within the polypeptide. These results represent the first evidence that the replication function associated with Zta may be dispensed with while maintaining the integrity of transcriptional activation activity. Furthermore, we demonstrate the ability of Rta to contribute to ori-Lyt replication by partially complementing a replication-impaired Zta protein. The property of Rta-mediated compensation of Zta function maps to the acidic activation domain. These findings underscore the importance of protein interactions with Zta and Rta for functional replication complex assembly.

TABLE 1. EBV replication proteins and expression plasmids

| Viral gene | Predicted function | Clone |
|---|---|---|
| Core machinery | | |
| BSLF1 | Primase | pRTS11[a] |
| BALF2 | Single-strand-DNA-binding protein | pRTS12 |
| BALF5 | DNA polymerase | pRTS13 |
| BMRF1 | Polymerase accessory | pRTS14 |
| BBLF2/3 | Primase-associated factor | pRTS25 |
| BBLF4 | DNA helicase | pRTS28 |
| Auxiliary components | | |
| BRLF1(Rta) | Regulatory | pRTS15 |
| BMLF1(Mta) | Regulatory, posttranscriptional | pRTS16 |
| Initiator protein | | |
| BZLF1(Zta) | Regulatory, origin-binding protein | pRTS21 |

[a] The open reading frames of the EBV replication loci were subcloned into an expression vector (pRTS2) containing the SV40 enhancer, β-globin intron, and SV40 polyadenylation signals as described previously (58).

## MATERIALS AND METHODS

**Plasmid constructions.** Plasmid pRTS2, a modified pSG5 vector (Stratagene), was constructed by destroying the *Xba*I site and placing the polylinker from pGH57 between the *Eco*RI and *Bgl*II sites. Oligonucleotide primers were synthesized to amplify the viral replication open reading frames extending from the ATG start site to the translation stop codon as indicated in Table 1. After PCR amplification, the DNA product was cleaved with the appropriate restriction endonucleases and subcloned into pRTS2 as follows: pRTS11, -12, -13, -14, -15, -16, -25, and -28 as *Xba*I-*Hin*dIII fragments and pRTS21 as a *Bam*HI-*Bgl*II fragment. These plasmid constructions have been described previously (58), as has the origin-containing plasmid, pSL77 (19).

The Zta deletions Δ2-141, Δ25-86, and Δ93-141 have been described elsewhere (45). Zta (Δ2-167) was constructed by PCR amplification using a 5′ oligonucleotide, AATGAATTCATATGGAGGAATGCGATTCTGAA, and a 3′ oligonucleotide specific for pBSKII vector sequences. The product was cleaved with *Eco*RI and ligated into the *Eco*RI site of pcDL-SRα-296. The open reading frame was checked by DNA sequencing, and expression was confirmed by Western blotting (immunoblotting). pRTS68 [Zta (Δ2-25)] was generated by using oligonucleotide-directed mutagenesis to delete nucleotides 103072 to 103143 from the Zta open reading frame. The parental construction was SVZwt (nls) (24), a gift from S. Speck (Harvard Medical School). The variants Zta (m12/13), Zta (m18/19), and Zta (m22/26) were generated by PCR mutagenesis (33) and cloned into the expression plasmid pcDL-SRα296 (67). Zta (Δ2-10) (pDH284) and Zta (Δ13-19) (pDH285) were originally cloned as cDNAs into the bacterial expression plasmid pQE8 (Qiagen, Chatsworth, Calif.). The coding sequences were then moved as an *Eco*RI fragment into the eukaryotic expression vector pcDL-SRα296. All Zta derivatives tested were controlled by the simian virus 40 (SV40) early promoter. The Rta activation domain mutants pLT24, pLT27, and pLT29 were obtained from J. M. Hardwick (Johns Hopkins School of Hygiene and Public Health). Plasmids pLT24 and pLT27 have been described elsewhere (32). The Rta protein encoded by pLT29 is truncated at codon 519, and activation domain II is replaced with the activation domain (codons 437 to 477) of EBV EBNA-2.

