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Identification of Cellular Target Genes of the Epstein-Barr Virus Transactivator Zta: Activation of Transforming Growth Factor βigh3 (TGF-βigh3) and TGF-β1

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The lytic switch transactivator Zta initiates the ordered cascade of Epstein-Barr virus gene expression that culminates in virus production. Zta is a sequence-specific DNA-binding protein that transactivates early viral promoters via *cis*-acting sequences. Activation of some of these genes is mediated through binding to consensus AP-1 promoter elements. This observation suggests that Zta may also regulate the expression of cellular genes. While many targets of Zta have been identified in the Epstein-Barr virus genome, putative host cell targets remain largely unknown. To address this issue, a tetracycline-regulated Zta expression system was generated, and differential hybridization screening was used to isolate Zta-responsive cellular genes. The major target identified by this analysis is a gene encoding a fasciclin-like secreted factor, transforming growth factor βigh3 (TGF-βigh3), that was originally identified as a gene that is responsive to the potent immunosuppressor TGF-β1. Northern (RNA) blot analysis demonstrated that induction of Zta expression results in a 10-fold increase in TGF-βigh3 mRNA levels. Zta was also found to increase TGF-β1 mRNA levels as well as the amount of active TGF-β1 secreted into the medium. Interestingly, α1-collagen IV, which has been shown to potentiate the effects of TGF-β1, is also a cellular target of Zta. These results suggest that Zta could play a role in modulating the host cell environment through activating the expression of secreted factors.

During primary infection, Epstein-Barr virus (EBV) actively replicates in epithelial cells of the oropharynx and subsequently infects recirculating B lymphocytes (42, 47). In some individuals, this primary EBV infection induces a lymphoproliferative disease, infectious mononucleosis, characterized by transient immunosuppression (40, 42) and an unusual expansion of atypical lymphocytes, the majority of which are not B cells but $CD8^+$ T cells (40, 52). While EBV replicates fully in differentiated pharyngeal epithelium (4, 55), infection of B lymphocytes is mostly nonproductive. Instead, the virus establishes a "latent" phase, during which only a few viral genes, which are required for maintenance of the viral genome, are expressed (51). Lytic replication can be activated in these cells, however, by the lytic switch transactivator Zta (also called BZLF1, EB1, and Zebra). Zta expression ultimately results in the expression of an estimated 100 or more viral replication-associated genes (3), including early antigens, viral capsid antigens, and membrane antigens. Of all the viral transactivators examined, Zta is unique in its ability to initiate this ordered cascade of EBV gene expression that culminates in virus production (12, 23).

Zta is a 34- to 38-kDa DNA-binding protein related to the bZip family of transcription factors (15). The carboxyl-terminal domain of Zta mediates homodimerization through a coiled-coil interaction (8, 20, 30), although Zta lacks the heptad repeats of leucine residues found in leucine zipper proteins. The basic region of Zta has sequence homology with the DNA binding domain of members of the AP-1 family of transcription factors (15). The amino-terminal domain of Zta has been shown to play a role in activation of transcription (9, 17, 31) and to be essential for in vitro association with the TATA box-binding protein TBP (32) and the general transcription factor TFIIA (9, 33).

Transcriptional activation of viral promoters by Zta occurs via binding to *cis*-acting AP-1 or ZRE (Zta-responsive elements) sites. EBV lytic cycle promoters that are activated by Zta in infected B cells and/or in HeLa epithelial cells include those encoding the transactivators BZLF1, BRLF1, and BMLF1 (15, 18, 24, 38, 54), the bcl2 homolog BHRF1 (34), BHLF1 (31), and the delayed early protein BMRF1 (25). While many targets of Zta have been identified in the EBV genome, until now only one cellular gene, the proto-oncogene c-*fos*, has been shown to be activated by Zta through AP-1 and AP-1-like sites (19, 45). However, characterization of cellular Zta-responsive genes is important to fully understand EBV-host cell interactions. For example, modification of cellular gene expression by Zta could influence spreading of the virus or recognition of infected cells by the immune system.

The objective of the present study was to identify the major cellular targets of Zta. For this purpose, an epithelial cell line that contains the Zta gene under the control of a tetracycline-responsive promoter was generated (22). By using this tetracycline-regulated Zta expression system and differential hybridization screening procedures, several Zta-induced gene transcripts were identified. The major target, transforming growth factor βigh3 (TGF-βigh3), which is induced about 10-fold, was previously isolated as the product of a TGF-β1-responsive gene (50). TGF-βigh3 is a 68-kDa secreted protein with homology to a putative neuronal cell-cell adhesion protein, fasciclin-I (50), and has been shown to affect cell growth as well as cell morphology (49). We also investigated TGF-β1 expression in the tetracycline-regulated Zta cell line and found that both TGF-β1 mRNA levels and the amount of biologically active TGF-β1 are increased in cells expressing Zta. These results suggest that besides playing a direct role in viral repli-

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cation, Zta may also be involved in altering the host cell environment through autocrine or paracrine pathways.

