Identification of Acidic and Aromatic Residues in the Zta Activation Domain Essential for Epstein-Barr Virus Reactivation

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Epstein-Barr virus (EBV) lytic cycle transcription and DNA replication require the transcriptional activation function of the viral immediate-early protein Zta. We describe a series of alanine substitution mutations in the Zta activation domain that reveal two functional motifs based on amino acid composition. Alanine substitution of single or paired hydrophobic aromatic amino acid residues resulted in modest transcription activation defects, while combining four substitutions of aromatic residues (F22/F26/W74/F75) led to more severe transcription defects. Substitution of acidic amino acid residue E27, D35, or E54 caused severe transcription defects on most viral promoters. Promoter- and cell-specific defects were observed for some substitution mutants. Aromatic residues were required for Zta interaction with TFIIA-TFIID and the CREB-binding protein (CBP) and for stimulation of CBP histone acetyltransferase activity in vitro. In contrast, acidic amino acid substitution mutants interacted with TFIIA-TFIID and CBP indistinguishably from the wild type. The nuclear domain 10 (ND10) protein SP100 was dispersed by most Zta mutants, but acidic residue mutations led to reduced, while aromatic substitution mutants led to increased SP100 nuclear staining. Acidic residue substitution mutants had more pronounced defects in transcription activation of endogenous viral genes in latently infected cells and for viral replication, as measured by the production of infectious virus. One mutant, K12/F13, was incapable of stimulating EBV lytic replication but had only modest transcription defects. These results indicate that Zta stimulates viral reactivation through two nonredundant structural motifs, one of which interacts with general transcription factors and coactivators, and the other has an essential but as yet not understood function in lytic transcription.

Epstein-Barr virus (EBV) is a human herpesvirus that replicates in the oropharynx and establishes a latent infection in memory B lymphocytes (reviewed in references 3, 26, and 43). Latent EBV infection is associated with several human malignancies, including endemic Burkitt's lymphoma, nasopharyngeal carcinoma, ≈50% of Hodgkin's disease cases, and lymphoproliferative disorders in the immunosuppressed. Lytic replication can be detected in rare opportunistic infections like oral hairy leukoplakia, but is largely restricted in immunologically healthy individuals (20). Infectious virus can be detected in most EBV-positive adults, and it is thought that lytic replication is required for the lifelong persistence of EBV (23). Additionally, high antibody titers to lytic antigens correlate with increase risk of nasopharyngeal carcinoma, suggesting that lytic replication may increase the probability of an EBV-associated malignancy (13).

Lytic replication requires the coordinated expression of two viral immediate-early proteins, Zta (also called BZLF1, ZEBRA, and EB1) and Rta (BRLF1) (16). Zta is a member of the basic leucine zipper (b-zip) family of DNA-binding proteins that stimulates transcription of numerous viral genes essential for lytic replication, as well as several cellular genes of unknown function (9, 12, 15, 33). Zta binds directly to the viral origin of lytic replication and recruits the virally encoded DNA primase and polymerase processivity factors that are essential for DNA replication (18, 33, 44, 45). Virus lacking Zta is incapable of

lytic cycle gene expression or DNA replication, indicating that Zta is essential for virus viability (16).

The Zta transcriptional activation domain has been mapped to the amino-terminal 100 amino acids (11, 17, 30). Replication function is also dependent on the transcription activation domain, and the two activities are thought to be tightly integrated (44). In addition to transcription and replication, Zta can arrest cell cycle progression by a mechanism dependent on the b-zip domain (6, 7). During lytic reactivation, Zta localizes and disrupts PML-associated nuclear domains (ND10/PODs) which are thought to function in viral DNA replication (2, 5). Zta is subject to several posttranslational modifications that regulate its function, including tetradecanoyl phorbol acetate (TPA)-inducible phosphorylation at serine 186, oxidation of cysteine 189, and SUMO-1 modification of lysine 12 (2, 4, 27).

The mechanisms of transcription activation by Zta have been examined in some detail. The amino-terminal transcription activation domain of Zta consists of three functionally redundant modules, but the specific function of each module has not been fully elucidated (11). Zta can stimulate the formation of the TFIIA and TFIID complex on naked DNA templates in vitro, and this activity correlates with transcription activation of a subset of viral promoters (10, 31). Zta binds to general transcription factors TFIIA, TBP, and at least one high-molecular-weight component of the TFIID complex (29, 32). Transcription activation is also stimulated by cotransfection of the CREB-binding protein (CBP) and p300, which function as coactivators for numerous promoter-specific transcription factors (1, 51; reviewed in 19). Zta binds strongly to the cysteine-histidine (C/H)-rich regions 1 and 3 of CBP (51).

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Both the activation domain and the DNA-binding domain of Zta have been implicated in the binding to CBP (1, 51). The interaction between Zta and CBP can potentially stimulate CBP nucleosome-specific histone acetyltransferase (HAT) activity (8). This activity was dependent on the Zta activation and DNA-binding domains and correlated with the ability of Zta to bind small oligonucleosomes (8). In addition to CBP binding, Zta alters the activity of several cellular transcription factors, including p53, NF- κ B, and c-Myb (22, 25, 52).

Transcriptional activation domains have been studied in some detail for several activators (40). Early studies identified amino acid compositions that correlated with transcription activities, including acidic stretches, hydrophobic clusters, glutamine- and proline-rich regions, and leucine motifs involved in coactivator binding (41). However, only a few of these activation regions have been characterized with biophysical techniques, and in these instances the domains were found to lack structure in the absence of a binding partner (28, 50). These results suggest that activation domains are flexible regions that can adapt a structure to accommodate multiple interactions with distinct targets.

To better understand the amino acid motifs and potential structure-function relationship in the Zta activation domain, we have generated a series of alanine substitution mutations throughout the first 90 amino acid residues in Zta. The activation domain of Zta consists of several hydrophobic aromatic amino acid clusters, interspersed with several acidic residues adjacent to glutamine or proline residues. These two classes of amino acid residues were targeted by site-directed mutagenesis and then assayed for transcription functions and biochemical activities that have been established previously for Zta. We found that the Zta activation domain consists of two functionally distinct amino acid compositions that target different cellular factors to regulate transcription in a promoter- and cell type-dependent manner.

MATERIALS AND METHODS

Cells. HeLa, 293, and D98/HR1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco-BRL), glutamine, and penicillin-streptomycin in a 5% CO₂ incubator at 37°C. DG75 and RAJI lymphoblastoid cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics in a 5% CO₂ incubator at 37°C. The 293 EBV-positive BZLF1-knockout cells (ZKO) were maintained in RPMI medium supplemented with 10% fetal bovine serum, 100 μ g hygromycin per ml, glutamine, and antibiotics in a 5% CO₂ incubator at 37°C. ZKO cells carry the gene for green fluorescent protein (GFP) under control of the human cytomegalovirus immediate-early promoter-enhancer (16).

