Evidence for DNA Hairpin Recognition by Zta at the Epstein-Barr Virus Origin of Lytic Replication

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The Epstein-Barr virus immediate-early protein (Zta) plays an essential role in viral lytic activation and pathogenesis. Zta is a basic zipper (b-Zip) domain-containing protein that binds multiple sites in the viral origin of lytic replication (OriLyt) and is required for lytic-cycle DNA replication. We present evidence that Zta binds to a sequence-specific, imperfect DNA hairpin formed by an inverted repeat within the upstream essential element (UEE) of OriLyt. Mutations in the OriLyt sequence that are predicted to disrupt hairpin formation also disrupt Zta binding in vitro. Restoration of the hairpin rescues the defect. We also show that OriLyt DNA isolated from replicating cells contains a nuclease-sensitive region that overlaps with the inverted-repeat region of the UEE. Furthermore, point mutations in Zta that disrupt specific recognition of the UEE hairpin are defective for activation of lytic replication. These data suggest that Zta acts by inducing and/or stabilizing a DNA hairpin structure during productive infection. The DNA hairpin at OriLyt with which Zta interacts resembles DNA structures formed at other herpesvirus origins and may therefore represent a common secondary structure used by all herpesvirus family members during the initiation of DNA replication.

The study of human herpesvirus replication has become increasingly important over the past few decades as the incidence of human immunosuppression has escalated. The prototypic gammaherpesvirus, Epstein-Barr virus (EBV), is the etiological agent underlying diseases such as infectious mononucleosis, Burkitt’s lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma, posttransplant lymphoproliferative disease, oral hairy leukoplakia, and AIDS immunoblastic lymphomas (59, 83). The incidence of EBV pathogenesis is greatly increased during immunosuppression, largely due to uncontrolled lytic-cycle replication. While much of the EBV-induced pathology has been attributed to viral latency, increases in viral load and viral DNA replication are known to increase the risk of virus-associated disease. In addition to viral burden, viral lytic proteins are thought to contribute directly to pathogenesis, including tumorigenesis (33, 34). Lytic replication during primary infection is also required for the establishment of latency within the host (83). Currently, the most effective treatments for herpesvirus infections are nucleoside inhibitors, which inhibit lytic replication by blocking the action of viral DNA polymerases. These drugs, however, have limited clinical efficacy against EBV infection and pathogenesis (1).

The EBV-encoded immediate-early protein Zta (also referred to as ZEBRA, EB1, BZLF1, and Z) is a multifunctional protein that initiates lytic-cycle gene expression and DNA replication (15, 16, 36, 55, 60). Zta is a member of the basic leucine zipper (b-Zip) family of DNA binding proteins and can interact directly with DNA recognition sites in many viral gene promoters to stimulate transcription (68). Zta also binds directly to the viral origin of lytic replication (OriLyt) and recruits core replication enzymes to the site of replication initiation (22, 23, 27, 46, 62, 64, 65). The direct interaction of Zta with OriLyt is essential for lytic-cycle replication, but it is not known if Zta possesses inherent DNA replication factor functions, such as single-strand DNA binding or strand-unwinding activity. Moreover, the mechanism of replication initiation at OriLyt, as well as those at other herpesvirus origins, remains enigmatic.

Each herpesvirus family member encodes a conserved “core” of lytic-replication enzymes consisting of a DNA polymerase, a processivity factor, a helicase-primase complex, and a single-stranded binding protein (10). This replication machinery is required for DNA synthesis. The best-characterized herpesvirus to date is herpes simplex virus type 1 (HSV-1) (9, 11, 42). Reports of studies using core HSV-1 replication proteins have shown that these enzymes are capable of replicating preinitiated templates (20, 28, 57, 69, 70). In addition to the core proteins, each viral species also encodes an essential origin-binding protein, though these proteins are quite divergent. HSV-1 encodes an origin-binding helicase, UL9, required for infection and replication initiation (2, 12, 13, 52, 54, 56, 78), while EBV relies on Zta as the virally encoded origin binding factor (14, 21, 24, 40, 45, 46, 67). The conserved core machinery seems to be interchangeable between viruses. For example, the HSV-1 core replication enzymes are capable of replicating a Zta-initiated EBV template (22), and the EBV core replication machinery is able to replicate a human cytomegalovirus (CMV) template initiated by the CMV origin binding protein UL84 (63). These observations demonstrate that the origin binding proteins mainly confer origin specificity and that origin binding proteins are the only viral proteins required for origin melting.

