In Vitro Transcriptional Activation, Dimerization, and DNA-Binding Specificity of the Epstein-Barr Virus Zta Protein

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The Epstein-Barr virus BZLF1 immediate-early gene encodes a transcriptional activator protein, Zta, which acts as a key regulatory switch in the transition between the latent and lytic viral life cycle. In this work, full-length Zta was expressed at high levels in *Escherichia coli* and purified to homogeneity by DNA affinity chromatography. The bacterial protein bound to specific target sequences (Zta response elements) and activated transcription in vitro from an Epstein-Barr virus early target promoter (BHLF1). Zta bound to DNA as a dimer. The formation of a heterodimer with a Zta deletion mutant was detected by gel electrophoresis mobility shift assays. Footprinting analysis on the BHLF1, BZLF1, and simian virus 40 control regions revealed multiple binding sites with no simple consensus sequence. Zta bound upstream from its own promoter at low concentrations, while at high concentrations it bound at the transcription start site, suggesting that it may activate and then autoregulate its own expression. These results demonstrate that Zta is a sequence-specific DNA-binding transcription factor.

Epstein-Barr virus (EBV) is a human herpesvirus which infects B lymphocytes and epithelial cells of the nasopharynx. Infection of B cells typically results in the establishment of a latent multicopy episome, while infection of the epithelium supports lytic-cycle replication (45, 52). Viral gene expression during the latent state is limited to six nuclear antigens (9, 21–24, 28, 32, 49, 50), two membrane proteins (13, 20, 26, 38), and two small polymerase III transcripts (1, 39). Spontaneous activation of the lytic cycle occurs at low frequency in B cells and is characterized by the temporally regulated expression of over 100 open reading frames. The switch to the lytic cycle can be induced by transfection and overexpression of a single viral gene, BZLF1 (2, 6, 7, 58). Treatment of latently infected B cells with pleiotropic signal-transducing chemicals, such as phorbol esters (66), calcium ionophores (10), or sodium butyrate (42), also increases the frequency of lytic-cycle replication. It has been postulated that these chemical inducing agents act by increasing the expression of the BZLF1 gene.

The BZLF1 gene *trans* activates a highly limited and specific set of viral promoters (33, 40). Cotransfection experiments have shown that the BZLF1 gene activates several early lytic-cycle viral promoters in EBV-infected lymphoblastoid cells and in uninfected HeLa cells (5, 6, 19, 33, 40, 43, 55, 58, 61). Dissection of these promoters has revealed a set of Zta response elements (ZREs) which were able to confer Zta inducibility when placed upstream of nonresponsive promoters. The highly responsive BHLF1 promoter, for example, consists of two Zta-responsive regions, a minimal promoter (–7 to –155, referred to as MinL in this study) and a distal enhancer (–650 to –900) which has cell type specificity (40). *Escherichia coli*-derived fusion proteins expressing Zta bind specifically to four sites in the minimal promoter and to two sites in the Zta-dependent enhancer region (41).

The Zta amino acid sequence has significant sequence homology with the basic region/DNA-binding domain of the Jun-Fos family of transcription factors (3, 12, 43). This

observation led to the discovery that the BZLF1-encoded protein binds to AP-1 sites (12). The Zta-binding sites in the BHLF1 promoter appear to be related to but clearly distinct from AP-1-binding sites. However, both AP-1-related and distinct Zta-binding sites placed upstream from nonresponsive promoters conferred Zta inducibility on these targets in cotransfection experiments (41, 61). It is unknown whether there are functional differences between the various DNA-binding sites in the virus, and it remains unclear how flexible the Zta recognition sequence may be.

Zta has been shown to have homology with the basic region of the Jun-Fos family of transcription factors (12), but has not been shown to possess an obvious leucine zipper dimerization domain (35). Recent studies show that dimerization of GCN4, Fos, Jun, and C/EBP is essential for DNA-binding activity (17, 25, 34, 36, 60). Dimerization through the leucine zipper is believed to involve the formation of a coiled-coil structure, which could be satisfied by a 4–3 repeat of hydrophobic amino acid residues (not necessarily leucines), with a precise spatial relationship to the basic domain (47, 62). As we discuss here, the Zta sequence does conform to these more general rules that predict it to be a member of the leucine zipper family of dimeric DNA-binding proteins. However, direct experimental evidence has not yet determined whether Zta binds DNA as a multimer or monomer.