Plasmid and recombinant proteins. The EBV Zta protein was expressed in transient-transfection assays from either ZtaSR α , a simian virus 40-based enhancer system (48), or Zta-pcDNA3, a cytomegalovirus-based promoter system (Invitrogen). The BZLF1 promoter-luciferase construct (ZpLuc) was generated by amplification of BZLF1 -220 to +12 as an *NheI*-*HindIII* fragment in pGL3BASIC (Promega). The BRLF1 promoter-luciferase construct (RpLuc) was generated by amplification of BRLF1 -178 to +28 as an *NheI*-*HindIII* fragment in pGL3BASIC (Promega). The Mp promoter-luciferase construct (MpLuc) was generated by amplification of BMRF1 -300 to +1 as an *NheI*-*HindIII* fragment in pGL3BASIC. Z₇E4TCAT (gift of M. Carey) and BHLF1 CAT have been described previously (34). Zta deletion mutants were generated by PCR mutagenesis and cloned as *EcoRI*-*Bam*HI fragments in pBKSII (Stratagene) for in vitro translation with T3 RNA polymerase. Zta alanine substitution mutants were generated by overlap PCR mutagenesis and cloned in the pSR α 296 vector for mammalian cell expression (48). Glutathione S-transferase (GST)-CBP C/H1 and C/H3 have been described previously (51).

Transfections and reporter assays. HeLa, DG75, D98/HR1, and 293 ZKO cells were transfected using Lipofectamine 2000 (LF2000) reagent (Gibco-BRL) according to the manufacturer's protocol. Briefly, 6×10^5 adherent cells (HeLa, D98/HR1, and 293 ZKO) were seeded in six-well plates 12 to 16 h prior to transfection. For suspension cells (DG75), 8×10^5 cells were passaged into 24-well plates immediately before transfection. Plasmid effector DNA was added at 0.5 to 1 μ g, depending on the experiment, and the BZLF1-Luc, BRLF1-Luc, and BMRF1-Luc reporter plasmids were added to 0.5 μ g. For each well of cells to be transfected, the DNA was diluted into 250 μ l of Opti-Mem I reduced serum medium (Gibco-BRL), and 2 μ l of LF2000 reagent was diluted into 250 μ l of Opti-Mem I medium separately. The diluted DNA was combined with the diluted LF2000 reagent. After incubation at room temperature for 20 min, the DNA-LF2000 reagent complexes were added to each well. Transfected cells were harvested at 48 h posttransfection. Luciferase assays were performed by using the luciferase assay system (Promega). The results of luciferase assays were based on experiments performed at least in triplicate on multiple independent transfections. The error bars show the standard error of the mean for three to nine separate determinations. Expression levels of all activator proteins were monitored by Western blot analysis.

EMSAs. The magnesium-agarose electrophoretic mobility shift assay (EMSA) was described previously (31). Approximately 50 to 150 ng of Zta derivative was incubated with ³²P-labeled Z₅E4T promoter (100 fmol) with 100 ng of GST-CBP(C/H1) or GST-CBP(C/H3) protein. Recombinant TFIIA and affinity-purified HeLa-derived TFIID were generated as previously described (39).

GST interaction assays. GST fusion proteins were purified by glutathione-Sepharose chromatography and dialyzed to remove free glutathione. Purified GST proteins were incubated with ³⁵S-labeled in vitro-translated Zta proteins and then precipitated with glutathione-Sepharose, washed four times, and eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, essentially as described (38). Zta mutants were assayed for interaction with GST-C/H1 and -C/H3 by the Mg-EMSA as described previously (8).

HAT assay. Zta mutants (300 ng) were expressed and purified as hexahistidine amino-terminal fusion proteins from pQE8 as described previously (31). Purified Zta proteins were incubated with His-tagged full-length baculovirus CBP (gift of D. Thanos) and 200 ng of small oligonucleosomes with 0.25 μ Ci of [³H]acetyl coenzyme A (Amersham) in a 30- μ l reaction containing HAT buffer (50 mM Tris [pH 8.0], 5% glycerol, 0.1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM sodium butyrate) at 30°C for 1 h. The reactions were resolved by SDS-15% PAGE. The gel was enhanced using Entensify (NEN) and analyzed by autoradiography.

Western blotting. Cells were harvested, washed once with phosphate-buffered saline (PBS), and lysed in SDS-PAGE loading buffer by boiling for 10 min. Equal amounts of protein in sample buffer were loaded on SDS-8% PAGE gel and separated by gel electrophoresis. The gels were transferred by electroblotting to a nitrocellulose membrane. The primary antibodies used include EBV p52/50 anti-EA-D monoclonal antibody (Advanced Biotechnologies), EBV anti-Rta (Argene), anti-HA (Boehringer), and a rabbit polyclonal antibody raised against Zta. Signals were visualized by enhanced chemiluminescence (Amersham).

Indirect immunofluorescence. D98/HR1 cells were transfected with Zta mutants using LF2000 on coverslips in 12-well plates and fixed in 1% paraformaldehyde at 48 h posttransfection. The fixed cells were permeabilized by incubation in 0.2% Triton (20 min on ice) and incubated with primary antibodies to Zta (rabbit polyclonal 1:1,000 dilution) or monoclonal antibody 1150 specific for the ND10 SP100 protein (1:100 dilution). Mouse antibody was visualized with fluorescein isothiocyanate, and rabbit antibody was visualized with Texas Red, essentially as described previously (5). Cells were then stained for DNA with Hoechst 33258 and mounted with Fluoromount G (Fisher Scientific). Cells were analyzed with a Leitz Fluovert inverted microscope equipped with a digital camera. Images were obtained using software from QED Imaging (Pittsburgh, Pa.).

Infection studies and FACS. Virus production was induced by transfecting 1 μ g of various Zta expression plasmids, including the vector pcDLSR α 296, into 293 ZKO cells. Supernatants were harvested from these cells 48 h posttransfection and passed into six-well plates through 0.8- μ m filter pores (16). About 2×10^5 Raji cells in 200 μ l of complete RPMI medium were added to each well containing the supernatants from different transfections. Approximately 100 ng/ml TPA was also added to each well to help to identify the GFP. The virus titers were determined by analyzing the percentage of green Raji cells by fluorescence-activated cell sorter (FACS) analysis 4 days after infection. Briefly, cells were collected by centrifugation for 5 min at 2,000 rpm and washed once with cold $1 \times$ PBS. About 5×10^5 cells were then resuspended in 0.5 ml of PBS for FACS analysis using an EPICS XL flow cytometer (Coulter Corporation, Hi-leah, Fla.).