Based on the heterogeneity of these disparate initiator proteins, one would expect that different herpesviruses should also have varied origin structures, but surprisingly, some intriguing similarities exist. Most herpesvirus lytic origins are located between divergent promoters containing multiple transcription
factor binding sites (6, 31, 66, 72, 73), and replication appears to be dependent on the transcription of these promoters (65, 77, 81, 82). Herpesvirus lytic origins also include inverted-repeat sequences that function as origin binding protein recognition elements. HSV-1 UL9 binds to three “boxes” within the lytic origin, OriL, including a BoxII/BoxI inverted repeat (18) where UL9 is able to induce structural changes in OriL (18, 38). Using electron microscopy (EM), one group observed that this ATP-independent alteration included DNA bending and produced a prereplicative initiation structure, later called “OriL,” which, after stabilization by photo-cross-linking, could be bound by a single-stranded binding protein with high affinity (5, 49, 50). Ficer mapping has revealed that UL9 is able to unwind BoxI, though this action is dependent on the presence of a single-stranded binding protein, an adjacent AT-rich sequence, and a single-strand tailed DNA substrate (32, 41). Further experiments have shown that OriL contains a hairpin in vitro, and it has been hypothesized that this structure is an important intermediate during the initiation of lytic replication (3–5, 48).

EBV does not encode an ATP-dependent helicase similar to UL9 that interacts with OriLyt. However, it is possible that Zta, which binds to OriLyt, may provide some of the functions that UL9 contributes to the initiation of DNA replication. To investigate this, we have explored the possibility that an imperfect inverted repeat containing the ZRE1 and ZRE2 binding sites in the upstream essential element (UEE) of OriLyt forms a structure similar to the DNA hairpin structure described at HSV OriL, formed by the UL9 binding sites Box III and BoxI (65, 66). We present evidence that ZRE1 and ZRE2 in the UEE can form a stable imperfect hairpin structure that is preferentially bound by Zta. We provide structural and genetic evidence that a DNA hairpin forms at the UEE during lytic replication and that Zta binding to this hairpin is important for the lytic-replication function.

MATERIALS AND METHODS

Prediction of DNA secondary structure. The minimum free energies and predicted secondary structures of DNA sequences were analyzed using the Vienna RNA website program RNAfold (http://rna.alive.univie.ac.at/cgi-bin/ RNAfold.cgi) with DNA parameters (29, 61).

EMSA.s. Electromobility shift assays (EMSA.s) were performed as previously described (74). Briefly, wild-type and mutant Zta proteins were expressed and purified from Escherichia coli as hexahistidine fusion proteins. Purified Zta proteins (25 nM) were then incubated with single- or double-stranded, methylated or unmethylated DNA oligonucleotides (1.3 nM) synthesized by Integrated DNA Technologies (IDT). DNA probes were radiolabeled with [γ−32P]ATP using polynucleotide kinase. Each EMSA was repeated at least three times to ensure reproducibility, and the results of a representative experiment are shown in Fig. 1, 2, 3, 4, and 6. Protein binding was quantified with ImageQuant TL software (version 2005; Amersham) using the following equation: percentage bound = (bound signal)/total lane signal. When more than one protein concentration was used, the result for each lane was calculated independently, and the quantification from a single concentration point taken from the linear part of the signal curve is given.