To begin to understand the mechanism of Zta transcriptional activation, we purified Zta protein after expression to high levels in engineered *E. coli* (b-Zta). Purified b-Zta was tested for its capacity to stimulate transcription from the BHLF1 minimal promoter in vitro when added to nuclear extracts prepared from uninfected HeLa cells. Zta protein was also tested for its ability to bind DNA in a dimeric form. To better assess the DNA-binding specificity, we analyzed Zta for its ability to footprint over the simian virus 40 (SV40) promoter-enhancer control region, which is known to contain consensus AP-1 sites. We also tested the ability of Zta to bind to its own promoter (BZLF1), which others have shown to respond positively (autostimulatory) to cotransfection of the BZLF1 gene (61).

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MATERIALS AND METHODS

Plasmids and synthetic oligonucleotides. Plasmids used for the in vitro transcription assay were derived from pAd1BLS-5/+5, which contains an *XhoI* linker insertion at the initiation site of the adenovirus E1B transcription unit (57, 64). Plasmid pPL200 was constructed by the addition of an 8-base-pair (bp) *SmaI* linker into the filled-in *XhoI* site of pAd1BLS-5/+5. pPL204 was constructed by the insertion of a filled-in *BamHI*-*HindIII* fragment of pPL98 (40) (containing BHLF1 sequences -7 to -158) into the filled-in *XhoI*-*HindIII* site of pAd1BLS-5/+5. Plasmid pPL205, used for DNase I footprinting assays, was constructed identically to pPL204, except that it was derived from pPL52 (40) and contains an additional 52 bp of BHLF1 leader sequence from +45 to -158.

Synthetic oligonucleotides designed for gel mobility shift probes were made as complementary 30-mers with *BamHI* and *BglIII* cohesive ends. The ZRE oligonucleotide was derived from the -704 to -684 BHLF1 site described previously as ZRE5 (5'-GATCTTCTAGACCAAATGTG CAAAGGTGAG-3' and its complement 5'-GATCCTCA CCTTTGCACATTTGGTCTAGAAGATC-3'). An oligonucleotide with a mutated ZRE5, designated Δ ZRE5, has the sequence 5'-GATCTTCTAGACCAAATGGTCAAAGGTG AG-3' (41). The control CAAT oligonucleotide used as a competitor in footprinting experiments was derived from the BHLF1 minimal promoter (-77 to -101) containing the sequence 5'-GATCCTGGGGCTTCTTATTGGTTAA TTCA-3'. The oligonucleotide sequence used for construction of the oligonucleotide affinity column was a 12-mer, 5'-GATCTGTGCAAG-3', and its complement, 5'-GATCC TTGCACAG-3'.

Overexpression and purification of b-Zta. A BZLF1 cDNA containing 30 bp of untranslated leader and polylinker sequence derived from pPL102 (41) was cloned as an *EcoRV*-*XbaI* filled-in fragment into the filled-in *BamHI* site of the expression vector pAS1. Vector pAS1 contains the bacteriophage lambda p_L promoter and *cII* protein ribosome-binding site (56). The resultant plasmid, pPL122, expresses the complete BZLF1 cDNA with an additional 11-amino-acid N-terminal fusion, consisting of the following amino acids: MDHRIPLSLLK—. pPL122 was used to transform bacterial strain N5151, containing the temperature-sensitive lambda repressor *cI857*, and overexpression of the b-Zta protein could be observed after 1 h of heat shock at 42°C. Bacterial cell lysates were prepared as described before (51). The high-salt extract was discarded, and the insoluble pellet, which was highly enriched for b-Zta protein, was suspended in 5 M urea-12.5% sucrose-10 mM Tris chloride (pH 7.7)-0.1 mM EDTA-1 mM phenylmethylsulfonyl fluoride. b-Zta was renatured out of urea by the method described for bacterial Sp1 (30). Renatured Zta protein was used directly in DNA-binding assays or applied to a DNA affinity column containing the multimerized oligonucleotide sequence 5'-GATCTGTGCAAG-3', covalently linked to Affiprep 10 activated resin (Bio-Rad Laboratories) (31, 65). Typically, 10 ml of urea-renatured crude Zta (0.5 mg/ml) was mixed with 3 ml of resin and mixed in batch for at least 1 h at 4°C. The resin was washed with at least 10 column volumes of D buffer (25 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-KOH [pH 7.9], 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl and then with 1 column volume of D buffer containing 250 mM KCl. The Zta protein was eluted from the affinity column at an unusually high salt

concentration, 1.5 M KCl, to prevent the otherwise broad elution profile. Eluted protein was analyzed by silver staining of polyacrylamide gels (63) and by immunoblotting with horseradish peroxidase as an indirect color detector (4).