**Transient expression and DNA replication assays.** DNA transfections, the transfection-replication assay, and chloramphenicol acetyltransferase (CAT) assays were performed as previously described (20, 46). Vero cells were maintained in Dulbecco modified Eagle medium plus 5% fetal calf serum at 37°C in 5% $CO_2$ and were transfected by calcium phosphate precipitation. Briefly, for replication assays $1.5 \times 10^6$ Vero cells per 100-mm-diameter dish were transfected with 3.5 to 5.0 μg of pSL77(ori-Lyt) DNA, 1.5 μg of Zta expression plasmid, and 700 ng of expression plasmids for each of the six core replication proteins as well as Mta and Rta. Cells were harvested 80 h after transfection. Autoradiograms of Southern blot analyses were quantified with a Kontes fiber optic scanner. For transient expression assays, $3 \times 10^5$ Vero cells were transfected with 2 μg of plasmid DNA in six-well dishes, and cells were harvested 48 h after transfection. Cell extracts were incubated with 0.2 μCi of [$^{14}$C]chloramphenicol (60 mCi/mmol) for 1 h at 37°C. The acetylated forms were separated by chromatography in chloroform-methanol (95:5) solvent. CAT activity was quantitated by phosphorimager analysis.

Raji lymphoblastoid cells were maintained at $5 \times 10^5$ cells per ml in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in 5% $CO_2$ and were transfected by the DEAE-dextran method. Induction of BMRF1 was assayed with $2 \times 10^6$ Raji cells per sample and 3 μg of each Zta expression plasmid. Cells were resuspended at $10^7$/ml in Ca–Tris-buffered saline (CaTBS; 0.1 g of

$MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2$, 8.0 g of NaCl, 0.38 g of KCl, 0.1 g of $Na_2HPO_4 \cdot 12H_2O$, and 3.0 g of Tris hydrochloride per liter [pH 7.5]). DNA was mixed with 300 μl of CaTBS and 300 μl of CaTBS-DEAE-dextran (1.0 mg/ml). Approximately $10^6$ cells in 100 μl of CaTBS were added to the DNA-containing mixture, inverted several times, and placed at 37°C for 1 h with occasional rocking. Cells were then pelleted at 3,000 rpm for 3 min, washed twice with Tris-buffered saline (minus $CaCl_2$), and then resuspended in 2.0 ml of RPMI 1640 medium containing 10% fetal calf serum. Cells were harvested 48 h after transfection.

**Western analysis.** Immunoblot assays were performed as previously described (51). Cell extracts were boiled in lysis buffer (4% sodium dodecyl sulfate [SDS], 160 mM Tris [pH 6.8], 200 mM dithiothreitol, 20% sucrose, 0.02% bromophenol blue, 10% β-mercaptoethanol), and the proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% (BMRF1) or 12% (Zta) gels. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) and incubated with either Zta-specific monoclonal antibody (MAb) (BZ1; Dako, Carpinteria, Calif.) at 1:1,500 or BMRF1-specific monoclonal antibody (p52/50; Advanced Biotechnologies, Inc., Columbia, Md.) at 1:2,000. Proteins were visualized by chemiluminescence (Dupont NEN, Boston, Mass.).

**Immunofluorescence assay.** Vero cells were plated at approximately $0.8 \times 10^5$ cells per well onto glass tissue culture chamber slides (Nunc). DNA transfections were done as described above, with a total of 1.5 μg of DNA transfected per well. After a change of medium, the slides were harvested for immunofluorescence assay as described previously (58) except that a MAb to Zta (BZ1; Dako) was used at a dilution of 1:2,000.