Plasmids and cell lines. Full-length BZLF1 genes were cloned into the BamHI site of a pQE8 (Qiagen) bacterial expression vector. Full-length BZLF1 and BRLF1 genes were cloned into the EcoRI-SalI sites of the p3×FLAG-myc-CMV24 vector (Sigma) for mammalian cell expression. Mutations in Zta were generated by PCR mutagenesis of both pQE8-Zta and p3×FLAG-myc-CMV24-Zta using the QuickChange site-directed mutagenesis kit (Stratagene). ZKO-293 generated by PCR mutagenesis of both pQE8-Zta and p3×FLAG-myc-CMV24 vector (Sigma) for mammalian cell expression. Mutations in Zta were described (74). Briefly, wild-type and mutant Zta proteins were expressed and purified from E. coli and used in EMSA. The minimum free energies and predicted secondary structures of DNA sequences were analyzed using the Vienna RNA website program RNAfold (http://rna.alive.univie.ac.at/cgi-bin/ RNAfold.cgi) with DNA parameters (29, 61).

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Southern blotting (71). The DNA was then detected by hybridization with a digoxigenin-labeled probe specific for OriLyt or other regions of the EBV genome by using digoxigenin EasyHyb reagents (Roche) according to the manufacturer’s instructions.

EBV replication assay. Transient plasmid transfection of ZKO-293 cells was carried out using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. A total of 5 μg ZTKO-293 cells were transfected with Zta, Rta, or vector control plasmids. At 48 h posttransfection, cells were washed once with PBS and were lysed with 1 ml lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris [pH 8]) for 10 min on ice. Lysates were sonicated on ice using a Bioruptor (Diagenode) on the high setting with 30-s on/off pulses for a total of 25 min until the DNA was reduced to an average length of 200 to 600 bp. Lysates were analyzed by Western blotting with an antibody against the early antigen diffuse component (EA-D). Lysates were then diluted 1:10 in dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris [pH 8], 167 mM NaCl) and treated with 120 μg/ml protease K for 2 h at 50°C, and DNA was phenol-chloroform extracted and ethanol precipitated. The DNA quantity was measured with an Applied Biosystems real-time PCR machine, model 7000, using 6 ng DNA and 1.5 mM primers in 12 μl of 1× SYBR Green master mix solution (Roche). The primers used were GCCGCGTCACCGAATGC (BFR1-fwd), GGAAGCTGTGGTCCTAACCTC (BFR1-rev), GCATGTTTGCCCTACCA (GAPDH-fwd), and GGCCAGGTCTCTTTTTATTTCGT (GAPDH-rev).

RESULTS

Prediction of a DNA hairpin in the OriLyt UEE. A common feature of herpesvirus lytic origins is the presence of inverted-repeat DNA sequences which serve as binding sites for viral, and possibly cellular, lytic-replication initiator proteins (reviewed in reference 58). In HSV, an inverted repeat within the essential part of the lytic origin (OriL) forms a DNA hairpin in vitro, which is bound by the initiator origin binding protein (OBP), encoded by the UL9 gene (3–5, 48). We were interested in examining the essential elements of the Epstein-Barr virus origin of lytic replication (OriLyt), previously identified (65, 66), for candidate sequences that may form DNA hairpins due to intrastrand base pairing. Several Zta response elements (ZREs) have been mapped (46), including two adjacent sites, ZRE1 and ZRE2 (ZRE1/2), located within the critical upstream essential element (UEE) (Fig. 1A). We examined the
nucleotide composition of this sequence using the Vienna RNA website program RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) with DNA parameters, and we found that the top strand, but not the bottom strand, of the OriLyt ZRE1/2 sequence was predicted to form an imperfect DNA hairpin due to intrastrand base pairing (Fig. 1B). The minimal free energy ($\Delta G$) of the top-strand hairpin is predicted to be $-1.10$ kcal/mol, compared to $-0.2$ kcal/mol for the bottom strand. Based on this information, we hypothesized that DNA secondary-structure formation within the OriLyt UEE may contribute to origin function during lytic replication.