MATERIALS AND METHODS

Cells and antibody. Human cervical carcinoma cells (HeLa cells) were grown in high-glucose Dulbecco modified Eagle medium (Cellgro) supplemented with 10% fetal bovine serum (GIBCO). HeLa-HtTA cells, stably expressing the chimeric *tet* repressor-VP16 transactivator protein (tTA), were a generous gift of H. Bujard (University of Heidelberg) (22) and were grown in the same medium as the parental HeLa cells. Stable HeLa-HtTA transfectants expressing Zta (HeLa-Zta cells) were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and tetracycline (1 μg/ml; Sigma). Induction of Zta expression in these cells was obtained 72 h after removal of tetracycline in the complete medium. The EBV-negative Burkitt's lymphoma cell line DG75 was maintained in RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum. The mouse purified 12CA5F monoclonal antibody recognizing the hemagglutinin (HA) epitope was purchased from BAbCO (Richmond, Calif.) (16).

Plasmids and constructions. Plasmids pUHD15-1, coding for tTA, and pUHD10, a tTA-responsive expression vector containing *tet* operator sequences, were obtained from H. Bujard (University of Heidelberg) (22). The Zta gene, with an in-frame HA tag sequence, was subcloned into pUHD10 by using *Xho*I and *Xba*I restriction sites. The CMV-Zta expression vector used in transient transfection experiments was generated by cloning the Zta gene downstream from the cytomegalovirus (CMV) promoter in the expression vector pRC-CMV (Invitrogen). The reporter plasmids 3xZIIIB-CAT and Zp-CAT (containing Zta promoter sequences from position −221 to +14) have been described previously (21). The phTG17 construct, containing TGF-β1 promoter sequences, has been described elsewhere (28) and was a generous gift of S.-J. Kim (National Institutes of Health, Bethesda, Md.). We subcloned the TGF-β1 promoter (positions +102 to +289) into plasmid pGL2-CAT (Promega); the fragment from +102 to +289 was amplified by PCR with oligonucleotides 5′-CCGGAAGCTTGCCAAGAG GTCCCCGCGC-3′ and 5′-ACCGAGCTCCTCGGCCCGGAGCGGAGG-3′, digested with *Sac*I and *Hin*dIII restriction enzymes, and inserted into the *Sac*I and *Hin*dIII sites of the multiple cloning sites of pGL2-CAT plasmid, which were described elsewhere (21). The oligonucleotides were synthesized at the Dana-Farber Cancer Institute Facility. The TGF-β1 cDNA used for Northern (RNA) blot experiments was purchased from the American Type Culture Collection.

Establishment of Zta-expressing cell lines. HeLa-HtTA cells were cotransfected by calcium phosphate with the pREP4 plasmid (Invitrogen), which contains a hygromycin B resistance gene and the HA-Zta expression vector (pUHD10-Zta) at a 1:10 ratio. Transfected cells were selected in medium containing hygromycin B (250 U/ml; Calbiochem) and tetracycline (1 μg/ml; Sigma). Several resistant colonies were picked and analyzed for the expression of Zta by Western immunoblot analysis, as described below.

Western blot and indirect immunofluorescence analyses. HeLa-Zta transfectants were tested for inducible Zta expression by Western blot analysis. Briefly, 10^6 cells incubated in the absence or presence of tetracycline (1 μg/ml) were lysed in sodium dodecyl sulfate (SDS) loading buffer containing β-mercaptoethanol and heated to 100°C for 5 min before being loaded on an SDS–10% polyacrylamide gel. Proteins were blotted on a nitrocellulose membrane (Bio-Blot-NC; Costar) by electrotransfer. The blot was then incubated with the anti-HA monoclonal antibody 12CA5F (2.5 μg/ml), washed, and incubated with a polyclonal goat anti-mouse antibody conjugated to alkaline phosphatase (0.2 μg/ml; Fisher Biotech). Western blot reactions were revealed with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (NBT/BCIP) (Promega) as described by the vendor (35). In order to locate Zta in these transfected cells, indirect immunofluorescence was performed with HeLa-Zta transfectants cultured with or without tetracycline in the medium. Cells, grown on a coverslip, were washed with phosphate-buffered saline (PBS), fixed for 10 min with formaldehyde (3.7% in PBS), washed again with PBS, and treated for 10 min with NH_4Cl (50 mM in PBS). After being washed, the cells were permeabilized with Triton X-100 (0.1% in PBS). Blocking was performed with fetal bovine serum (20% in PBS, 15 min) prior to incubation with the anti-HA monoclonal antibody 12CA5F (40 μg/ml, 1 h). After three washes with PBS, cells were incubated for 30 min with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (20 μg/ml; Zymed) and washed again with PBS (three times). Slides were mounted with Mowiol, and the localization of Zta was visualized with a Zeiss microscope under ×20 magnification.