RESULTS

Promoter-specific defects of Zta alanine substitution mutants. A series of alanine substitution mutations in the Zta amino-terminal activation domain (amino acids 1 to 90) were generated by site-directed mutagenesis and assayed for transcription activation function on a series of Zta-responsive reporter genes by transient-transfection assay in HeLa cells. A highly sensitive synthetic reporter construct containing seven Zta binding sites upstream of the adenovirus E4 TATA box region (Z₇E4TCAT) was assayed. Alanine substitutions at amino acids D27/Q28, Q34/D35, and P53/E54 caused the most pronounced transcription defects (Fig. 1A). These amino acids are notable for containing a single acidic residue in a hydrophobic patch. Alanine substitution of Y33/Q34 had no effect on transcription, indicating that the glutamine at position 34 was not as important as the aspartic acid at position 35.

The same set of alanine substitution mutations were assayed for transcription activation of the EBV BHLF1 promoter. The Zta binding sites in the BHLF1 promoter are an essential component of OriLyt and direct transcription of a repetitive transcript that encodes a protein of unknown function. Alanine substitution mutations at K12/F13, F22/F26, L48/W49, and W74/F75 caused defects most notably in activation of BHLF1-CAT (Fig. 1B). Single amino acid substitutions at positions W74 and F26 had less dramatic effects on transcription, and the combination F22/F26/W74/F75 caused the most extreme defect in transcription. These results suggest that hydrophobic aromatic amino acid residues function cooperatively to stimulate transcription from BHLF1. Western blotting of Zta protein levels after a typical transfection indicated that most alanine substitution mutants were expressed at similar levels (Fig. 1B).

To further explore the observation that the Zta activation domain had promoter-specific activation functions, we compared several other EBV-derived promoters for their responsiveness to Zta mutants (Fig. 2). The BZLF1 promoter (Zp), the BMRF1 promoter (Mp), and the BRLF1 promoter (Rp) have been shown previously to be Zta responsive in the transient-transfection assay. Transcription activation of Zp was most significantly reduced by alanine substitution mutations in L48/W49 and F22/F26/W74/F75 (Fig. 2A). However, mutations in Q34/D35 and P53/E54 reduced transcription to less than 20% of the wild-type level, indicating that Zp depended on both acidic and hydrophobic residues in the Zta activation domain. Mp and Rp were similar to Zp, although they showed a greater dependence on amino acid residues D27/Q28 and Q34/D35 (Fig. 2B and C). Mp was more sensitive to mutations in W74/F75 than was Zp or Rp. In general, mutations in both acidic and aromatic residues resulted in strong transcription defects. These results suggest that the Zta activation domain consists of two motifs differentiated by the composition of acidic or hydrophobic aromatic amino acid residues. These motifs appear to have promoter-specific activation functions that cooperate for full activation of complex viral promoters such as Zp, Rp, and Mp.

Zta activation domain properties in various cell types. Lytic reactivation by Zta requires transcription stimulation of viral genes embedded in a chromatin-repressed latent viral genome. Transiently transfected reporter plasmids may not recapitulate chromatin structure, and therefore may not reveal all of the

transcription properties required by Zta for reactivation of latent virus. Consequently, we assayed the ability of Zta to stimulate viral gene expression in latently infected D98/HR1 cells.

Zta alanine substitution mutants were transfected into D98/HR1 cells and assayed by Western blotting for the expression of the viral proteins Rta, EA-D, and Zta, encoded by the viral genes BRLF1, BMRF1, and BZLF1, respectively (Fig. 3A). We found that Zta acidic amino acid residue mutants Q34/D35 and P53/E54 were significantly reduced for activation of Rta and EA-D. In contrast, the aromatic residue substitution mutants L48/W49 and F22/F26/W74/F75 were not significantly reduced for Rta and EA-D activation. The acidic residue substitution mutants were expressed at lower levels in D98/HR1 cells, while the aromatic residue substitution mutants were expressed at higher levels, suggesting that these residues may contribute to the stability of Zta in these cell types (Fig. 3A, lower panel).

EBV latently infects lymphoblastoid cells, and it is possible that Zta activation functions may differ in the various cell types that it infects. To determine whether Zta activation domain function was different in B lymphocytes, we assayed Zta substitution mutant transcription activation properties in the EBV-negative lymphoblastoid cell line DG75 (Fig. 3B). We found that the acidic amino acid substitution mutants D27/Q28, Q34/D35, and P53/E54 were significantly reduced for activation of Mp-Luc, similar to what was observed in HeLa cells. We also found that aromatic residue substitution mutants L48/W49 and F22/F26/W74/F75 were also reduced, although not as extensively as was observed in HeLa cells. W74/F75 was significantly more reduced for activation in HeLa cells than in DG75 cells (compare Fig. 2 and 3B). As was found for D98/HR1 cells, acidic residue substitution mutants had substantially reduced protein expression levels, while the aromatic substitution mutants had increased Zta expression levels (Fig. 3B, lower panel). Similar patterns of viral gene activation and Zta mutant stability were observed in EBV-positive Burkitt's lymphoma cell lines Raji and Akata, as well as in EBV-negative Akata cells (data not shown). The decreased stability of acidic residue substitution mutants Q34/D35 and P53/E54 is unlikely to account fully for the reduced transcription activation of these mutants, since similar low expression of wild-type Zta did not correlate with target gene activation levels (Fig. 3B lower panel, and data not shown). These findings suggest that the Zta activation domain may interact with cell-specific factors that regulate Zta transcription activation and Zta protein stability.

Zta aromatic amino acid residues confer CBP binding. We and others have shown that Zta binds the C/H1 and C/H3 domains of CBP (1, 51). To determine what amino acid residues in the Zta activation domain confer this binding, we assayed in vitro-translated Zta proteins for their ability to bind purified GST-C/H1 or GST-C/H3 in vitro (Fig. 4). Full-length Zta bound efficiently to GST-C/H1 and GST-C/H3, but did not bind significantly to GST alone (Fig. 4A). Deletion of the amino-terminal activation domain (Δ 2-141) completely eliminated CBP binding. Deletion of the amino-terminal half (Δ 3-68) or the carboxy-terminal half of the activation domain (Δ 66-141) eliminated CBP binding. Deletion of amino acids 94 to 140 (Δ 94-140) had no significant effect on Zta-CBP binding. Similarly, deletion of the extreme C-terminal domain of Zta (Δ 198-245) had little effect on CBP binding. These results

FIG. 1. Zta activation domain mutants have promoter-specific defects. Alanine substitution mutations in the Zta activation domain were assayed for transcription activation of Z₇E4TCAT (A) or BHLF1 CAT (B) in transient-transfection assays in HeLa cells. (C) Zta mutants were analyzed by Western blot of transfected cell extracts with polyclonal rabbit antisera directed against Zta. (D) Amino acid sequence of Zta activation domain. Amino acid substitutions causing defects in Z₇E4TCAT are noted in bold, and those defective in BHLF1 CAT are underlined.

indicate that Zta amino acid residues 2 to 94 were required for CBP binding in vitro.