**Zta binds specifically to the top strand of the OriLyt UEE.**

The lytic origin binding protein for HSV, UL9 (also known as OBP), has been shown to have single-stranded DNA binding capability (5). Given the functional homology between UL9 and the EBV lytic origin binding protein Zta (22), we decided to investigate whether or not Zta could bind preferentially to the top strand of the EBV OriLyt UEE, which was predicted to form a hairpin (Fig. 1B). To test Zta binding, we used an electromobility shift assay (EMSA) (Fig. 1C) employing radio-labeled single-stranded oligonucleotides taken from the OriLyt ZRE1/2 sequence or from other known ZREs from different parts of the EBV genome (Fig. 1D). We found that Zta specifically binds to the top (+) strand of OriLyt ZRE1/2 but not to single-stranded DNA sequences from the BMRF1 or BRLF1 promoter, each of which contains only a single ZRE. Zta does bind to the bottom (−) strand of OriLyt ZRE1/2, but to a much lesser extent (20% binding) than to the top strand (100% binding). This is likely due to low-level intrastrand base pairing in the bottom strand, which may be further stabilized by Zta protein. From these data we hypothesized that in order for Zta to bind a single strand of DNA, two ZREs within the sequence were required, and their sequence complementarity was important for Zta binding.

**Zta binding to the top strand of the OriLyt UEE is dependent on a ZRE1/2 hairpin.** To investigate the effect of hairpin
formation on Zta binding, we engineered mutations within the ZRE1/2 imperfect hairpin that would increase the intrastrand complementarity of the nucleotide sequence (Fig. 2A and B). We found that alteration of the sequence to ZRE1/1 (probe 3; predicted ΔG_{H9004}G, 10.9 kcal/mol) or ZRE2/2 (probe 2; predicted ΔG_{H9004}G, 8.7 kcal/mol) perfect hairpins led to a decrease in observed Zta binding from that with the wild type ZRE1/2 sequence (probe 1; predicted ΔG_{H9004}G, 1.10 kcal/mol) (Fig. 2A). This suggests that Zta preferentially binds to an imperfect hairpin sequence rather than to a perfect DNA duplex, which mimics double-stranded DNA. Zta binding was specific for a ZRE/ZRE hairpin, as evidenced by the fact that Zta did not bind to a hairpin that lacked Zta recognition sites (probe 4; predicted ΔG_{H9004}G, 16.6 kcal/mol). Zta also did not bind to a wild-type ZRE1/2 sequence split between two DNA oligonucleotides (probe 5), suggesting that the hairpin structure contributes to DNA binding affinity.

To further investigate the contributions of the ZRE1/2 hairpin to Zta binding, we engineered mutations across the wild-type ZRE1/2 sequence (Fig. 2C and D). Mutations predicted to disrupt hairpin formation (mutants 2 [predicted ΔG_{H9004}G, 0.00 kcal/mol] and 5 [predicted ΔG_{H9004}G, 0.00 kcal/mol]) and/or ZRE recognition (mutants 2, 6, and 7) significantly impair Zta hairpin binding, while mutations in the intervening sequence (mutants 3 and 4) do not have as great an effect (Fig. 2C). In addition, Zta does not bind to a nonspecific hairpin DNA, as evidenced by the fact that it does not bind to the scrambled sequence (mutant 8) that can form a hairpin but lacks Zta binding sites.