cDNA library construction, differential screening, and Southern blot analysis. A unidirectional cDNA library was constructed in ZAPII (Stratagene) by using poly(A)$^+$ RNA from HeLa-Zta cells grown in the absence of tetracycline for 3 days. First-strand cDNAs from HeLa-Zta and HeLa-HtTA cell mRNAs were synthesized with the Superscript preamplification system (Gibco-BRL). The screening of this library was performed by differential hybridization with HeLa-Zta cDNA as the plus probe and HeLa-HtTA cDNA as the minus probe. Probes were ^{32}P labelled by random priming (Gibco-BRL). Duplicate filter lifts (Amersham) from 11 plates (5,000 plaques each) were prehybridized for 2 h at 42°C and then hybridized overnight at 42°C in 50% formamide–5× Denhardt solution–0.5% SDS–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 μg of tRNA per ml–50 μg of salmon sperm DNA per ml. The filters were washed twice in 1× SSC–0.1% SDS at room temperature for 15 min and twice with 0.1× SSC–0.1% SDS at 55°C for 30 min. Plaques showing differential hybridization with the plus and minus probes were picked up, and the corresponding pBluescript II SK− phagemids were rescued by in vivo excision with Exassist helper phage (Stratagene). The secondary screening was performed by Southern blot analysis. *Eco*RI-*Xho*I inserts from the plasmid DNA were loaded in duplicate on a 1% agarose gel and transferred to a nylon membrane (Amersham). Filters were hybridized as described above. Clones showing differential hybridization after the secondary screening were subjected to DNA sequencing.

DNA sequence analysis. DNA sequencing of the differential clones was performed with the Sequenase version 2.0 kit (United States Biochemical) and the T3 oligonucleotide primer. The program BLASTN (1) was used to compare the cDNA sequences with all sequences present in the National Center for Biotechnology Information nonredundant nucleic acid database.

Bioassay of TGF-β1 activity. In order to quantitate active and/or latent TGF-β1 secreted in tissue culture supernatants of the HeLa-Zta transfectants, sandwich enzyme-linked immunosorbent assays (ELISAs) were performed according to the manufacturer's recommendations (Transforming Growth Factor β1 ELISA system; Promega). Cells grown for 2 days in the presence of tetracycline (1 μg/ml) or in its absence were washed and incubated in serum-free medium. Supernatants from cultures incubated for 24 h were collected and either directly tested in ELISAs for the quantification of the naturally processed TGF-β1 or subjected to acid activation according to the instructions of the manufacturer, to detect both active and latent forms of TGF-β1.

Transfections and CAT assays. Transient transfection experiments with HeLa-Zta cells were performed by the calcium phosphate precipitation procedure, while a DEAE-dextran method was used for the transfection of DG75, as previously described (21). Thirty-microgram quantities of the different reporters were used to transfect approximately 10^6 HeLa-Zta cells. DG75 cells (10^7) were cotransfected with 2-μg quantities of the reporter plasmids together with 1 μg of Zta expression vector (CMV-Zta) or the control vector (CMV). Cells were harvested 48 h after transfection, and extracts were assayed for chloramphenicol acetyltransferase (CAT) activity, which was measured with a Betascope (Betagen).

Northern blot analysis. Total RNAs from HeLa-HtTA and Hela-Zta cells grown in the presence or absence of tetracycline were isolated by acid guanidium thiocyanate-phenol-chloroform extraction (10). Poly(A)$^+$ RNAs were prepared by passage over an oligo(dT)-cellulose column (Collaborative Research). The poly(A)$^+$ RNAs (1 μg) were denatured, separated by electrophoresis through 1% agarose-formaldehyde gels, and transferred to nylon membranes (Hybond-N; Amersham). RNAs were cross-linked to the membrane by UV irradiation. Blots were prehybridized for 2 h and hybridized overnight at 42°C in 50% formamide–6× SSC–0.5% SDS–5× Denhardt solution–10 mM EDTA (pH 8)–100 μg of salmon sperm DNA per ml. The blots were subjected to high-stringency washes, first at room temperature in 2× SSC–0.5% SDS for 2 min, then at room temperature in 2× SSC–0.1% SDS for 15 min, and finally at 68°C in 0.2× SSC–0.5% SDS for 2 h. The blots were then exposed to autoradiography film (X-Omat; Kodak) at −70°C. The fold induction was determined from signal intensities as measured with a Betascope.