DNA-binding assays. Both DNase I footprinting and gel mobility shift assays used the same reaction conditions for DNA binding. Zta protein was incubated with 10 kcpm of radiolabeled probe (typically 2 to 5 kcpm/fmol) in 10 mM HEPES (pH 7.9)-1 mM EDTA-60 mM KCl-0.05% Nonidet P-40-50 μ g of bovine serum albumin per ml-10 μ g of poly(dIdC):poly(dIdC) (Pharmacia) per ml. Gel mobility shift assays were done in a 12- μ l volume, and footprinting experiments were conducted with fourfold more probe and protein in a 50- μ l reaction volume. In DNase I protection experiments (15), the DNA-protein complex was allowed to form for 30 min at room temperature and then made 10 mM $MgCl_2$, 3.5 mM $CaCl_2$, and 100 ng of DNase I per ml for 15 s. Reactions were stopped by the addition of an equal volume of stop buffer containing 20 mM EDTA, 200 mM NaCl, and 1% sodium dodecyl sulfate (SDS) (29). The quenched reaction mixes were phenol extracted, ethanol precipitated, and denatured in formamide-dye prior to electrophoresis on 8% sequencing gels. Polyacrylamide gels (6%) containing 1 $\times$ TBE buffer (0.089 mM Tris-borate, 0.089 mM sodium borate, 0.002 mM EDTA) were used for gel electrophoresis mobility shift assays (14, 16).

The footprinting probes used for the BHLF1 minimal promoter (MinL) were generated by end-labeling plasmid pPL205 at the *EcoRI* site, either by T4 polynucleotide kinase or the Klenow fragment "fill-in" reaction, and then cut with *HindIII* to generate a 300-bp fragment. The SV40 early regulatory region and the BZLF1 promoter probes were produced from end-labeling the *HindIII* site of plasmid pPL17 (19) and cutting with *PvuII* and *BalI*.

Methylation interference experiments were conducted with 10⁶ cpm of dimethyl sulfate-treated BZLF1 probe and 10 μ l of b-Zta in a preparative gel electrophoresis mobility shift assay. Free and bound species were excised and electroeluted from wet gels into buffer containing 10 mM Tris chloride (pH 7.5) and 1 mM EDTA, and ethanol precipitated with 0.3 M sodium acetate (pH 5.0). The pellets were suspended in 1 M piperidine, heated at 95°C for 15 min, and precipitated with 2.5 volumes of ethanol. Dried samples were resuspended in formamide dye and loaded onto 7% polyacrylamide-8 M urea sequencing gels.

In vitro transcription. Affinity-purified b-Zta protein dialyzed into D buffer with 100 mM KCl was added to a 50- μ l (final volume) transcription reaction mix containing 250 ng of supercoiled template DNA, 1 μ g of pUC18 carrier DNA, 75 μ g of HeLa cell nuclear extract protein (prepared by the method of Dignam et al. [8]), 12 mM HEPES-KOH (pH 7.9), 60 mM KCl, 12% (vol/vol) glycerol, 6 mM $MgCl_2$, 0.6 mM dithiothreitol, 0.5 mM each of four ribonucleoside triphosphates, 5 mM creatine phosphate, and 8.25 μ g of creatinine kinase per ml. Reaction mixes were incubated at 30°C for 90 min. RNA was extracted and quantitated by primer extension analysis as described before (57). The length of the primer extension product from control E1B promoter was 115 nucleotides. The length of the correctly initiating primer extension product from the MinL promoter was 105 nucleotides.

RESULTS

Overexpression and purification of Zta protein from *E. coli*. The Zta protein containing an 11-amino-acid N-terminal

FIG. 1. SDS-polyacrylamide gel analysis of b-Zta purification. (A) The high-salt soluble (10 μ l [10 μ g], lane 1) and insoluble (1 μ l [0.5 μ g], lane 2) fractions were examined on a 12% polyacrylamide-SDS gel and visualized by silver staining. The insoluble protein was solubilized in urea, renatured, and applied to a ZRE affinity column. The 1.5 M eluate (70 μ l) from the affinity column is shown in lane 3. Lane M, high-molecular-mass markers (sizes shown in kilodaltons). (B) Insoluble fraction (0.5 μ l, lane 2) and affinity-purified b-Zta (10 μ l, lane 3) were transferred to nitrocellulose from 12% polyacrylamide-SDS gels, incubated with rabbit antiserum raised against a Zta-specific peptide, and then visualized with horseradish peroxidase-conjugated secondary antibody. (C) Same as panel B except nitrocellulose blots were incubated with antiserum raised against purified b-Zta protein.