To further map the interaction of the Zta activation domain with CBP and to better correlate transcription properties with CBP binding, we assayed several of the Zta substitution mu-

nants for binding to GST-CBP-C/H1 and -C/H3 (Fig. 4B). We found that F22A and F26A had reduced but detectable binding to both domains of CBP, while the combination F22A/F26A reduced CBP binding to nearly undetectable levels. Alanine substitution of W74 alone eliminated CBP binding, and all

FIG. 2. Effect of Zta activation domain mutants on EBV early lytic promoters. (A, B, and C) The BZLF1 (Zp-Luc), BMRF1 (Mp-Luc), and BRLF1 (Rp-Luc) constructs were assayed for Zta transcription activation in transient-transfection assays in HeLa cells. Zta alanine substitution mutants are indicated below. (D) Zta mutants were analyzed for expression levels by Western blotting analysis.

combinations of mutants containing W74, including W74/F75 and the quadruple substitution mutant F22/F26/W74/F75, had undetectable binding to CBP. In contrast, substitution mutations in L48/W49 did not eliminate CBP binding, nor did substitution of the acidic amino acids Q34/D35 or P53/E54. These

results indicate that combinations of hydrophobic amino acids contribute to stable interaction with both the C/H1 and C/H3 domains of CBP. The interaction of in vitro-translated Zta with CBP domains may not reflect the more selective binding required for tran-

FIG. 3. Cell-specific defects of Zta activation domain mutants. (A) Zta alanine substitution mutants were transfected into D98/HR1 cells and assayed 72 h posttransfection by Western blot for expression of Rta (upper panel), EA-D (middle panel), and Zta (lower panel). (B) Zta mutants were assayed for transcription activation of the Mp-Luc reporter plasmid in DG75 lymphoblastoid cells.

scription activation in vivo. To better address this concern, we assayed the ability of GST-C/H1 and GST-C/H3 to interact with Zta bound to the Z₇E4T promoter using EMSA (Fig 5A). The panel of Zta alanine substitution mutations assayed in Fig.

1 and 2 were compared for their ability to form stable EMSA-resolvable complexes with GST-C/H1 (top panel) and GST-C/H3 (middle panel). Zta binding with GST alone is shown in the lower panel. Quantitation of the binding reactions indi-

FIG. 4. Zta activation domain mutants disrupt CBP binding in solution. (A) Zta deletion mutants were ³⁵S labeled by in vitro translation and assayed for binding to GST-CBP-C/H1 or -C/H3 domain. (B) Zta alanine substitution mutants were assayed for binding to GST-CBP-C/H1 or -C/H3 proteins. (C) Schematic of Zta functional domains.

cated that multiple aromatic amino acid residues were essential for CBP complex formation (Fig. 5D). In particular, we found that K12/F13, F22/F26, L48/W49, and W74/F75 were significantly reduced for CBP binding. The quadruple mutant F22/F26/W74/F75 showed completely disrupted interaction with CBP in this assay, again suggesting that the combinations of aromatic amino acids cooperate for stable binding to CBP. The binding to CBP correlated well with the transcriptional activation of the BHLF1 promoter (compare Fig. 1B and 5D).

Amino acid residues required for stimulation of CBP HAT activity. We have found previously that Zta stimulates the

HAT activity of CBP directed towards small oligonucleosomes (8). This assay requires full-length CBP and may better reflect the in vivo transcription activation functions of Zta than the binding assays with CBP fragments. The panel of Zta alanine substitution mutants were assayed for their ability to stimulate HAT activity of affinity-purified full-length CBP (Fig. 5B). Quantitation of histone acetylation (Fig. 5D) indicated that the same aromatic residues required for EMSA binding were important for HAT stimulation. Specifically, K12/F13, F22/F26, L48/W49, W74/F75, and F22/F26/W74/F75 were most significantly reduced for stimulation of HAT activity. Recombinant

FIG. 5. Aromatic residue substitutions disrupt CBP-promoter recruitment and stimulation of HAT activity. (A) Zta alanine substitution mutants were assayed by Mg-agarose EMSA for binding to GST-CBP-C/H1 (top panel), GST-CBP-C/H3 (middle panel), or GST alone (lower panel) in a complex with the Z₇E4T promoter probe. (B) Zta substitution mutants were assayed for the ability to stimulate CBP nucleosome-directed HAT activity in vitro. Acetylated nucleosomes were visualized by fluorography of SDS-PAGE gels. (C) Coomassie brilliant blue staining of recombinant Zta substitution mutants used in panels A and B. (D) Quantitation of CBP EMSA (black bars) and HAT activity (grey bars) shown in A and B above.

FIG. 6. Formation of the Zta-TFIID-TFIIA-promoter complex with Zta activation domain mutants. (A) Mg-agarose EMSA was used to analyze Zta stimulation of TFIIA-TFIID complex formation on the Z₇E4T promoter DNA probe. Complexes were formed in the absence (–) or presence (+) of Zta mutants as indicated above each lane. (B) The average values for at least three independent Z-D-A complex assays were quantitated for each Zta substitution mutant.

Zta protein abundance was similar in all cases (Fig. 5C). These results indicate that hydrophobic aromatic residues are essential for stimulation of CBP HAT activity. This experiment also demonstrates that acidic residues Q34/D35 and P53/E54 stimulate HAT activity like wild-type Zta despite their transcription defect on several reporter plasmids.

Amino acid residues required for TFIIA-TFIID promoter complex formation. Zta can also stimulate the formation of a stable interaction between core promoter sequences and the TFIIA-TFIID general transcription factor complex. The panel of Zta mutants were assayed by EMSA for their ability to stimulate the Z-D-A complex with the Z₇E4T promoter (Fig. 6). A representational EMSA for each mutant is shown (Fig. 6A). The average of at least three independent EMSA experiments was quantitated and represented graphically (Fig. 6B). Z-D-A complex formation was dependent on aromatic amino acid residues K12/F13, F22/F26, L48/W49, Y64/H65, W74/F75, and F22/F26/W74/F75. The substitution mutations that dis-

rupted Z-D-A complex formation were similar to those that disrupted CBP binding and BHLF1 transcription activation (compare Fig. 6B, 5D, and 1B).