RESULTS

Tetracycline-regulated Zta expression in epithelial cells. Since the major site of in vivo EBV replication is epithelial tissue, either in the oropharynx (48) or in the cervix (46), we have employed an epithelial cell line derived from a cervical carcinoma, HeLa, to identify cellular genes induced by Zta. An expression system developed by Gossen and Bujard (22) was employed to generate HeLa cell lines that conditionally express Zta. The Zta gene, with an in-frame sequence encoding the HA epitope tag, was cloned downstream from a minimal CMV promoter containing bacterial *tet* operator (*tetO*) sequences. This plasmid was cotransfected with a plasmid containing a hygromycin resistance gene into HeLa cells that constitutively expresses the chimeric transactivator tTA. tTA contains the DNA binding domain from the bacterial *tet* repressor (*tetR*) fused to the activation domain from the herpesvirus VP16 protein. In the presence of the antibiotic tetracycline, tTA cannot interact with the operator and therefore cannot activate the gene of interest. The withdrawal of tetracycline from the medium allows tTA to bind to the *tetO* sequences and leads to high-level expression.

Hygromycin-resistant colonies were selected and tested for Zta expression in the presence and absence of tetracycline. Western blot analysis demonstrated tetracycline-regulated expression of Zta in two independent clones (Fig. 1A). No Zta is

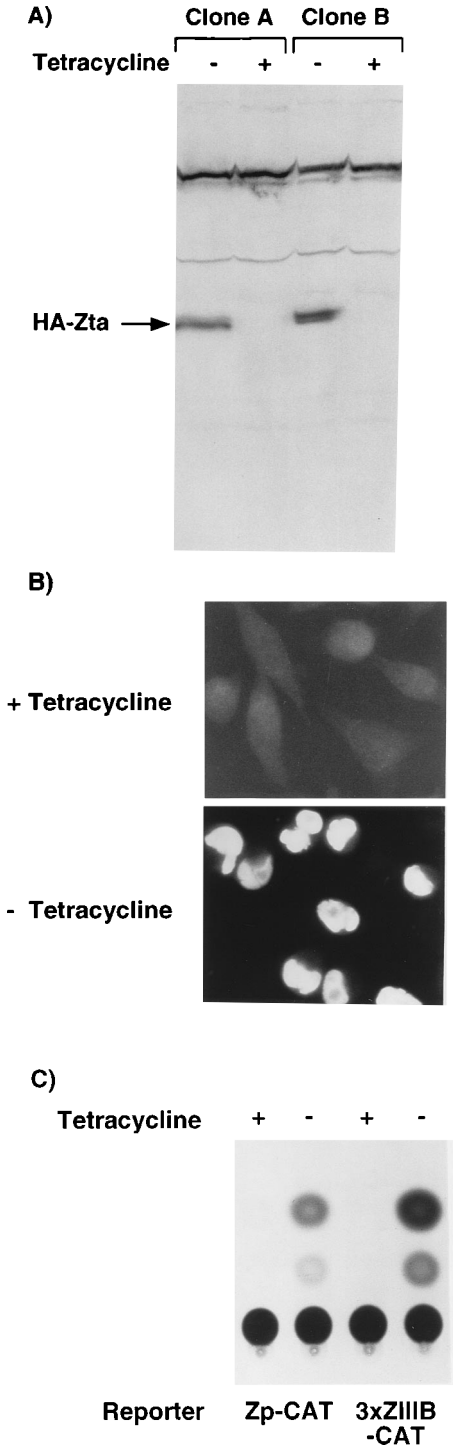

FIG. 1. Generation of stable HeLa transfectants expressing Zta under the control of a tetracycline-regulated promoter. (A) Western blot analysis of Zta expression in two independent HeLa-Zta clones grown in the presence of tetracycline (+) or grown for 3 days after removal of tetracycline (−), using an anti-HA monoclonal antibody (12CA5F). (B) Indirect immunofluorescence analysis of HeLa-Zta cells with the anti-HA 12CA5F monoclonal antibody. Nuclear staining was observed with cells incubated in the absence of tetracycline, while only background staining was obtained in the presence of tetracycline. (C) Transactivation of CAT reporter constructs containing Zta-responsive elements (3×ZIIIB) or the Zta promoter (Zp) in HeLa-Zta transfectants expressing Zta (without tetracycline [−]) or not expressing Zta (with tetracycline [+]).