fusion (b-Zta) was expressed to very high levels after heat inactivation of the temperature-sensitive lambda repressor $cI857$, which controls expression from this vector. Although soluble extracts with DNA-binding activity could be prepared from high-salt washes of lysed *E. coli* cells, the majority of the protein remained as an insoluble inclusion body. The insoluble pellet from the high-salt wash was solubilized in urea and slowly dialyzed to yield a partially purified, concentrated, and active source of the Zta protein (Fig. 1A, lane 2). Zta protein functional for DNA binding was then purified with an oligonucleotide affinity column. After the column was washed with 0.1 and 0.25 M KCl, elution with 1.5 M KCl yielded highly purified b-Zta protein, which migrated as a 33-kilodalton (kDa) band on a 12% polyacrylamide-SDS gel (Fig. 1A, lane 3). Gel mobility shift assays following each chromatographic step demonstrated that the active material was retained and had DNA-binding specificity for the ZRE5 site (TGTGCAA) but not for an otherwise identical oligonucleotide, Δ ZRE5, containing a double point mutation in the Zta recognition sequence (TGGTCAA), consistent with recent findings (Fig. 2) (41). This polypeptide was shown to be immunologically related to the EBV-encoded Zta protein by immunoblotting with an antiserum raised against a synthetic BZLF1 peptide (anti-pep, Fig. 1B). Antiserum raised against the purified fusion protein (anti-b-Zta) had the same reactivity as the antiserum generated from the synthetic peptide (Fig. 1B and C). We also observed some reactivity with a band migrating at approximately 63 kDa which we believe to be a disulfide-linked dimer, since its appearance was variable and reduced after extensive boiling in β -mercaptoethanol (not shown; see below).

Purified b-Zta protein stimulates transcription in vitro. The affinity-purified Zta protein was tested for its ability to

FIG. 2. Gel electrophoresis mobility shift analysis of DNA affinity chromatography of b-Zta. A 1- μ l amount of urea-renatured insoluble b-Zta (load), the flowthrough (flow), or various KCl concentration elutions (0.1, 0.25, and 1.5 M) were incubated with radiolabeled oligonucleotide probe ZRE5 (a) or Δ ZRE5 (b). All elutions consisted of 1 column volume. Bound (B) and free (F) complexes were resolved in 6% polyacrylamide gels.

stimulate transcription from the BHLF1 minimal promoter of EBV in an in vitro transcription reaction with HeLa cell nuclear extract. The BHLF1 minimal promoter (MinL) responds to the BZLF1 gene product in cotransfection experiments performed in VERO cells (40). The MinL promoter was also shown to contain four Zta-binding sites in earlier studies (41), which was confirmed here with highly purified b-Zta protein (see Fig. 5). Primer extension assays of in vitro transcription reactions are shown in Fig. 3. In the absence of Zta protein, there was a low level of transcription initiating from the nucleotide corresponding to the in vivo-determined cap site of the MinL promoter (27, 37, 40). Addition of 10 μ l of purified b-Zta produced a ninefold increase in the correctly initiated RNA from the MinL promoter, while no increase in transcription was observed with the control adenovirus E1B promoter (Fig. 3A). This stimulation of transcription from the MinL promoter was dose dependent, increasing with the addition of increasing amounts of Zta protein. No increase in E1B transcription was detected at any concentration of Zta (Fig. 3B). These results demonstrate that Zta specifically stimulates transcription from a promoter to which it binds.

Zta binds DNA as a dimer. We used the method introduced by Hope and Struhl (25) to test whether the Zta protein bound to its DNA recognition site in an oligomerized form. A deleted form of Zta was made by removing a *Hind*III fragment encoding 62 amino acids from the amino-terminal third of the protein (Δ Zta25/87) and tested for its ability to form heterodimers in gel mobility shift assays with the wild-type 33-kDa protein. When the wild-type b-Zta protein was incubated with the ZRE5 oligonucleotide probe, it produced a slower-migrating species (bands AA) than did the Δ Zta25/87 deletion mutant (bands BB) in a mobility shift assay (Fig. 4, lanes 1 and 2, 5 and 6). When the two proteins were incubated together at 22°C for 1 h, only two bands corresponding to the separate proteins (AA and BB) were observed (Fig. 3, lane 3). However, when the two proteins were incubated at 37°C for 1 h or renatured together out of 5 M urea, a third intermediate band (AB) was observed (Fig. 3, lanes 4, 7, and 8). We interpret this intermediate band to correspond to a heterodimer formed between the wild type b-Zta (A) and the smaller Δ Zta25/87 deletion mutant (B).