Zta activation domain function in ND10 dispersion. EBV reactivation induced by Zta transfection correlates with the dispersion of ND10-associated proteins from the characteristic patterns of punctate nuclear bodies (2, 5). Zta activation domain mutants were assayed for their ability to disrupt ND10 bodies using the SP100 protein as a marker for ND10 integrity. We focused on a subset of Zta mutants that were found to have biochemical and functional defects in the previous set of experiments. D98/HR1 cells transfected with control vector pcDNA3 had ≈10 to 20 SP100-positive nuclear domains characteristic of ND10 (Fig. 7, top panel). Cell transfected with wild-type Zta resulted in a diffusion of ND10 and enhanced staining of SP100. Substitution K12/F13 resulted in weak dispersion of ND10 and no enhanced staining of SP100. F22/F26 had a staining pattern similar to the wild type. Acidic residue

FIG. 7. Dispersion of ND10 protein SP100 by Zta activation domain mutants. (A) A subset of Zta alanine substitution mutants (indicated to the left of each panel) were transfected into D98/HR1 cells and assayed 48 h posttransfection by indirect immunofluorescence. Antibodies specific for Zta (left panel) were detected with Texas Red, and antibodies specific for SP100 (middle panel) were detected in green with fluorescamine. The merge of the two images is shown in yellow (right panel).

mutations Q34/D35 and P53/E54 caused SP100 dispersion but did not enhance SP100 expression levels. In contrast, aromatic residue mutations L48/W49 and F22/F26/W74/F75, which were defective for binding to CBP and TFIIA-TFIID and for BHLF1 transcription activation, caused a massive increase in SP100 staining and no discernible ND10 structure (Fig. 7). These results suggest that Zta activation domain functions contribute to the dispersion of ND10 and the accumulation of nuclear SP100 protein.

Transcription activation and DNA replication of BZLF1-null EBV. The Zta alanine substitution mutants were next assayed for their ability to stimulate lytic gene expression and viral DNA replication in the absence of potential interfering effects of virally encoded Zta. 293 cells harboring a recombinant viral genome lacking Zta coding sequences (ZKO) were used for transcription reactivation and replication studies (16). Transfection of wild-type Zta into ZKO cells resulted in strong

stimulation of EA-D and Rta gene expression, as assayed by Western blotting (Fig. 8A). Alanine substitution at acidic amino acid residues D27/Q28, Q34/D35, and P53/E54 gave the most dramatic reductions in activation of EA-D and Rta. We also observed that aromatic amino acid substitution L48/W49 and the quadruple substitution F22/F26/W74/F75 were diminished relative to wild-type Zta activation levels. In contrast to our studies with D98/HR1 and DG75 cells (Fig. 3), most Zta mutants were expressed to similar levels (Fig. 8A, top panel). These results indicate that in the context of a latent virus lacking endogenous wild-type Zta, transcription activation requires both the acidic and hydrophobic activation surfaces of Zta.

The ability of Zta substitution mutants to stimulate viral replication and infectious virus production was assayed by the GFP-positive conversion of Raji cells. Recombinant EBV genomes have an integrated GFP gene that can be used to assay

FIG. 8. Reactivation of BZLF1-knockout EBV. (A) Zta substitution mutants were transfected into 293 ZKO cells and assayed by Western blotting for expression of Zta, EA-D, and Rta, as indicated. (B) Supernatants from transfected 293 ZKO cells were measured for infectious virus production by superinfection of Raji cells. Raji cell superinfection was quantitated by FACS analysis of GFP-positive cells.

infected cell number (16). Filtered supernatants from transfected ZKO cells were used to superinfect Raji cells, which were quantitated by FACS analysis. As expected, acidic residue substitutions that abrogated transcription activation caused defects in progeny virus production (Fig. 8B). Similarly, the quadruple aromatic substitution F22/F26/W74/F75 was significantly reduced for progeny virus production relative to wild-type Zta. However, L48/W49 did not show significant defects in progeny virus production, although its transcription was reduced to less than 30% of wild-type activity. These results indicate that the transcription activation function of the acidic amino acid residues (D27, D35, and E54) are most critical for

viral replication in 293 cells. Interestingly, K12/F13, which did not show significant defects in transcription activation of EA-D and Rta (Fig. 8A), was reduced to less than 10% of wild-type Zta for production of progeny virus. This is consistent with a DNA replication-specific function for Zta through amino acids K12 and F13 (44).

DISCUSSION

Zta is a multifunctional protein essential for EBV lytic cycle gene expression and viral DNA replication (36, 47). Lytic cycle gene expression requires Zta transcription activation of numerous and diverse gene promoters, in various cell types and conditions. These requirements have resulted in a complex and multifaceted transcription activation domain. Previous truncation mutagenesis revealed that the Zta activation domain consists of three functionally redundant modules that reside within the first 90 amino acid residue of Zta (11). Mutagenesis studies of other transcription activators, including herpes simplex virus VP16 and *Saccharomyces cerevisiae* GCN4, reveal that both bulky hydrophobic clusters and acidic amino acid residues are important for activation function (24, 37, 42). We have observed that the Zta activation domain consists largely of hydrophobic amino acids with a repetitive pattern of aromatic residues and patches of acidic residues adjacent to glutamine or proline. In this work, we found that alanine substitution of aromatic and acidic residues disrupted Zta transcription activation function in a promoter- and cell type-dependent manner. Specifically, we found that combinations of hydrophobic residues were important for transcription activation of the BHLF1, BZLF1, BMRF1, and BRLF1 promoters in HeLa and 293 cells. In contrast, acidic residues were important for activation of the synthetic construct Z₇E4T and the viral BMRF1 and BRLF1 promoters in most cell types. Acidic residues were most important for transcription activation and reactivation of latent virus in D98/HR1 (Fig. 3) and 293 ZKO infected cells (Fig. 8). Biochemical analysis of Zta mutants revealed that combinations of hydrophobic aromatic residues were important for interactions with CBP and the TFIIA-TFIID core promoter binding factors (Fig. 2 to 5). No obvious biochemical defect was found for acidic amino acid substitution mutants, suggesting that interaction with factors other than CBP and TFIIA-TFIID was disrupted. Taken together, these results suggest that Zta has two distinct activation interfaces with non-redundant functions essential for lytic cycle gene expression and replication (Fig. 9).