## RESULTS

**Identification of a replication function within the Zta transactivation domain.** We initially evaluated four Zta variants possessing deletions in the Zta activation domain for their effects on ori-Lyt replication in the transient cotransfection-replication assay. Such an assay measures DNA amplification by exploiting the ability of the *Dpn*I restriction enzyme to discriminate between methylated and unmethylated DNA sequences. Basically, only target origins which undergo amplification upon transfection into eukaryotic cells will lose the bacterially imposed methylation pattern and become resistant to cleavage by *Dpn*I. All input molecules unable to replicate in transfected tissue culture cells will remain methylated and be digested by *Dpn*I. The replication gene expression plasmids listed in Table 1 were cotransfected into Vero cells along with pSL77, the EBV *Bam*HI-H origin-bearing plasmid (Fig. 1A), and one of the Zta expression derivatives presented in Fig. 1B. Transfected cell DNA was isolated, digested with *Bam*HI-*Dpn*I, and examined by Southern blot analysis for DNA amplification. In this transient assay, Zta variants lacking aa 2 to 141 or 2 to 167 were impaired for replication function (Fig. 1C, lanes 6 and 7). Amino acids from 25 to 86 have been shown to be important for transcriptional activation (45), leading to speculation that this region of Zta may affect replication efficiency. Surprisingly, however, Zta carrying a deletion encompassing this region, Δ25-86, mediated ori-Lyt DNA replication (lane 4). Additionally, a Zta variant deleted for aa 96 to 141 was also replication competent (lane 5). All Zta derivatives were tested for the ability to localize to the nucleus upon transfection as determined by indirect immunofluorescence with a MAb to Zta. Indeed, all Zta expression plasmids described in Fig. 1B localize to the nucleus, and a subset of these derivatives are shown in Fig. 1D.

Since a Zta polypeptide deleted for aa 2 to 141 lost replication activity but deletion of aa 25 to 86 and 96 to 141 did not affect replication function, we set out to determine whether aa 2 to 25 might represent a specific subdomain through which replication function is mediated. The transient replication assay system was used to examine the replication property of a Zta (Δ2-25) derivative, encoded by pRTS68. This analysis revealed that deletion of the first 25 aa culminated in the abrogation of replication function, thus potentially delineating a replication-indispensable Zta domain (Fig. 2, lane 3).

**Functional dissection of the replication domain within aa 2 to 25 of Zta.** In an attempt to further narrow the replication-specific region of Zta, we used two smaller deletions of pRTS68, Zta (Δ2-10) and Zta (Δ13-19), along with three Zta variants containing double-point mutations within the first 26 aa (Fig. 3A). Western analysis with an anti-Zta MAb demonstrated that all of the Zta variants were expressed in transfected cells and expressed at comparable levels (Fig. 3B).

The Zta mutant derivatives were first tested for transcriptional activity in a transient transfection assay. Both the BHLF1 promoter controlling the *Not*I repeat transcript and the upstream enhancer region of ori-Lyt contain Zta-binding sites, and the BHLF1 promoter is activated by Zta in transient expression assays (40, 44). The ability of the Zta variants to activate CAT expression in a transient expression assay was examined by using the target plasmid pDH123, which contains sequences extending from the BHLF1 promoter through the enhancer region. All Zta expression derivatives were competent to activate CAT expression from pDH123 (Fig. 4A). Zta (Δ2-25) induced a response comparable to and slightly greater than that of the wild-type (wt) polypeptide. Similarly, Zta (Δ2-10), Zta (m12/13), Zta (m18/19), and Zta (m22/26) showed wild-type transactivation activity, while Zta (Δ13-19) activated to levels within twofold of that observed with wt Zta.

Transcription factors essential to the replication process may possess a multiplicity of functional roles. One key factor in the enhancement of origin activation may reside in the ability to displace template-tethered nucleosomes (9, 39). The removal of such proteins can serve to allow access of key regulatory or initiator polypeptides to the DNA origin. Introduction of Zta reactivates EBV lytic gene expression from endogenous, nucleosome-associated EBV genomes, presumably in part by mediating removal of such template-bound nucleosomes (62). The ability of Zta variants to activate targets in transient assays does not always correlate with the ability to activate expression from an endogenous template (36). We therefore examined the histone displacement potential of the Zta derivatives. Raji cells were transfected with the Zta expression plasmids and assayed for activation of the endogenous early BMRF1 gene and subsequent expression of the BMRF1 gene product, the polymerase processivity factor. Raji cells transfected with only pSG5 (vector) failed to express BMRF1 as assayed by immunoblot analysis. All Zta derivatives tested were functional for induction of BMRF1 protein expression (Fig. 4B).