Zta binds to consensus AP-1 sites in the SV40 enhancer and

FIG. 3. Primer extension analysis of in vitro-transcribed RNA. (A) RNA synthesized in vitro with HeLa cell nuclear extract and pPL204 (MinL) or pPL200 (E1B) was analyzed by primer extension analysis. Reactions were supplemented with 10 μ l of affinity-purified b-Zta (+) or with 10 μ l of buffer (–). Dideoxy sequencing markers of the MinL template (pPL204) orient the BHLF1 GATAA at –30 and the initiation site of MinL transcription at +1. (B) Same as in panel A except that equimolar amounts of templates pPL200 (E1B) and pPL204 (MinL) were added to the same reaction mixture. Increasing amounts of affinity-purified b-Zta were added as indicated above each lane (in microliters). The E1B- and MinL-specific transcriptional initiation sites are indicated to the right. The major unmarked higher-molecular-weight extension products were specific for the E1B template.

to AGCAAT-like motifs in its own BZLF1 promoter. The Zta protein has been shown to bind to a series of degenerate sites in the BHLF1 promoter by DNase I protection assays (TGTGCAA, TGTGTAA, TGAGCAA, and TGTGTCA) (41) (Fig. 5). In addition, Zta protein was shown to bind to the collagenase TRE (TGAGTCA) in mobility shift assays (12, 41). To examine Zta binding to the prototype AP-1 site, we footprinted b-Zta on the SV40 enhancer-promoter. We found that b-Zta protected the consensus AP-1 sites positioned by standard SV40 map units (59) at 35 to 29 (TGACTAA) and

FIG. 4. Electrophoresis mobility shift assay of heterodimer formation. Radiolabeled ZRE5 oligonucleotide was incubated with 1 μ l of wild-type b-Zta (lanes 1 and 5), 1 μ l of deletion mutation Δ Zta25/87 (lanes 2 and 6), 0.5 μ l of both b-Zta and Δ Zta25/87 (lanes 3 and 7), or 1 μ l of a 1:1 mixture of wild-type b-Zta and Δ Zta25/87 renatured out of 5 M urea (lanes 4 and 8). Lanes 1 to 4 were incubated at 22°C for 1 h, and lanes 5 to 8 were incubated at 37°C for 1 h. Homodimers AA and BB, shown on the right, correspond to the wild-type and deletion mutation Δ Zta25/87, respectively. Species AB corresponds to the presumed heterodimer species.

FIG. 5. DNase I footprinting of b-Zta over the BHLF1 minimal (MinL) promoter. Radiolabeled probe was incubated with 0, 2, or 5 μ l of urea-renatured b-Zta as indicated above each lane. Footprinted regions are indicated on the left as ZRE1 (–42 to –60), ZRE2 (–60 to –77), ZRE3 (–98 to –115), and ZRE4 (–132 to –148). The first guanosine of the GATAA sequence is at position –30, and the transcription initiation site is indicated on the left by the arrow. Lanes G and A/G are chemical sequencing reaction marker lanes of the coding strand.

114 to 120 (TGACTAA) (Fig. 6A, Tables 1 and 2). In addition, b-Zta partially protected bases 128 to 147 adjacent to the distal AP-1 site. Surprisingly, this partially protected region from 128 to 147 (Tables 1 and 2) had little similarity to the previously observed binding sites. b-Zta also protected equivalent sequences present in the second copy of the 72-bp repeat (sequences 170 to 200 and 200 to 219).

In the BZLF1 promoter, b-Zta protected three major blocks of sequence stretching from +43 to +15 (region I), from +13 to –14 (region II), and from –105 to –138 (region III) (Fig. 6B). In addition, weak protection was observed over the region –57 to –72 containing the putative AP-1 site [(–66)TGACATCA(–59)] (61). The sequences protected at region III could be divided into two partially overlapping subdomains, IIIa and IIIb, which were resolved by their differential affinity for b-Zta during titration and competition

FIG. 6. DNase I footprinting analysis of b-Zta over the SV40 early promoter-enhancer and the BZLF1 promoter. Either 0, 2, or 5 μ l of urea-renatured b-Zta (indicated above each lane) was incubated with radiolabeled fragments containing the SV40 promoter-enhancer (A) or the BZLF1 promoter (B). The SV40 promoter-enhancer fragment contained the sequences 5171 to 270 (standard SV40 map units [59]). The BZLF1 promoter fragment spans the regions +114 to -350, relative to the mRNA transcriptional initiation site (+1), which was set at EBV map unit 103,195 (3, 61). Lanes A/G and G are chemical sequencing marker lanes. Footprinted regions are indicated on the right by brackets. Weakly protected regions are indicated by brackets set farther to the right.

experiments described below (Fig. 7A and B). Since none of these footprinted regions contained obvious recognition sequences similar to those observed previously, we performed methylation interference studies on the coding strand to identify specific nucleotide sequences recognized by Zta (Fig. 7C). This method allowed us to identify three guanine residues in region III which, when methylated, interfered with b-Zta binding in a gel mobility shift assay. Comparison of the bound versus free forms of the BZLF1 promoter probe indicated that methylation of the G -113 residue interfered most dramatically with b-Zta binding. Partial interference was observed with the two G residues at positions -125 and -127 relative to the BZLF1 mRNA initiation site (3, 61).