The most notable conclusion from these studies was that acidic amino acid residues (D27, D35, and E54) were essential for Zta transcription activation of Rta and EA-D from latently infected cells, but had no obvious disruption of CBP and TFIIA-TFIID complex formation (Fig. 8). Acidic amino acid residue substitutions were similarly reduced in Burkitt's lymphoma cells (data not shown) and for the Z₇E4T promoter in HeLa cells (Fig. 1). Acidic amino acid substitutions had no effect on CBP binding (Fig. 4 and 5), stimulation of CBP HAT activity (Fig. 5), or TFIIA-TFIID promoter complex formation (Fig. 6). Thus, it is likely that Zta interacts through these acidic residues with some other component of the transcriptional machinery besides CBP or TFIIA-TFIID. It is possible that these acidic residues promote conformational changes in these

FIG. 9. Distinct targets of the acidic and aromatic residues in the Zta activation domain. Aromatic residues were important for CBP and TFIIA-TFIID interactions, Hp transcription activation, and Zta protein degradation. Acidic residues were essential for Rp, Mp, and Zp transcription and viral DNA replication and increase Zta stability. K12 and F13 were essential for replication and SUMO-1 modification (2).

target factors that alter their activity in transcription, but have no detectable effect on their stable association with Zta as measured in these studies. It seems unlikely that mutations in acidic residues cause global changes in Zta structure, since these mutations have nearly wild-type transcription activity on the BHLF1 promoter in HeLa cells (Fig. 1). The strong dependence on acidic residues in 293 and Burkitt's lymphoma cells may reflect an inactivation of the CBP pathway in these cells. 293 cells contain adenovirus E1A, which can inactivate CBP, potentially rendering the hydrophobic activation module of Zta ineffective. Thus, in E1A-positive cells such as 293, the dependence on the acidic module may be more pronounced. A similar inactivation of CBP may occur in Burkitt's lymphoma cells, but this has not been established.

The hydrophobic residues in the Zta activation domain affected transcription when mutated in combination, but not when mutated individually. A similar cumulative effect of hydrophobic amino acid mutations was observed for the activation domain of yeast GCN4. Mutation of hydrophobic clusters reduced transcription weakly, while combination of hydrophobic mutations resulted in more dramatic transcription defects. Combinations of hydrophobic mutations in GCN4 led to the loss of interaction with TFIID, SAGA, and holo-RNA polymerase (14). A very similar observation was made for Zta; combinations of hydrophobic aromatic residues led to the loss of interaction with TFIID-TFIIA and coactivator CBP. In both cases, the same amino acids mediated interactions with distinct transcription factor targets. Precisely how the same activation surface recruits these two biochemically distinct targets remains unclear. One possibility is that the same surface recruits these factors mutually exclusively and sequentially, which has been shown for the estrogen receptor recruitment of the nuclear hormone family of coactivators (46). Alternatively, Zta may recruit both factors simultaneously through a large activation surface.

The requirements for lytic DNA replication and production of infectious virus correlated well with transcription activation function, indicating that transcription of viral genes is a principal function of Zta (Fig. 8). However, hydrophobic mutation L48/W49 reduced transcription to less than 30% of wild type,

yet produced similar levels of infectious virus, suggesting that production of virus occurs efficiently even with reduced levels of some viral transcripts. Interestingly, substitution mutations at K12 and F13 resulted in a severe loss of viral replication and infectious virus production but only minor defects in transcription activation. This substitution mutant has been shown to be defective for lytic replication in a reconstituted transfection system, consistent with our results using recombinant virus lacking Zta (44). K12/F13 was found to be important for recruitment of virally encoded DNA replication proteins (18, 44). K12/F13 had minimal transcription defects in transient-transfection assays, but was reduced for CBP-C/H1 binding and TFIIA-TFIID complex formation (Fig. 2, 4, and 6). Thus, mutation of K12/F13 may have additional effects on the folding of the Zta activation domain that reduce most biochemical activities. However, the severe reduction in replication is most consistent with the loss of recruitment of EBV replication proteins.

Zta amino acid residue K12 has been shown to be the site of modification by SUMO-1, a ubiquitin-like protein that can be conjugated to lysine residues on proteins associated with ND10 formation and viral replication domains (2, 35). We analyzed several Zta proteins for their effect on ND10 structure by assaying the ND10-associated protein SP100 after transient transfection in D98/HR1 cells (Fig. 7). The K12/F13 mutant was attenuated for SP100 dispersion, suggesting a correlation between Zta SUMO-1 modification of Zta, ND10 dispersion, and EBV lytic replication. However, acidic substitution mutants Q34/D35 and P53/E54, which failed to stimulate transcription in D98/HR1 cells and were replication defective in ZKO cells, dispersed SP100 structures. This indicates that dispersion of SP100 is not sufficient for viral reactivation and replication. Interestingly, the aromatic substitution mutations L48/W49 and F22/F26/W74/F75 led to an increase SP100 levels in Zta-positive cells, suggesting that Zta also regulates SP100 accumulation. Additional studies will be required to determine if Zta alters the stability of SP100 and whether Zta has a similar effect on other ND10-associated proteins, like PML.

SUMO-1 modification has been proposed to compete with ubiquitin modification to regulate the stability of target proteins. Interestingly, we found that several Zta activation domain mutants had substantially different stability in some cell types. Specifically, the acidic residue substitution mutants had reduced stability while the aromatic substitutions had increased stability in D98/HR1 and in lymphoblastoid cells (Fig. 3). Interactions between activation domains and basal transcription factors can mark activators for ubiquitin-mediated degradation (49). While we have not identified a ubiquitination site on Zta, our data are consistent with a model in which the activation domain contributes to protein stability through amino acid residues involved in transcription activation function. A similar observation has been made for the ubiquitin-mediated degradation of p53, which is targeted by CBP C/H1 domain binding to the p53 activation domain (21). Mutations in Zta aromatic residues L48/W49 and F22/F26/W74/F75 that abrogate binding to CBP led to a large increase in Zta stability in D98/HR1 and lymphoblastoid cells (Fig. 3), suggesting that Zta association with CBP may similarly regulate Zta stability.