The ability of the Zta mutants to promote replication of EBV ori-Lyt was examined in the presence and absence of Rta in a cotransfection transient assay. In the absence of Rta, wt Zta, the double mutants Zta (m18/19) and Zta (m22/26), and the deletion Zta (Δ2-10) were found to possess significant replication ability (Fig. 5A, lanes 1, 6, 7, and 8, respectively). The Zta (Δ2-25) and Zta (Δ13-19) deletions as well as the Zta (m12/13) mutant were defective for replication (lanes 4, 5, and 9). In the presence of Rta, a replication signal became detectable for Zta (m12/13) and weakly detectable for Zta (Δ13-19) (lanes 11 and 15). Relative levels of replication, expressed as the replication index, are shown in Fig. 5B and C. Moreover, we have determined that the Zta derivatives tested in Fig. 5A are capable of localizing to the nucleus upon transfection. The immunofluorescence assay data for a subset of the Zta deletions are shown in Fig. 5D.

**Rta rescue of the replication-impaired Zta (m12/13) mutant requires activation domain II.** Rta acts as an ancillary factor to boost replication efficiency but is not essential (20). To further explore the contribution of Rta to ori-Lyt replication, Rta variants with altered activator domains were tested for the ability to complement the Zta (m12/13) mutant. The Rta activation domain can be divided into two subdomains. Domain

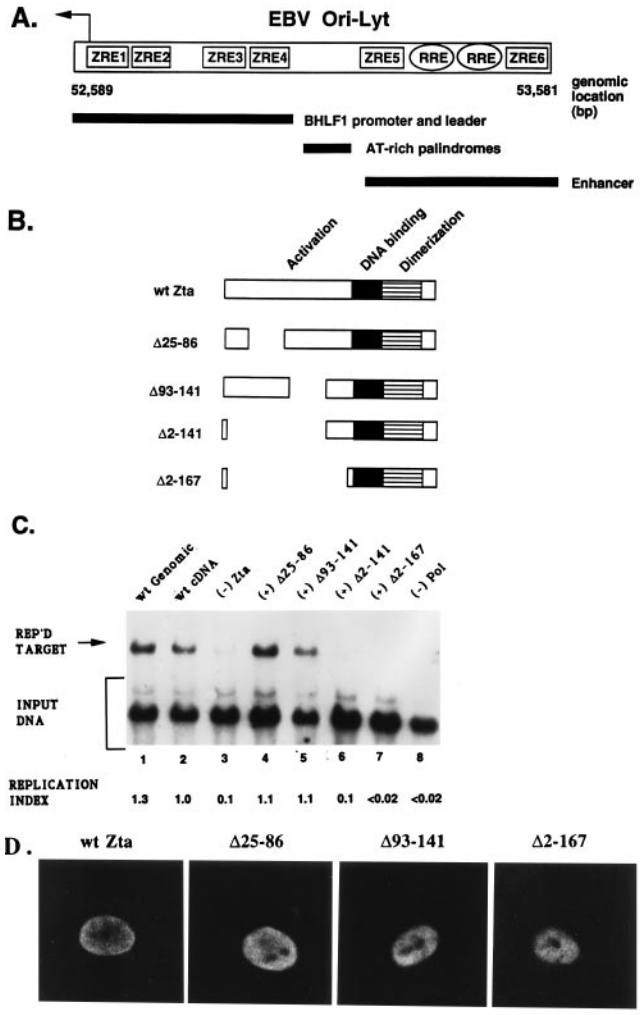


FIG. 1. Effects of deletions within the Zta activation domain on transient replication of EBV ori-Lyt. (A) Organization of ori-Lyt. The relative positions of Zta-binding sites (ZRE1 to -5), Rta-binding sites (RRE), the BHLF1 promoter and leader region, and AT-rich palindromes with adjacent polypurine-polypyrimidine tract and enhancer domain are shown. (B) Locations of deletions introduced into the Zta transactivation domain. (C) Transient replication assay. Vero cells were cotransfected with the *Bam*HI-H target and the expression plasmids for the replication genes listed in Table 1. The wt Zta expression plasmid was replaced with plasmids expressing Zta deletion variants as indicated. Isolated cell DNA was digested with *Bam*HI and *Dpn*I, and a Southern blot of the separated DNA fragments was probed with $^{32}$P-labeled ori-Lyt DNA. Replicated *Dpn*I-resistant *Bam*HI-H target and unreplicated input DNA are indicated. Lane 1, wt genomic-expressed Zta supplied by pPL17; lane 2, wt cDNA-expressed Zta supplied by pPL220; lane 3, minus Zta; lane 4, Zta (Δ25-86); lane 5, Zta (Δ93-141); lane 6, Zta (Δ2-141); lane 7, Zta (Δ2-167); lane 8, minus DNA polymerase, BALF5. The replication index corresponds to a densitometric analysis of the ratio of the *Dpn*I-resistant band relative to the first *Dpn*I-sensitive (input DNA) band. This value was set at 1.0 for the positive control (lane 1). (D) Immunofluorescence assay. Zta expression constructs were transfected into Vero cells to verify nuclear localization by using a fluorescein isothiocyanate-labeled mouse MAb to Zta.