The data from Fig. 2A suggest that Zta preferentially binds to an imperfect hairpin sequence. To further test this hypothesis, we designed a competition assay in which a perfectly complementary, double-stranded ZRE sequence from the EBV R promoter (Rp) (dsZRE2) was radiolabeled and prebound to Zta protein, followed by incubation with an unlabeled (cold) competitor. The competitors used were either the top strand of OriLyt ZRE1/2 alone [ssZRE1/2(\text{top})], the bottom strand alone [ssZRE1/2(\text{bottom})], or a duplex consisting of both the top and bottom strands (dsZRE1/2). While each cold competitor was able to compete off some Zta, the most effective competitor contained the imperfect hairpin (Fig. 3A and B). As expected, the bottom strand was the least efficient competitor. This indicates that Zta preferentially binds to single-stranded ZRE1/2(\text{top}) relative to double-stranded ZRE1/2 or to double-stranded Rp ZRE2 probe DNA.

To see if Zta could bind to two noncomplementary ZREs on a single DNA strand, we then changed the ZRE2 sequence into an AP1 site, which Zta is known to bind in the double-stranded form, by making a three-nucleotide TGC-to-GAG change (Fig. 4A). Zta was unable to bind this ZRE1/AP1 mutant, which is incapable of forming a hairpin (predicted ΔG_{H9004}G, 4.50 kcal/mol) (Fig. 4B). Zta was, however, able to bind to an AP1/AP1 rescue hairpin (predicted ΔG_{H9004}G, –4.50 kcal/mol) engineered by subsequent mutation of the ZRE1 site to a com-

![FIG. 2. Zta binds to an OriLyt single-stranded DNA hairpin. (A) Zta was assayed for its ability to bind oligonucleotide substrates with different capacities to form stable hairpins. Oligonucleotide probes 2 and 3 improve the complementarity of the ZRE sites. Probe 5 contains the wild-type sequence but is split into two oligonucleotide molecules that were annealed prior to the EMSA binding reaction. (B) Oligonucleotide probe sequences. (C and D) Zta was assayed for its ability to bind wild-type (probe 1) or mutant (probes 2 to 7) OriLyt hairpin sequences. Mutations 2 and 5 disrupt the hairpin, and mutations 2, 6, and 7 disrupt ZRE recognition. Mutations 3 and 4 alter mostly intervening sequence between the inverted repeats. Mutation 8 is a scrambled sequence that is capable of forming a hairpin but lacks a consensus ZRE. The percentage of each probe bound to Zta is indicated beneath the corresponding lane. The asterisk indicates a nonspecific band. These data are representative of three independent experiments.](http://jvi.asm.org/ on October 2, 2017 by guest)
plementary API1 site. These data suggest that Zta binding to the top strand of the OriLyt UEE involves the formation of a partially complementary duplex ZRE recognition site, consistent with the data from Fig. 2 and 3, where the observed Zta binding was greater when the complementation was imperfect.

Nuclease sensitivity of OriLyt during lytic replication. Computational and in vitro biochemical experiments support the notion that the OriLyt UEE forms a top-strand DNA hairpin that can be specifically recognized by Zta. To investigate the possibility that a hairpin structure forms at the UEE during cell infection, we assayed EBV-positive cells for the formation of nuclease-sensitive structures at OriLyt during lytic replication (Fig. 5). EBV-positive B95-8 cells were treated with sodium butyrate (NaB) and 12-O-tetradecanoyl phorbol-13-acetate (TPA) for 48 h to stimulate lytic-cycle replication, which was monitored by Western blotting for expression of the EBV early gene product EA-D (Fig. 5B). DNA from latent and lytic cells was isolated rapidly, digested with restriction enzymes, and then enriched for DNA replication intermediates by purification over benzoylated napthoylated DEAE (BND)-cellulose (Fig. 5B). Caffeine-eluted, BND-cellulose-purified DNA was then treated with increasing concentrations of S1 nuclease, which has specificity for single-stranded and nicked DNA. DNA was then assayed by Southern blotting with probes specific for the OriLyt UEE or the OriLyt downstream essential element (DEE) (Fig. 5B). We found that the region covering the UEE was more sensitive to S1 nuclease digestion than the region covering the DEE. S1 sensitivity was not detected in DNA derived from latently infected cells (Fig. 5C, left lanes) but was readily detected in DNA derived from lytically induced cells (Fig. 5C, right lanes). This suggests that S1-sensitive DNA structures form during lytic replication. In a second experiment, we compared the UEE to several other regions of the EBV genomes, including DNA fragments similar in size to OriLyt (Fig. 5D). We found that the UEE was selectively sensitive to S1 nuclease treatment, while DNAs from regions outside OriLyt, including the BMRF2, BXRF1, and BALF2 open reading frames, were relatively insensitive to S1 nuclease. Because the OriLyt UEE contains a promoter, we wanted to rule out the possibility that S1 sensitivity was simply the result of BHLF1 promoter activity. We probed the same Southern blot with a probe for another EBV promoter region covering the BRLF1 promoter (BRLF1p), which is also active during lytic replication. Unlike the OriLyt UEE region, the BRLF1p region was insensitive to S1 in our assay (Fig. 5D, top). These findings suggest that the OriLyt UEE forms a specific S1 nuclease-sensitive DNA structure during lytic replication in living cells.