TABLE 1. Summary of Zta-responsive cDNAs

Gene	No. of cDNAs isolated	Fold induction[a]
Actin	2	2
Vimentin	1	2
α1-Collagen IV	1	4
HLA class I	6	3
TGF-βigh3	3	10

[a] Calculated as the ratio of signal intensities (HeLa-Zta probe to HeLa-HtTA probe) divided by the ratio of signal intensities for the tubulin probe, in Southern blot analysis (for actin, vimentin, and α1-collagen IV) or in Northern blot analysis (for HLA class I and TGF-βigh3).

detectable in extracts from cells incubated in the presence of tetracycline, while Zta is readily detected in extracts from cells cultured in the absence of tetracycline for 3 days. Indirect immunofluorescence analysis was performed to verify that the HA-tagged Zta protein was correctly localized in the nucleus. A strong nuclear signal is observed in a HeLa-Zta cell line grown for 3 days in the absence of tetracycline, while only minimal background staining is observed in cells grown in the presence of tetracycline (Fig. 1B). Similar results were obtained with a polyclonal rabbit antibody that recognizes the dimerization domain of Zta (data not shown). Finally, to ensure that the Zta expressed in these cells is functional, the activities of two different Zta-responsive promoters were tested in cells cultured with or without tetracycline. As shown in Fig. 1C, both the Zta promoter (Zp) and a synthetic Zta responsive promoter (3×ZIIIB) are active in cells cultured without tetracycline but are inactive in cells cultured in the presence of tetracycline. We conclude that this stable epithelial cell line, HeLa-Zta, conditionally expresses functional Zta and should be a useful tool for isolating Zta-responsive cellular genes.

Identification of cellular Zta target genes by differential hybridization screening. A cDNA library was constructed in λZAPII with poly(A)$^+$ RNAs from HeLa-Zta cells cultured in the absence of tetracycline for 3 days. Duplicate filter lifts (55,000 plaques plated at a low density) were screened by differential hybridization with cDNA probes synthesized from either HeLa-Zta cells expressing Zta (plus probe) or the parental HeLa-HtTA cells (minus probe). cDNA clones showing differential hybridization after the primary screening were subjected to secondary screening. This was performed by Southern blotting of restriction enzyme-digested recombinant plasmid clones and probing with labelled cDNAs from HeLa-Zta and control HeLa-HtTA cells. Thirteen cDNA clones that consistently hybridized differentially with the plus and minus cDNA probes were selected for nucleotide sequencing. This analysis revealed that they correspond to five different previously characterized genes (Table 1). Two independent clones matching β-actin and one corresponding to vimentin, two cytoskeletal proteins, were only slightly induced (twofold). The expression of β-actin and vimentin has been shown previously to be induced after in vitro EBV infection of Burkitt's lymphoma cells (5). Another target of Zta, α1-collagen type IV, an extracellular matrix macromolecule, was found to be induced fourfold by Zta in Southern blot experiments (data not shown). Six independent clones encoding major histocompatibility complex (MHC) class I molecules were also isolated. Northern blot analysis revealed that MHC class I mRNA levels are ca. threefold higher in induced than in uninduced HeLa-Zta cells, while tubulin expression is not affected by Zta expression (Fig. 2). The most highly induced gene identified in this study is the

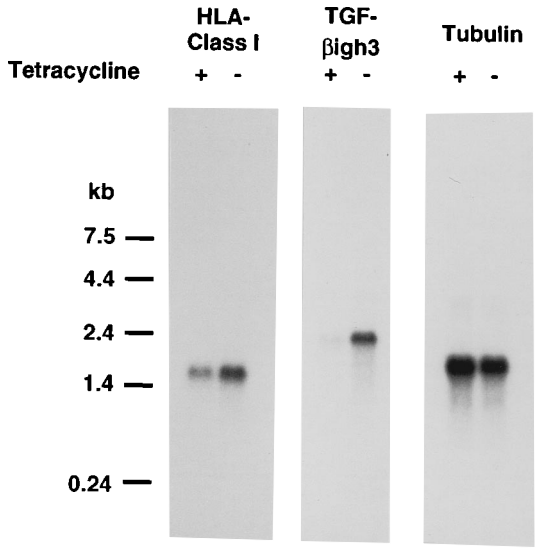

FIG. 2. Northern blot analysis of Zta-responsive gene mRNA expression in HeLa-Zta transfectants. One microgram of polyadenylated mRNA from HeLa-Zta cells grown in the presence of tetracycline (+) or in the absence of tetracycline (−) was fractionated on an agarose-formaldehyde gel, transferred to nylon filters, and hybridized to ^{32}P-labelled HLA class I, TGF-βigh3, and tubulin probes. The tubulin mRNA level is not affected by Zta and serves as a control of mRNA loaded in each lane. Numbers at the left indicate sizes of RNA markers.

TGF-βigh3 gene, a TGF-β1-responsive gene (50). Three independent clones corresponding to this gene were isolated. Northern blot analysis showed that TGF-βigh3 mRNA levels were induced ca. 10-fold in HeLa cells expressing Zta (Fig. 2).