II is an acidic, potent activation domain. Domain I is a proline-rich domain which possesses weak activity when assayed independently and synergizes with domain II in B cells but not in epithelial cells (32, 49). Deletion of domain I resulted in Rta complementation of Zta (m12/13) that was as efficient or better than that observed with wt Rta (Fig. 6, lane 3). Deletion of domain II abrogated the ability of Rta to complement this Zta mutant (lane 4). Further, replacement of domain II with the activation domain from EBNA-2 resulted in a chimeric protein that was unable to complement the Zta (m12/13) mutant (lane 5) in the replication assay. This Rta–EBNA-2 chimeric protein, however, functioned in a transactivation assay in Vero cells with 80% of the activity of wt Rta (data not shown). Each of the Rta variants retains DNA-binding ability, and hence binding of Rta to ori-Lyt is itself insufficient to rescue Zta function.

## DISCUSSION

*cis*-acting elements which function as origins of DNA replication frequently comprise an essential core component and a dispensable auxiliary component, often composed of promoter and enhancer elements (17, 29). Initiation typically begins within the central core region adjacent to an AT-rich motif capable of being easily unwound, while the auxiliary elements determine replication efficiency. Well-characterized viral origins include those of HSV-1 and SV40. Alphaherpesvirus replication origins such as those from HSV-1 possess a comparatively simple structure containing a palindrome with a central AT-rich region plus recognition sites for a viral origin-binding protein UL9 (6, 47, 52). Replication in SV40 requires a single multifunctional *trans*-acting viral factor, the large T antigen, and utilizes a multicomponent *cis*-acting origin domain which harbors several specific binding sites for T antigen as well as containing an AT-rich palindrome (1). The EBV lytic origin consists of three genetically separate domains: an enhancer, and a pair of AT-rich palindromes alongside a polypyridimine tract and the BHLF1 promoter (30, 61). Recently our laboratory demonstrated that EBV lacks an equivalent of the HSV-1 UL9 protein. Instead, the lytic cycle transactivator Zta serves an essential origin-binding function in transient ori-Lyt replication assays (20). Zta binds to six sites within ori-Lyt, and the integrity of such ZRE sequences must be maintained in order to retain replication activity (60).

Interaction of origin-binding proteins such as HSV-1 UL9, SV40 T antigen, and papillomavirus E1 with the DNA template initiates the replication process by facilitating distortion of the origin DNA and localized melting. Moreover, such viral polypeptides possess intrinsic helicase activities to begin the initial unwinding prior to the assembly of the remaining replication machinery (2, 15, 70). The mechanism by which Zta contributes an essential function to lytic replication remains poorly characterized. Transcription initiation complexes adjacent to, or within, origins of replication can stimulate origin

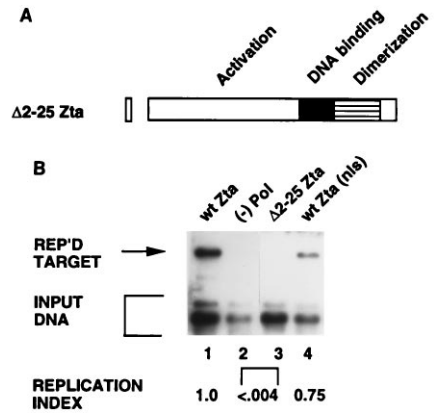


FIG. 2. A Zta mutant deleted for aa 2 to 25 lacks replication competency. (A) Schematic of the Δ2-25 protein. (B) Southern blot analysis of a transient replication assay performed as described for Fig. 1, using Δ2-25. Lane 4 contains a wt Zta polypeptide with a second nuclear localization signal (24).