FIG. 3. The OriLyt ZRE1/2 top strand is able to competitively bind Zta. (A) Double-stranded DNA containing the ZRE2 sequence from the EBV R promoter (dsZRE2) was radiolabeled and incubated with or without Zta in the presence of cold competitor double-stranded ZRE1/2 (dsZRE1/2) or single-stranded ZRE1/2 from the top [ssZRE1/2(+)] or bottom [ssZRE1/2(−)] of OriLyt. (B) The percentage of binding of Zta to the radiolabeled dsDNA ZRE2 was quantified for each lane.

FIG. 4. Zta binding to the ZRE1/2 top strand is dependent on hairpin formation. (A and B) The ZRE1/2 OriLyt sequence was changed to either a ZRE1/API1 or an API1/API1 sequence and was assayed for Zta binding via EMSA. Mutation of the ZRE2 site to an API1 sequence disrupts hairpin formation, while the API1/API1 rescue is predicted to restore hairpin formation. Zta protein was added at 3-fold increasing concentrations ranging from 15 to 135 nM. The percentage of each probe bound to Zta is given beneath the corresponding lane. The asterisk indicates a nonspecific band. These data are representative of three independent experiments.
Zta mutants deficient for OriLyt hairpin binding are also replication incompetent. To determine whether hairpin binding by Zta correlated with lytic-replication function, we assayed a series of Zta mutants that were known to have defects in DNA replication but no apparent defects in binding to double-stranded ZRE DNA. Wild-type and mutant Zta proteins with single amino acid changes within the basic region of the protein (Fig. 6A) were expressed in and purified from E. coli and were assayed by EMSAs for DNA binding properties. We found that several of these Zta mutants exhibited reduced binding to the ZRE1/2 hairpin compared to that of wild-type Zta (Fig. 6A and B, lanes 1 to 7). These include the C171S, Y180A, S186A, C189A, and V184A S186A R187A (VASR) mutants. Importantly, these mutations did not disrupt Zta binding to a control double-stranded ZRE2 DNA probe from the EBV Rp (Fig. 6A and B, lanes 8 to 14). The VASR mutant was significantly less efficient at DNA binding than the other mutants for all of the probes tested. The same Zta mutants were cloned into a mammalian expression vector, transfected into 293-ZKO cells, and assayed for their abilities to induce lytic replication (Fig. 6C). These findings indicate that Zta mutants that are compromised for binding to the ZRE1/2 hairpin are also compromised for stimulating DNA replication.