To compare the affinity of Zta for the multiple recognition sites in the BZLF1 promoter, decreasing amounts of b-Zta

TABLE 1. b-Zta protected sequences in DNase I footprinting analyses^a

Gene	Protected sequences
SV40	(42)CCATGGCTGACTAATTTT(25) (106)ATGGTTGCTGACTAATTGAGATG(128) (147)CAGAAGTATGCAAAAGCATGC(128)
BZLF1	I (+43)CATCTTCAGCAAAAGATAGCAAAGGTGGCC(+15) II (+13)GCAAGGTGCAATGTTTGTGAAGTTAC(-14) III (-105)TACATTAGCAATGCCTGTGGCTCATGCATAGTTT(-138) (-72)AAACCATGACATCACA(-57)
BHLF1	(-42)GGGTCTCTGTGTAATACT(-59) (-77)TCCTGAGCAAACCTTA(-62) (-98)TTCAGGTGTGTCATTTTA(-115) (-132)TAAGGTGTGTCAACAGG(-148) (-698)CCAAATGTGCAAAGGAG(-682) (-859)GGCTTAGTGTGCATGGTG(-840)

^a For SV40, the numbers to the right and left indicate the positions of sequences in the SV40 genome (59). For BZLF1 and BHLF1, the numbers represent the positions relative to the start site of transcription. Underlined bases are candidate recognition sequences (see Table 2).

protein were analyzed in the footprinting assay. When threefold dilutions of the b-Zta protein were made, we found that protection over region IIIa occurred at the lowest amount of 0.074 μ l (Fig. 7A, lane 3). Partial protection of site IIIb occurred at 0.22 μ l (Fig. 7A, lane 4); 0.67 μ l protected site IIIb completely and produced partial protection of sites I and II, which were completely protected with 2 μ l (Fig. 7A, lanes 5 and 6). Competition for b-Zta binding with a 30-bp oligonucleotide containing the BHLF1 ZRE5 (TGTGCAAA) demonstrated that region IIIa required the highest concentrations (200 ng) for the inhibition of DNase I protection (Fig. 7B, lane 2 and 3). This was consistent with the b-Zta footprint titration, which also indicated that site IIIa had the highest affinity for b-Zta.

Since region IIIa contained a putative recognition sequence with some resemblance to a CCAAT box [(-109)TTAGCAATG(-117)], we tested the ability of a control CCAAT oligonucleotide to compete with b-Zta binding to region IIIa. The 30-bp oligonucleotide containing the AACCAAT sequence derived from the BHLF1 promoter (MinL, sequences -84 to -101) did not compete for the b-Zta binding to any of the BZLF1 sites (Fig. 7B, lanes 5 and

TABLE 2. Classification of candidate recognition sequences^a

Group	Recognition sequence	Site
I	(-109)TTAGCAATG(-117) (+29)ATAGCAAAG(+21) (+38)TCAGCAAAG(+30) (+9)GGTGCAATG(+1) (141)TATGCAAAG(133) (-74)TGAGCAAAC(-66) (-693)TGTGCAAAG(-685)	BZLF1 (IIIa) BZLF1 (I) BZLF1 (I) BZLF1 (II) SV40 (octamer) BHLF1 BHLF1
II	(-49)TGTGTAATA(-57) (-104)TGTGTCATT(-112) (-137)TGTGTCACC(-145) (-851)TGTGTCATG(-843) (-120)TGTGGCTCA(-128)	BHLF1 BHLF1 BHLF1 BHLF1 BZLF1 (IIIb)
III	(35)TGACTAATT(27) (114)TGACTAATT(122) (-66)TGACATCAC(-58) (-8)TCACTAAAC(+1)	SV40 (AP-1) SV40 (AP-1) BZLF1 BZLF1 (II)

^a See Table 1, footnote a.