Induction of TGF-β1 by Zta. Since TGF-βigh3 was isolated previously as an upregulated gene in an adenocarcinoma cell line treated with TGF-β1 (50), we tested whether TGF-β1 expression might also be affected by Zta expression. An additional impetus for addressing this question is the results of previous studies demonstrating the induction of TGF-β1 expression by the cellular AP-1 binding transcription factor c-JUN (27). We first tested the levels of active TGF-β1 in the supernatants of HeLa-HtTA and HeLa-Zta cells cultured for 3 days in either the presence or absence of tetracycline (Fig. 3). Similar amounts of active TGF-β1 were released in supernatants from HeLa-HtTA cells incubated in the presence or absence of tetracycline. Similar amounts were also observed in supernatants from HeLa-Zta cells cultured in the presence of tetracycline. In contrast, supernatants from induced HeLa-Zta cells had a twofold increase in the level of biologically active TGF-β1 compared with supernatants from HeLa-HtTA cells or uninduced HeLa-Zta cells (Fig. 3A). Since TGF-β1 is also secreted from cells in an inactive form (latent TGF-β1), we performed ELISAs after acid treatment of the supernatants, which activates latent TGF-β1. This allows the detection of both active and latent forms of TGF-β1. Induced HeLa-Zta cells produced twofold more acid-activated TGF-β1 than did HeLa-Zta cells cultured in the presence of tetracycline (Fig. 3B). These results indicate that Zta expression increases the levels of both active and latent forms of TGF-β1 secreted in the medium.

To investigate whether increased TGF-β1 production by Zta was due to an increase of mRNA levels, Northern blot analysis was performed with polyadenylated RNA isolated from HeLa-Zta cells cultured in the presence or absence of tetracycline. A three- to fourfold increase of TGF-β1 transcript was observed in induced versus uninduced HeLa-Zta cells (Fig. 4), correlating with the observed increase in TGF-β1 protein secreted into the culture medium (Fig. 3).

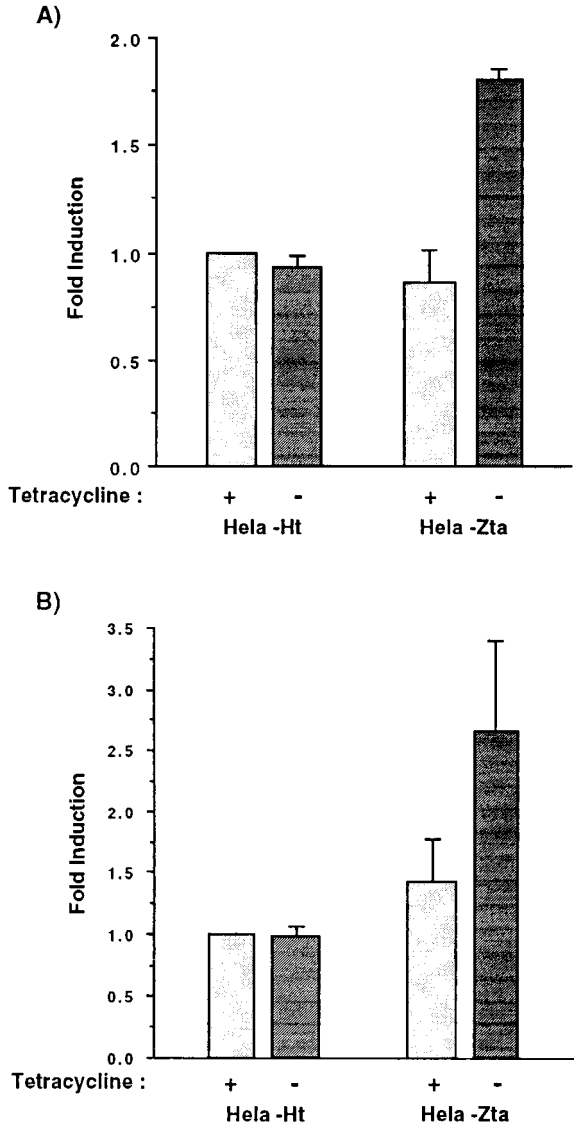

FIG. 3. Induction of TGF-β1 by Zta, as assayed by ELISA. (A) HeLa-HtTA and HeLa-Zta cells cultured in the presence (+) or absence (−) of tetracycline were grown in serum-free medium for 24 h. The culture supernatants were harvested, and the amount of biologically active TGF-β1 secreted from the cells (10^6 cells) was directly assayed by ELISA, according to the manufacturer's protocol (Promega). (B) Similar experiments were also performed after acid treatment of the samples, allowing the measurement of both latent and active forms of TGF-β1, as described in Materials and Methods. Results are presented as means of two triplicate determinations and are expressed as fold induction compared with HeLa-HtTA control supernatants. Error bars indicate maximal variation of activity.