Zta has been shown to bind preferentially to DNA containing methylated cytosine, and Zta amino acid residues S186 and C189 have been implicated in this recognition (7, 8, 17, 37). The binding of Zta to methylated DNA is thought to be critical for its ability to activate lytic reactivation, but recognition of alternative DNA conformations, such as hairpin DNA, was not directly compared in these earlier studies. We therefore assayed the effects of these Zta mutations on binding to the methylated ZRE3 from Rp compared to binding to the ZRE1/2 hairpin. We found that most Zta mutants that were defective for ZRE1/2 hairpin binding were similarly defective for binding to methylated ZRE3 from Rp (Fig. 6A and B). To rule out the possibility that the replication deficiency of Zta mutants was an indirect consequence of an inability to activate Rta expression, which is also essential for viral DNA replication, we cotransfected an Rta expression plasmid in the replication experiments to rescue any Rta-specific deficiencies (Fig. 6C and D). Addition of ectopic Rta stimulated some viral early genes (e.g., EA-D and BALF2) (Fig. 6D), indicating that it compensates for some defects in Zta transcription activation. However, ectopic Rta expression did not rescue DNA replication from any of the Zta mutants (Fig. 6C). These findings indicate (i) that Rta cannot compensate for the replication deficiency of Zta mutants that fail to bind efficiently to the ZRE1/2 hairpin and (ii) that the amino acids used by Zta to recognize the ZRE1/2 hairpin are similar to those used to recognize methylated DNA.

**DISCUSSION**

In this work, we explore whether the EBV-encoded immediate-early protein Zta shares properties with origin binding factors of other *Herpesviridae* family members. We show that a region of the EBV OriLyt contains an inverted repeat of ZRE1 and ZRE2 that is predicted to form a stable hairpin structure on the top strand. We then show that the top strand of the UEE is a relatively high affinity DNA binding substrate for purified Zta protein *in vitro* (Fig. 1 to 3). The requirement for Zta binding sites and hairpin DNA was demonstrated by mutagenesis studies of the UEE top-strand DNA *in vitro* (Fig. 4). Evidence for the *in vivo* formation of a DNA hairpin structure was provided by S1 nuclease sensitivity assays on DNA replication intermediates isolated from lytic B95-8 cells (Fig. 5). Finally, the functional significance of hairpin binding by Zta was provided by a strong genetic correlation between Zta hairpin binding and replication activity (Fig. 6). Taking these findings together, we conclude that the UEE of OriLyt is capable of forming a DNA hairpin on the top strand that is bound preferentially by Zta, and that Zta binding to this hairpin is important for EBV lytic replication.

A number of studies have shown that the HSV origin binding protein UL9 is able to bind a single-stranded DNA hairpin formed in the HSV lytic origin (3, 5). Although UL9 and Zta are structurally unrelated, they may be considered functional homologues. Each is able to recognize its own virus’s lytic origin (19, 46, 56), and each confers origin-binding specificity on the conserved, core herpesvirus replication machinery (27, 53).
We also noted some sequence homology between the Zta basic domain (Fig. 6A) and the UL9 helicase Ia motif, known to bind single-stranded DNA (53, 58). Furthermore, both Zta and UL9 are known to interact with single-stranded DNA binding proteins (ssBP). UL9, a superfamily II ATP-dependent helicase, cooperates with the HSV-encoded ssBP, ICP8, to accomplish strand separation and unwinding (30, 38, 49–51). Unlike UL9, Zta does not appear to be a helicase and has no known enzymatic activity; however, it is able to bind and recruit the cellular RecQL1 protein (also a superfamily II helicase) to OriLyt (76). Zta also brings the cellular mitochondrial ssBP to OriLyt (80), as well as members of the EBV core replication machinery, which include the ssBP BALF2 (27, 43, 44, 84). These similarities between UL9 and Zta support the model that Zta may contribute to the formation of DNA structural changes at OriLyt.