FIG. 7. Titration of b-Zta binding, specific oligonucleotide competition of DNase I footprinting, and methylation interference over the BZLF1 promoter. (A) Titration of b-Zta binding over the BZLF1 promoter. The BZLF1 promoter was incubated with 0, 0.02, 0.07, 0.22, 0.67, or 2 μ l of urea-renatured b-Zta (lanes 1 to 6, respectively). Footprinted regions I, II, IIIa, and IIIb are indicated on the right, with the boundaries of the protected region given as units with respect to the BZLF1 mRNA start site (3, 61). The transcription initiation site is marked by an arrow at position +1. (B) Oligonucleotide competitor challenge to b-Zta binding over the BZLF1 promoter. The BZLF1 promoter probe was incubated with 0 (lane 1) or 2 (lanes 2 to 7) μ l of b-Zta. Oligonucleotide competitor ZRE5 (lanes 2, 3, 4) or CAAT (lanes 5, 6, 7) was included in the binding reaction mix at 0 (lanes 2 and 5), 20 (lanes 3 and 6), or 200 (lanes 4 and 7) ng, as indicated above each lane. Chemical sequencing markers appear in lanes labeled A and A/G. (C) Methylation interference of b-Zta binding to the BZLF1 promoter. Bound (B) and free (F) methylated BZLF1 probes were purified from gel electrophoresis mobility shift assay, cleaved with 1 M piperidine, and analyzed by electrophoresis on 7% sequencing gels. Chemical sequencing lanes G and A/G are indicated. Strong interference by methylated G residue -113 is indicated by open circles (○), and weak interference by residues -125 and -127 is indicated by solid circles (●). The BZLF1 nucleotide sequence interfering with binding is written on the right and corresponds to the center of regions IIIa and IIIb in panel A.

6). The control AACCAAT oligonucleotide, which did not bind to b-Zta in gel mobility shift assays or DNase footprinting (Fig. 5), did bind avidly to a cellular factor present in HeLa and Raji lymphoblastoid cells (41).

Table 1 shows a list of sequences protected from DNase I by b-Zta. In Table 2, 9-bp candidate recognition sequences from protected regions were selected based on previous studies with mutant oligonucleotides (41), methylation interference (Fig. 7C), and visual comparison. As shown, they can be arranged into three groups of related sequences, but only a very degenerate consensus sequence fits all three groups.

DISCUSSION

This study demonstrates that Zta protein directly stimulates transcription in vitro from a promoter to which it binds. Purified Zta protein bound to and increased transcription ninefold from the BHLF1 minimal promoter (MinL) when

added to HeLa cell nuclear extracts. Earlier results have shown that the Zta gene, BZLF1, stimulates BHLF1 transcription when the two genes are cotransfected into HeLa cells (5, 6, 40, 41, 58). The results presented here indicate that Zta acts as a typical transcription factor that binds directly to specific sequences upstream of responsive promoters and stimulates transcription through one or more activation domains. The ability to obtain large amounts of functional Zta from engineered *E. coli*, as described here, should facilitate future investigations of the mechanism by which this factor stimulates RNA polymerase II transcription.

Evidence presented here shows that the Zta protein binds to its DNA recognition site as a dimer. This is analogous to the leucine zipper class of transcription factors, including GCN4, C/EBP, Jun, and Fos (17, 25, 34-36, 47, 60). A homology has been pointed out (12) between Zta and the basic region of these leucine zipper transcription factors,

Consensus:	
Zipper	-BB-BN--AA-B-R-BB-----L-----L-----L-----L-----L-
Coiled-coil	-BB-BN--AA-B-R-BB-----X---X---X---X---X---X---X---X---X-
Sequence:	
C/EBP	288- RR-RN--AV-K-R-KA-----T---V---L---N---L---V---L---L---L-
Jun	267- KR-RN--AA-K-R-RK-----L---V---L---N---L---A---L---V---L-
Fos	143- RR-RN--AA-K-R-RR-----L---T---L---K---L---I---L---K---L-
GCN4	231- KR-RN--AA-R-R-RK-----L---V---L---N---L---V---L---V---R-
Zta	177- KR-KN--AS-K-R-KF-----Y---A---K---N---L---L---M---L---D-

FIG. 8. Amino acid consensus sequence alignment of leucine zipper model (35, 62) and coiled-coil structure (47). The 4-3 repeat of hydrophobic residues is indicated by an X in the coiled-coil model, and B represents either basic amino acid K or R. Comparison of the C/EBP, Jun, Fos, GCN4, and Zta sequences demonstrates that both proteins conform to the coiled-coil structural constraints (47, 62). The position of the first residue of the basic domain of each protein is indicated by the number to the left of the sequence. The single-letter amino acid code is used to denote specific amino acid residues. Dashes represent unspecified amino acid residues.