The mutational analysis of the Zta activation domain provides some insight into the mechanism of Zta-stimulated tran-

scription and DNA replication. Our data indicate that Zta contains two compositionally and functionally distinct activation motifs (Fig. 9). Hydrophobic aromatic residues were essential for CBP and TFIIA-TFIID complex formation in vitro and had significant effects on Zta stability in vivo. Although we did not see dramatic effects of aromatic residue substitutions in the replication and reactivation studies in vivo, this may be a result of the relatively conservative mutation to alanine. More radical substitutions of these aromatic residues are more likely to reveal significant replication and reactivation defects in vivo. The acidic residues gave clear phenotypes in replication and reactivation but were not important for interactions with CBP or TFIIA-TFIID. Acidic residue mutations led to decreased stability of Zta in lymphoblastoid cells, suggesting that these residues stabilize wild-type Zta. The cellular targets that mediate the acidic residue activation and replication function and whether these are distinct from the targets of the Zta aromatic residues remain important unsolved questions for future studies.

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The first two authors contributed equally to this work.

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REFERENCES

- Adamson, A. L., and S. Kenney. 1999. The Epstein-Barr virus BZLF1 protein interacts physically and functionally with the histone acetylase CREB-binding protein. *J. Virol.* **73**:6551–6558.
- Adamson, A. L., and S. Kenney. 2001. Epstein-barr virus immediate-early protein BZLF1 is SUMO-1 modified and disrupts promyelocytic leukemia bodies. *J. Virol.* **75**:2388–2399.
- Babcock, G. J., L. L. Decker, M. Volk, and D. A. Thorley-Lawson. 1998. EBV persistence in memory B cells in vivo. *Immunity* **9**:395–404.
- Baumann, M., H. Mischak, S. Dammeier, W. Kolch, O. Gires, D. Pich, R. Zeidler, H. J. Delecluse, and W. Hammerschmidt. 1998. Activation of the Epstein-Barr virus transcription factor BZLF1 by 12-*O*-tetradecanoylphorbol-13-acetate-induced phosphorylation. *J. Virol.* **72**:8105–8114.
- Bell, P., P. M. Lieberman, and G. G. Maul. 2000. Lytic but not latent replication of Epstein-Barr virus is associated with PML and induces sequential release of nuclear domain 10 proteins. *J. Virol.* **74**:11800–11810.
- Cayrol, C., and E. Flemington. 1996. G₀/G₁ growth arrest mediated by a region encompassing the basic leucine zipper (bZIP) domain of the Epstein-Barr virus transactivator Zta. *J. Biol. Chem.* **271**:31799–31802.
- Cayrol, C., and E. K. Flemington. 1996. The Epstein-Barr virus bZIP transcription factor Zta causes G₀/G₁ cell cycle arrest through induction of cyclin-dependent kinase inhibitors. *EMBO J.* **15**:2748–2759.
- Chen, C.-J., Z. Deng, A. Y. Kim, G. A. Blobel, and P. M. Lieberman. 2001. Stimulation of CREB binding protein nucleosomal histone acetyltransferase activity by a class of transcriptional activators. *Mol. Cell. Biol.* **21**:476–487.
- Chevallier, G. A., E. Manet, P. Chavrier, C. Mosnier, J. Daille, and A. Sergeant. 1986. Both Epstein-Barr virus (EBV)-encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. *EMBO J.* **5**:3243–3249.
- Chi, T., and M. Carey. 1996. Assembly of the isomerized TFIIA-TFIID-TATA ternary complex is necessary and sufficient for gene activation. *Genes Dev.* **10**:2540–2550.
- Chi, T., and M. Carey. 1993. The ZEBRA activation domain: modular organization and mechanism of action. *Mol. Cell. Biol.* **13**:7045–7055.
- Countryman, J. K., L. Heston, L. Gradoville, H. Himmelfarb, S. Serdy, and G. Miller. 1994. Activation of the Epstein-Barr virus BMRF1 and BZLF1 promoters by ZEBRA in *Saccharomyces cerevisiae*. *J. Virol.* **68**:7628–7633.
- Dardari, R., M. Khyatti, A. Benider, H. Jouhadi, A. Kahlain, C. Cochet, A. Mansouri, B. El Gueddari, A. Benslimane, and I. Joab. 2000. Antibodies to the Epstein-Barr virus transactivator protein (ZEBRA) as a valuable biomarker in young patients with nasopharyngeal carcinoma. *Int. J. Cancer* **86**:71–75.
- Drysdale, C. M., B. M. Jackson, R. McVeigh, E. R. Klebanow, Y. Bai, T. Kokubo, M. Swanson, Y. Nakatani, P. A. Weil, and A. G. Hinnebusch. 1998. The Gcn4p activation domain interacts specifically in vitro with RNA polymerase II holoenzyme, TFIID, and the Ada-Gcn5p coactivator complex. *Mol. Cell. Biol.* **18**:1711–1724.

15. Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides. 1989. Epstein-Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos. *EMBO J.* **8**:127–132.
16. Feederle, R., M. Kost, M. Baumann, A. Janz, E. Drouet, W. Hammerschmidt, and H. J. Delecluse. 2000. The Epstein-Barr virus lytic program is controlled by the cooperative functions of two transactivators. *EMBO J.* **19**:3080–3089.
17. Flemington, E. K., A. M. Borrás, J. P. Lytle, and S. H. Speck. 1992. Characterization of the Epstein-Barr virus lytic transactivator Zta interacts with the helicase-primase replication proteins. *J. Virol.* **72**:8559–8567.
18. Gao, Z., A. Krithivas, J. E. Finan, O. J. Semmes, S. Zhou, Y. Wang, and S. D. Hayward. 1998. The Epstein-Barr virus lytic transactivator Zta interacts with the helicase-primase replication proteins. *J. Virol.* **72**:8559–8567.
19. Goldman, P. S., V. K. Tran, and R. H. Goodman. 1997. The multifunctional role of the coactivator CBP in transcriptional regulation. *Recent Prog. Horm. Res.* **52**:103–119.
20. Greenspan, J. S., D. Greenspan, E. T. Lennette, D. I. Abrams, M. A. Conant, V. Petersen, and U. K. Freese. 1985. Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. *N. Engl. J. Med.* **313**:1564–1571.
21. Grossman, S. R., M. Perez, A. L. Kung, M. Joseph, C. Mansur, Z. X. Xiao, S. Kumar, P. M. Howley, and D. M. Livingston. 1998. p300/MDM2 complexes participate in MDM2-mediated p53 degradation. *Mol. Cell* **2**:405–415.
22. Gutsch, D. E., E. A. Holley-Guthrie, Q. Zhang, B. Stein, M. A. Blonar, A. S. Baldwin, and S. C. Kenney. 1994. The bZIP transactivator of Epstein-Barr virus, BZLF1, functionally and physically interacts with the p65 subunit of NF- κ B. *Mol. Cell. Biol.* **14**:1939–1948.
23. Ikuta, K., Y. Satoh, Y. Hoshikawa, and T. Sairenji. 2000. Detection of Epstein-Barr virus in saliva and throat washings in healthy adults and children. *Microbes Infect.* **2**:115–120.
24. Jackson, B. M., C. M. Drysdale, K. Nataran, and A. G. Hinnebusch. 1996. Identification of seven hydrophobic clusters in GCN4 making redundant contributions to transcriptional activation. *Mol. Cell. Biol.* **16**:5557–5571.
25. Kenney, S. C., E. Holley-Guthrie, E. B. Quinlivan, D. Gutsch, Q. Zhang, T. Bender, J. F. Giot, and A. Sergeant. 1992. The cellular oncogene c-myc can interact synergistically with the Epstein-Barr virus BZLF1 transactivator in lymphoid cells. *Mol. Cell. Biol.* **12**:136–146.
26. Kieff, E. 1996. Epstein-Barr virus and its replication, p. 2343–2396. *In* D. Knipe and P. M. Howley (ed.), *Field's virology*, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
27. Kouzarides, T., G. Packham, A. Cook, and P. J. Farrell. 1991. The BZLF1 protein of EBV has a coiled coil dimerisation domain without a heptad leucine repeat but with homology to the C/EBP leucine zipper. *Oncogene* **6**:195–204.
28. Kussie, P. H., S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A. J. Levine, and N. P. Pavletich. 1996. Structure of the MDMs oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **274**:948–953.
29. Lieberman, P. 1994. Identification of functional targets of the Zta transcriptional activator by formation of stable preinitiation complex intermediates. *Mol. Cell. Biol.* **14**:8365–8375.
30. Lieberman, P. M., and A. J. Berk. 1990. In vitro transcriptional activation, dimerization, and DNA-binding specificity of the Epstein-Barr virus Zta protein. *J. Virol.* **64**:2560–2568.
31. Lieberman, P. M., and A. J. Berk. 1994. A mechanism for TAFs in transcriptional activation: activation domain enhancement of TFIID-TFIIA-promoter DNA complex formation. *Genes Dev.* **8**:995–1006.
32. Lieberman, P. M., and A. J. Berk. 1991. The Zta trans-activator protein stabilizes TFIID association with promoter DNA by direct protein-protein interaction. *Genes Dev.* **5**:2441–2454.
33. Lieberman, P. M., J. M. Hardwick, J. Sample, G. S. Hayward, and S. D. Hayward. 1990. The zta transactivator involved in induction of lytic cycle gene expression in Epstein-Barr virus-infected lymphocytes binds to both AP-1 and ZRE sites in target promoter and enhancer regions. *J. Virol.* **64**:1143–1155.
34. Lieberman, P. M., J. Ozer, and D. B. Gursel. 1997. Requirement for TFIIA-TFIID recruitment by an activator depends on promoter structure and template competition. *Mol. Cell. Biol.* **17**:6624–6632.
35. Maul, G. G. 1998. Nuclear domain 10: the site of DNA virus transcription and replication. *Bioessays* **20**:660–667.
36. Miller, G. 1990. The switch between latency and replication of Epstein-Barr virus. *J. Infect. Dis.* **161**:833–844.
37. Moriuchi, H., M. Moriuchi, R. Pichyangkura, S. J. Triezenberg, S. F. Straus, and J. I. Cohen. 1995. Hydrophobic cluster analysis predicts an amino-terminal domain of varicella-zoster virus open reading frame 10 required for transcription activation. *Proc. Natl. Acad. Sci. USA* **92**:9333–9337.
38. Ozer, J., A. H. Bolden, and P. M. Lieberman. 1996. Transcription factor IIA mutations show activator-specific defects and reveal a IIA function distinct from stimulation of TBP-DNA binding. *J. Biol. Chem.* **271**:11182–11190.
39. Ozer, J., P. A. Moore, A. H. Bolden, A. Lee, C. A. Rosen, and P. M. Lieberman. 1994. Molecular cloning of the small (γ) subunit of human TFIIA reveals functions critical for activated transcription. *Genes Dev.* **8**:2324–2335.
40. Ptashne, M., and A. Gann. 1997. Transcriptional activation by recruitment. *Nature* **386**:569–577.
41. Ptashne, M., and A. A. Gann. 1990. Activators and targets. *Nature* **46**:329–331.
42. Regier, J. L., F. Shen, and S. J. Triezenberg. 1993. Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional activator. *Proc. Natl. Acad. Sci. USA* **90**:883–887.
43. Rickinson, A. B., and E. Kieff. 1996. Epstein-Barr Virus, p. 2397–2446. *In* D. Knipe and P. M. Howley (ed.), *Field's virology*, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
44. Sarisky, R. T., Z. Gao, P. M. Lieberman, E. D. Fixman, G. S. Hayward, and S. D. Hayward. 1996. A replication function associated with the activation domain of the Epstein-Barr virus Zta transactivator. *J. Virol.* **70**:8340–8347.
45. Scheepers, A., D. Pich, and W. Hammerschmidt. 1993. A transcription factor with homology to the AP-1 family links RNA transcription and DNA replication in the lytic cycle of Epstein-Barr virus. *EMBO J.* **12**:3921–3929.
46. Shang, Y., X. Hu, J. DiRenzo, M. A. Lazar, and M. Brown. 2000. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103**:843–852.
47. Speck, S. H., T. Chatila, and E. Flemington. 1997. Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene. *Trends Microbiol.* **5**:399–405.
48. Takebe, Y., M. Seiki, J.-I. Fujisasa, P. Hoy, K. Yokota, K.-I. Arai, M. Yoshida, and N. Arai. 1988. SR α promoter: an efficient and versatile mammalian cDNA promoter and R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8**:466–472.
49. Tansey, W. P. 2001. Transcriptional activation: risky business. *Genes Dev.* **15**:1045–1050.
50. Uesugi, M., O. Nyanguile, H. Lu, A. J. Levine, and G. L. Verdine. 1997. Induced alpha helix in the VP16 activation domain upon binding to a human TAF. *Science* **277**:1310–1313.
51. Zerby, D., C.-J. Chen, E. Poon, D. Lee, R. Shiekhattar, and P. M. Lieberman. 1999. The amino-terminal C/H1 domain of CREB binding protein mediates Zta transcription activation of latent Epstein-Barr virus. *Mol. Cell. Biol.* **19**:1617–1626.
52. Zhang, Q., D. Gutsch, and S. Kenney. 1994. Functional and physical interaction between p53 and BZLF1: implications for Epstein-Barr virus latency. *Mol. Cell. Biol.* **14**:1929–1938.