Zta, like UL9, is able to bind specifically to a single-stranded imperfect DNA hairpin formed within the top strand of a critical element within the lytic origin (Fig. 1). In EBV, this cis-acting component of OriLyt, the UEE, contains two adjacent, inverted Zta response elements (ZRE1/2) (65, 66) reminiscent of the BoxIII/BoxI hairpin formed in HSV OriS (39, 43, 47, 84).
The Zta protein promotes an open conformation at the UEE by stabilizing the intrastrand base pairing in the top strand of the OriLyt ZRE1/2 hairpin (Fig. 1). This may facilitate single-strand DNA binding and protein interactions with the bottom strand of the OriLyt UEE and may potentially promote DNA access to the viral core replication machinery (Fig. 7B). This model is supported by the increase in the S1 nuclease sensitivity of the UEE during lytic but not latent EBV replication (Fig. 5).

Evidence for the functional requirement for hairpin binding by Zta was provided by correlation studies with Zta mutants. We showed that Zta mutants defective for binding to OriLyt hairpin DNA were significantly impaired in their ability to support replication (Fig. 6). These mutants were not impaired for binding to double-stranded ZRE DNA probes. Several of these mutants have been characterized previously and are known to have additional defects, which may contribute to their defect in DNA replication. For example, both the C171 and S186 residues are thought to be required for binding to and activation of a subset of early gene promoters (25, 26, 75). This explains why these mutants, which are able to bind the OriLyt hairpin to some extent, are still severely compromised for replication. On the other hand, a mechanism for the importance of the Y180 residue has yet to be described. Our data indicate that even in the presence of Rta and early gene expression, these mutants, including the Y180A mutant, were unable to stimulate DNA replication (Fig. 6C). The correlation between hairpin binding and DNA replication function suggests that Zta hairpin binding is a critical, albeit not a sufficient, component of EBV lytic replication. Certainly, Zta has other functions at OriLyt, and these mutants may help to further separate the many roles of this important protein.

Each of the replication-deficient mutants assayed was also attenuated for binding to methylated Rp-ZRE3 (Fig. 6B). However, the defects in DNA replication were unlikely to be caused exclusively by the failure to bind to methylated DNA, since neither the exogenous expression of Rta nor the addition of 5'-azacytidine (data not shown) was able to rescue the induction of replication by Zta mutants. The relationship between hairpin binding and methylated Rp-ZRE3 binding may be explained by some structural similarities of the two ZRE substrates. The OriLyt imperfect hairpin contains three thymine bases in positions within the ZRE1/2 hairpin that correspond to the methylated cytosines in Rp-ZRE3 (Fig. 7A). We speculate that a combination of DNA mismatching and thymine positioning in the DNA hairpin may mimic cytosine methylation and enhance Zta recognition.

The precise mechanism of herpesvirus lytic-cycle DNA replication is not completely understood. However, the highly conserved nature and interchangeability of the core replication enzymes, such as the DNA polymerases and polymerase processivity factors, suggest that a common mechanism is used for all herpesviruses. Furthermore, all known herpesvirus lytic origins contain inverted-repeat sequences bound by viral or cellular proteins (58). Although the origin DNA sequences and DNA-binding proteins differ among herpesvirus family members, it is possible that a common higher-order structure is formed, which can be recognized by the conserved core replication machinery. Although this idea remains speculative, in this work we provide evidence that a hairpin structure can form at the EBV OriLyt in vitro that may functionally resemble the
hairpin formed at HSV OriS, also identified in vitro. We also provide evidence that this region of OriLyt is nucleosome sensitive during lytic replication and that mutants in Zta that are defective in binding to this inverted repeat are also defective in stimulating DNA replication. Taking these findings together, we conclude that Zta can bind and potentially stabilize a hairpin-like structure at OriLyt that is likely important for the initiation of lytic-cycle DNA replication. Future research, including further genetic experiments, will be required to confirm a role for DNA secondary structure in the lytic origins of herpesviruses and to examine the extent to which the EBV OriLy binding of Zta is functionally related to similar structures located within the essential elements of the lytic origins of other herpesviruses.

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REFERENCES

18. Liao, F. Y., W. D. Hayward. 2001. Interaction with the Epstein-