which is postulated to interact with DNA (62). However, Zta does not contain a region with leucines at every seventh position following the basic region, as found in the leucine zipper family of factors. This leucine repeat region is required for dimerization of these proteins (17, 34-36, 47, 60), and there is strong experimental evidence that the region conforms to the more general structure of a coiled coil (47). As discussed by O'Shea et al. (47), the more general requirement of a protein sequence for the formation of a coiled coil is the presence of a 4-3 repeat of hydrophobic residues. Figure 8 shows that while the Zta sequence does not contain the repeating leucines characteristic of leucine zipper transcription factors, it does contain the 4-3 repeat of hydrophobic residues required for the formation of a coiled-coil region. The single lysine can be considered a hydrophobic residue for this discussion because the $-(CH_2)_4-$ region of the side chain can provide hydrophobic contacts, as proposed for the coiled-coil region of myosin (44). Moreover, the spacing between the 4-3 hydrophobic repeat and the basic region conforms precisely to the spacing in the consensus sequence for leucine zipper transcription factors (61). This spacing is a critical aspect of the current structural model because it determines the alignment between the basic region and DNA (61). Based on these considerations of the Zta sequence and the experimental evidence that Zta binds DNA as a dimer (Fig. 4), it seems very likely that the structures of both the Zta DNA-binding and dimerization domains are analogous to those found in the leucine zipper class of transcription factors.

Zta was found to bind to multiple sites with disparate sequences within the BHLF1 and BZLF1 promoters of EBV and within the SV40 enhancer. Comparison of 13 footprinted regions containing an apparent 16 recognition sequences suggests that Zta does not have a simple consensus binding sequence (Tables 1 and 2). In a previous study, Zta was shown to footprint over six sites in the BHLF1 upstream control region and to bind to an oligonucleotide containing the collagenase AP-1 site but not to several control oligonucleotides when assayed by gel electrophoresis mobility shift (41). In this study, we noted footprints over the BZLF1 promoter protecting sequences with a seemingly unrelated GCAATG-like consensus (group I, Table 2). Despite the similarity of this sequence to a conventional CAAT box promoter element, the Zta protein showed no affinity for the

AACCAAT element in the BHLF1 promoter, nor did it footprint over the CCAAT sequence of the herpes simplex virus type 1 thymidine kinase (TK) promoter (not shown). Thus, Zta does not appear to be a CAAT-box-binding factor. It is interesting that the related C/EBP protein, which was originally described as a CAAT-box-binding protein, had a higher affinity for the GCAAT -82 transversion of the herpes simplex virus type 1 tk CCAAT sequence, even though this mutation did not confer greater transcriptional activity (18). The similarity between this latter GCAAT sequence and the Zta-binding sites (AGCAATG) suggests that C/EBP and Zta may share certain structural features that may be absent from the Jun and Fos proteins.

At present, we do not know whether there is any functional difference between the various Zta-binding sites, since only the collagenase TRE (TGAGTCA), BHLF1 ZRE5 (TGTGCAA), and the BZLF1 (TGAGCCACA) sequences have been shown to confer transcriptional responsiveness as single or multiple Zta-binding sites in promoters of reporter genes in cotransfection experiments (41, 61). It will be interesting to determine whether the other sequences, especially the high-affinity TTAGCAATG recognition site at -109 in BZLF1 and the related weaker site at position 147 to 128 in the SV40 enhancer, are responsive to Zta in vivo. Curiously, the candidate recognition sequence from the SV40 weak-affinity site is the octamer motif (11, 48). The possibility that Zta interacts with cellular AP-1- or octamer-binding sites may have interesting implications for our understanding of host cell responses to EBV infection.

DNase I footprinting analysis of the BZLF1 promoter, which expresses Zta protein, demonstrated a particularly interesting order to the affinity of the several binding sites. Zta bound with highest affinity to the site at -105 to -125, intermediate affinity to the site at -125 to -138, and lowest affinity to the sites at -14 to +13 and +15 to +43. These weaker sites fall directly over the transcriptional initiation site. In the case of herpes simplex virus type 1 IE175 and SV40 T antigen, binding to the cap site results in negative autoregulation of immediate-early gene expression (53, 54). The differential affinities and specific locations of Zta-binding sites on the BZLF1 promoter suggest that complex autoregulation may control BZLF1 gene expression. We hypothesize that the Zta protein autostimulates its own expression at low concentrations where upstream binding is favored, but represses its own transcription at higher concentrations where binding to low-affinity regions covering the mRNA initiation site is favored. In this way, once Zta expression was initiated, it would reinforce its own expression and then maintain a carefully regulated level of Zta protein, which would, in part, govern entry into a lytic replicative cycle.

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