Since TGF-β1 mRNA levels are clearly induced by Zta, we were surprised not to have isolated TGF-β1 cDNA clones during the differential hybridization screening of the HeLa-Zta cDNA library. Therefore, we investigated the relative abundances of TGF-β1 and TGF-βigh3 cDNAs in the HeLa-Zta cDNA library. These experiments revealed that while TGF-βigh3 belongs to the class of intermediate-abundance cDNAs (321 positive clones were detected among 350,000 plaques, representing 0.09% of total mRNAs), TGF-β1 is expressed at lower levels (17 positive clones were detected among 350,000

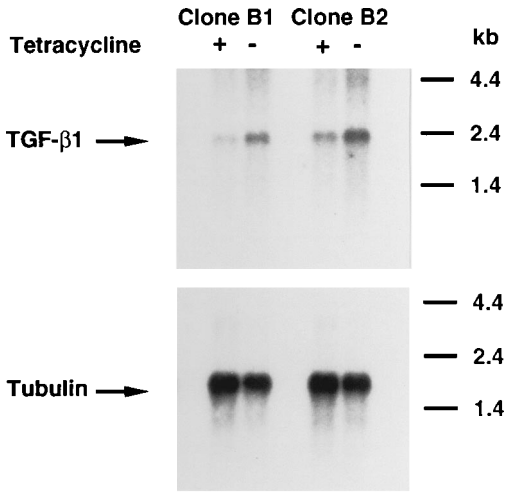

FIG. 4. Northern blot analysis of TGF-β1 mRNA expression in HeLa-Zta transfectants. One microgram of polyadenylated mRNA from two independent HeLa-Zta clones cultured in the presence (+) or absence (−) of tetracycline was separated by electrophoresis through a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a TGF-β1 cDNA probe as described in Materials and Methods. After the removal of bound probe, the blot was reprobed with a ^{32}P-labelled tubulin cDNA fragment to standardize the amount of mRNA loaded in each lane. Numbers at the right indicate sizes of RNA markers.

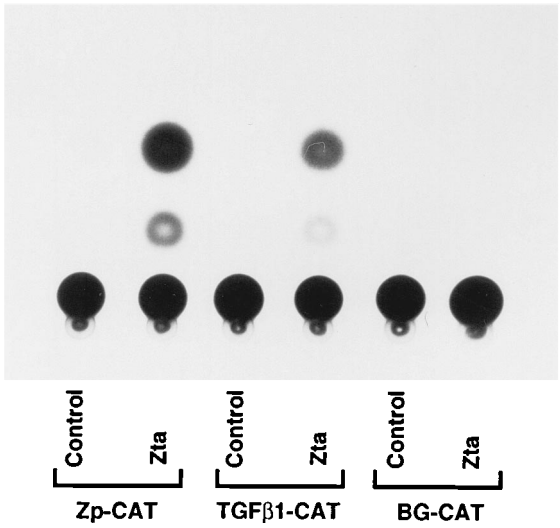

FIG. 5. Transactivation of the TGF-β1 promoter by Zta. Two micrograms of plasmid bearing the TGF-β1 promoter-CAT chimeric gene (TGF-β1-CAT) was cotransfected by the DEAE-dextran method in the EBV-negative Burkitt's lymphoma cell line DG75 with 1 μg of Zta expression vector or control vector, and CAT activity was assayed as described in Materials and Methods. Similar experiments were performed with the negative and positive control reporter plasmids BG-CAT and Zp-CAT, respectively.

plaques, which represents 0.004% of total mRNAs) (data not shown). This level is too low to be detected by differential screening, a method that allows the detection of abundant mRNAs constituting more than about 0.05% of the total mRNA (35).

Zta transactivates the TGF-β1 promoter in B cells. It has been shown previously that TGF-β1 expression is activated during the lytic cycle in B cells and that progression of the lytic cascade is dependent on TGF-β1 secreted into the medium (41). It is not known whether the induction of TGF-β1 expression is due to the inducing agents used to activate the lytic cycle or whether it might be mediated by an EBV-encoded transcription factor. As an initial effort to investigate whether Zta might contribute to activated TGF-β1 expression during the lytic cycle in B cells, we have tested a reporter plasmid containing a TGF-β1 promoter for responsiveness to Zta in the EBV-negative Burkitt's lymphoma cell line DG75. Cotransfection of the TGF-β1-CAT reporter with a Zta expression plasmid, CMV-Zta, in DG75 cells resulted in a 16-fold increase in activity compared with cotransfection with a control plasmid (CMV) (Fig. 5). In parallel, activation of the Zta promoter, Zp, was tested and found to be ca. 27-fold. A reporter plasmid containing the minimal β-globin promoter, BG-CAT was not affected by Zta. We have also performed similar experiments with HeLa cells, but we have not observed activation of TGF-β1-CAT by Zta. At this time we do not know whether this result can be attributed to technical issues or whether it reflects differential regulation. The results obtained with B cells, however, suggest that Zta might contribute to the observed activation of TGF-β1 expression during the lytic cycle in B cells. A determination of whether this translates to activation of the endogenous TGF-β1 gene will require the generation of B-cell lines that conditionally express Zta.