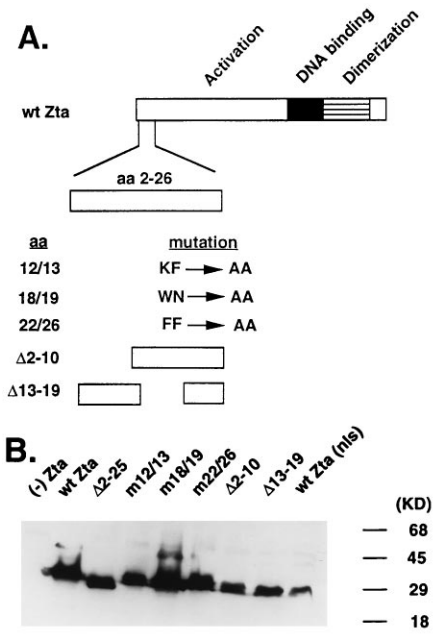


FIG. 3. Structures and expression of Zta variants carrying alterations in the aa 2 to 26 region of the activation domain. (A) Schematic representation of the Zta double-point mutants and small deletions tested. (B) Demonstration of protein expression by the panel of Zta derivatives in a Western blot analysis using an anti-Zta MAb. The positions of molecular mass markers are indicated on the right.

function. These schemes have been proposed for these complexes: (i) they may prevent nucleosome formation and hence increase access of DNA replication proteins to the origin; (ii) a transcription factor or competent transcription assembly complex may either facilitate unwinding of the DNA duplex or result in the generation of a secondary structure critical for initiation of replication; and (iii) polypeptides indispensable for transcriptional activation may directly recruit, stabilize, or serve as core replication machinery within the replisome complex. A goal of this study was to begin to evaluate which of these roles Zta plays in lytic-cycle replication.

One of the first observations made when the Zta transactivation domain deletions were tested for replication function in the transient transfection replication assay in Vero cells was that Δ25-86 retained replication competency comparable to wt Zta replication levels. The amino acids removed by this deletion have previously been shown to be critical for transcriptional activation of the BHLF1 promoter in a transient expression assay (45). Therefore, the transactivation function of this Zta variant did not directly correlate with replication activity. Conversely, Zta (Δ2-25) and Zta (Δ13-19) were transcriptionally active on the BHLF1 promoter in transient CAT assays and on the BMRF1 promoter located on the endogenous EBV genomes in Raji cells but were inactive in the replication assay. Thus, as measured by the cotransfection-replication assay, the replication activity of Zta is not solely a reflection of its transcriptional activation function. The contribution of certain other transcription factors to replication has been demonstrated to be independent of transactivation function. For example, in vitro replication of the SV40 origin of replication remains insensitive to α-amanitin (9). Moreover, the interaction of NF1 with adenovirus DNA polymerase to mediate formation of a preinitiation complex does not require the proline-rich activation domain for function (8, 50).

Examination of the replication ability possessed by the Zta deletion variants suggested that the N terminus may contain a subdomain through which replication function is mediated. Efficient ori-Lyt replication, but not transactivation function, was dependent on the presence of the first 25 aa of Zta. Previous studies have also reported that deletion of the N-terminal 25 aa of Zta has minimal effects on Zta transactivation activity in cotransfection and in vitro transcription assays (10, 24). Our finding represents the first demonstration of a genetic separation of transactivation function from replication activity within the transactivation domain. The replication-specific region within the transactivation domain was further narrowed by mutagenesis. Zta polypeptides deleted for aa 13 to 19 or mutated at aa 12 or 13 failed to replicate the ori-Lyt target in transfected Vero cells yet were able to mediate activation of both the BHLF1 promoter and the BMRF1 promoter. Moreover, such transactivation activity indicates that the mutant Zta polypeptides were able to interact normally with their cognate DNA-binding sites. This would be expected since the mutations are not within the DNA-binding domain of Zta.

T-antigen-directed SV40 DNA replication and E1-mediated replication of bovine papillomavirus in vitro are repressed by nucleosome assembly (9, 39). In EBV latency within B cells, early promoters are normally quiescent and nucleosome-associated prior to Zta expression and can be activated by Zta. Wild-type Zta, when bound to the BHLF1 promoter and enhancer, could also activate replication by functionally displacing nucleosomes present on the ori-Lyt template. We demonstrated that the entire set of Zta variants and wt Zta were competent to activate expression of the polymerase accessory protein (BMRF1) from the endogenous EBV genomes in Raji cells. Therefore, the inability to displace ori-Lyt-associated nucleosome complexes from the DNA template most likely does

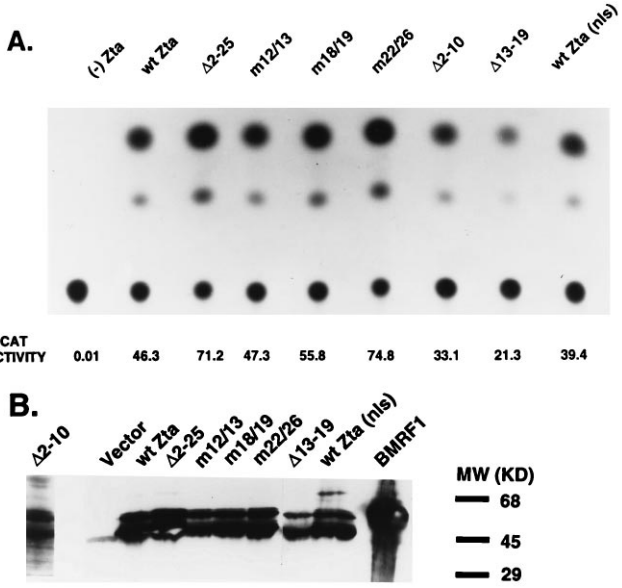


FIG. 4. The Zta derivatives are functional transactivators in transient assays. (A) Transient transactivation assay. The reporter plasmid, pDH123, contains the BHLF1 promoter and leader region driving the CAT gene. The reporter DNA (800 ng) was cotransfected with 1.5 μg of the Zta derivatives indicated at the top. Shown below is the percent acetylation as determined by quantitation with a phosphor imager. (B) Immunoblot showing induction of endogenous EBV BMRF1 gene expression in Raji cells upon transfection of the set of Zta derivatives. The membrane was probed with an anti-BMRF1 MAb. The last lane contains BMRF1-transfected cell extract as a positive control. Vector corresponds to pSG5-transfected Raji cell extract.

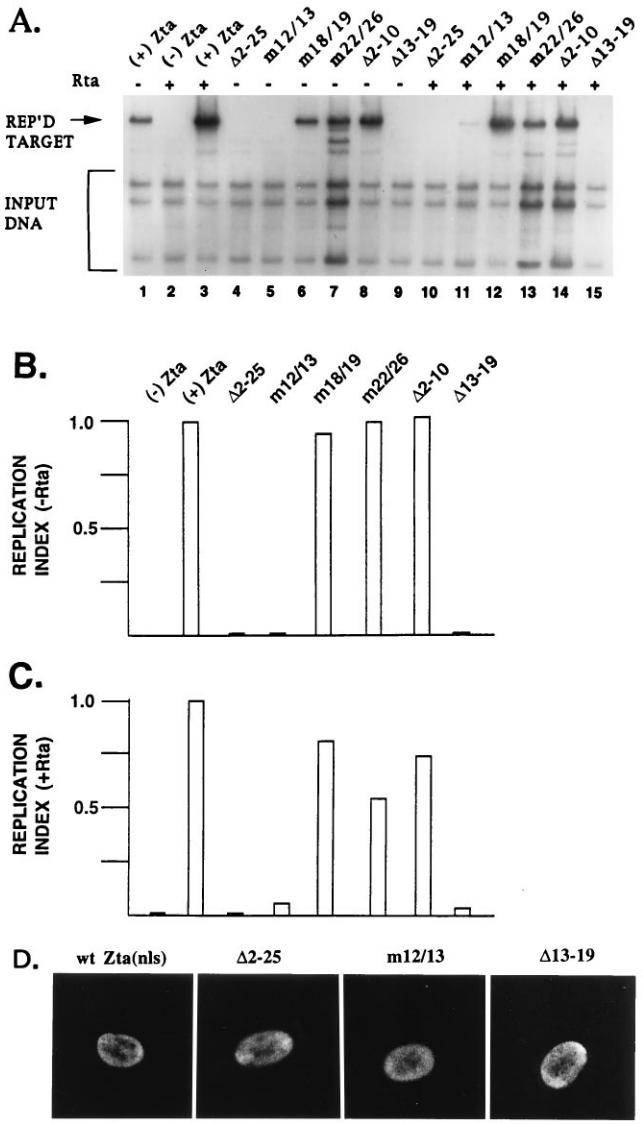


FIG. 5. Effects of mutations within the aa 2 to 26 region of Zta on replication function. (A) Southern blot analysis of a transient replication assay examining mutant Zta function in the presence and absence of Rta. Quantitation of the assay is shown in (B and C). All replication index values for lanes containing Rta are relative to that for the +Zta +Rta lane, which was set to 1.0. All replication index values for lanes lacking Rta are relative to that for the +Zta −Rta lane, which was set to 1.0. (D) Immunofluorescence assay. Zta expression constructs were transfected into Vero cells to verify nuclear localization by using a fluorescein isothiocyanate-labeled mouse MAb to Zta.

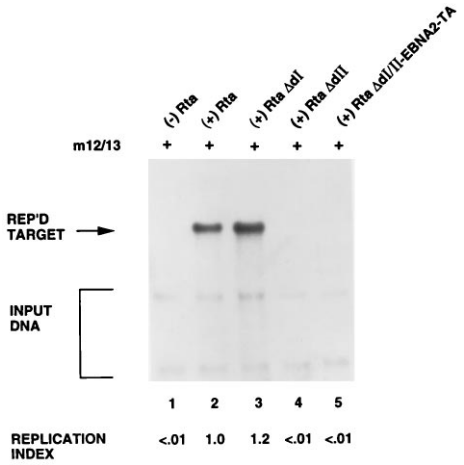


FIG. 6. Complementation of the Zta (m12/13) replication-deficient mutant by Rta maps to acidic activation domain II. Shown is Southern blot analysis of a transient replication assay examining the ability of Rta activation domain mutants to complement the replication deficit of Zta (m12/13). The replication index was calculated as described for Fig. 1.

not account for the replication-negative phenotype of Zta (Δ2-25) and Zta (Δ13-19). Zta apparently possesses a replication activity distinct from nucleosome disruption.

One interesting finding is the ability of Rta to compensate for the replication deficient Zta (m12/13) mutant protein. Zta and Rta both interact with DNA sequences within ori-Lyt. Rta is not essential for replication; it serves to augment replication activity in the presence of Zta (20). This function of Rta maps to the potent transactivation domain, domain II. DNA binding by Rta, in the absence of transactivation domain II, did not complement mutant Zta replication function, nor did transactivation mediated by a chimeric Rta protein in which domain II was replaced by the activation domain of EBNA-2. The implication is that domain II possesses amino acid sequences specific for the mutant Zta replication compensation function. Our data suggest that the amino terminus of Zta may interact with a cellular or viral polypeptide which is essential for efficient replication. The Zta (m12/13) mutant may have reduced affinity for this factor. Stabilization of the Zta-replication factor interaction by Rta contacting the replication complex could be sufficient to allow replication to ensue. The potential ability of Rta domain II to interact with such a replication factor as part of a replisome complex is not incompatible with Rta's inability to directly mediate ori-Lyt replication. [Rta was unable to aid the replication-deficient Zta (Δ2-25) polypeptide.] However, the ability of Rta to partially compensate for Zta function may be a complicating factor in analysis of Zta activity in other replication systems in which the endogenous EBV genomes are induced and also express Rta.

The lack of a truly dedicated virus-encoded origin-binding protein common to most viral systems, the absolute dependence on the Zta transcription factor, and the partial restoration of inefficient replication by a second transcription factor, Rta, encourage speculation that initiation of replication at EBV ori-Lyt may have more in common with a cellular origin of replication than most other viral counterparts.

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