DISCUSSION

In this report, we describe the identification of cellular target genes of the EBV transactivator Zta by employing a HeLa cell line that conditionally expresses Zta. The major target of Zta identified by this analysis is the TGF-βigh3 gene, which was previously isolated as a gene induced by TGF-β1 in different cell types (50). TGF-βigh3 is a secreted extracellular matrix protein that could participate in mediating some effects of TGF-β1 (49). This suggests that TGF-β1 and TGF-βigh3 may act in concert to elicit certain biological effects on cellular metabolism. We therefore tested whether TGF-β1 levels might also be activated by Zta and found that the amounts of both the mRNA and the secreted protein were elevated by Zta expression. Moreover, α1-collagen IV, which has been shown to potentiate the effects of TGF-β1 (39), is also a Zta-responsive gene (induced approximately fourfold). Collagen IV binds TGF-β1 specifically with a high affinity and may protect TGF-β1 from proteolytic degradation and/or allow it to adopt a more stable conformation (39). The coordinate induction of TGF-β1 and collagen IV expression by Zta could be a mechanism for enhancing the biological activity of TGF-β1 secreted by cells expressing Zta.

At this time, we do not know whether the observed increase in active TGF-β1 secreted into the medium is responsible for the observed activation of TGF-βigh3 levels or whether Zta can activate TGF-βigh3 through more direct means. We do not know whether the TGF-β1 signal transduction pathway leading to TGF-βigh3 induction is functional in HeLa cells and/or whether the observed increase of active TGF-β1 would be sufficient to elicit this response. However, it is likely that Zta-induced TGF-β1 levels might at least contribute to activation of TGF-βigh3 expression in some cell systems.

The induction of TGF-β1 by Zta could have many implications for EBV-host relationships. Biologically active TGF-β1 has previously been shown to be secreted by EBV-positive Burkitt's lymphoma B-cell lines expressing Zta, after treatment with agents that induce the EBV lytic cycle (41). Our results suggest that Zta could contribute to the activation of TGF-β1 expression in EBV-positive B cells during the lytic cycle. Moreover, Zta could also contribute to the induction of TGF-β1 expression that is observed in tonsillar epithelial cells during

infectious mononucleosis (2). Primary EBV infection can elicit a transient, but profound, immunodepression in immunocompetent individuals (14). This general depression of cell-mediated immune functions, which is particularly evident for patients in the acute phase of infectious mononucleosis, has been attributed to a broad-ranging suppressor T-cell activity (40, 42). However, TGF-β1 is a potent immunosuppressor (44), and release of active TGF-β1 by lytically infected epithelial cells of the oropharynx could also contribute to the transient immunosuppression observed in patients with acute infectious mononucleosis. Induction of TGF-β1 has recently been proposed to play a role in the immunosuppression observed during human T-lymphotropic virus type 1 and CMV infections (29, 36). This suggests that induction of TGF-β1 could be a general mechanism used by human viruses to downregulate host immune responses.

Induction of TGF-β1 by Zta could also play a role in the spreading of EBV in the epithelium. TGF-β1 is a potent costimulator for IgA production (11, 26), and IgA promotes infection of the epithelium by allowing EBV entry in epithelial cells (48). Moreover, TGF-β1 has been shown to act synergistically with the lytic EBV BCRF1 gene product, encoding a viral homolog of interleukin-10, to enhance IgA production (13). Together these results suggest that two EBV lytic proteins, Zta (by inducing TGF-β1) and BCRF1, could cooperate to enhance IgA production and thus promote spreading of EBV in the epithelium.

Another gene that we have identified as a cellular target of Zta encodes an MHC class I molecule which plays an important role in the immune system by presenting short peptides from viral antigens to $CD8^+$ T cells. Induction of MHC class I gene expression has previously been reported in the cases of many other viruses (43), including human CMV (7) and hepatitis B virus (56). The induction of MHC class I expression by Zta in lytically infected epithelial cells of the oropharynx or recirculating B lymphocytes could be one of the factors contributing to the massive expansion of $CD8^+$ T cells that is observed during acute EBV infection (40, 52). Although this stimulation of $CD8^+$ T cells was initially thought to be detrimental to the virus, recent studies have shown that most of these activated $CD8^+$ T cells undergo apoptotic cell death and are thus eliminated (53). Therefore, intense stimulation of $CD8^+$ T cells during acute EBV infection could be an important mechanism that helps to allow persistence of EBV in an immunocompetent host, as has been shown in the cases of other viruses (37).

In conclusion, we have used a powerful tetracycline-regulated expression system to identify cellular target genes of the EBV lytic swich transactivator Zta. A similar strategy was successfully used to identify p21/WAF1, a cyclin-dependent kinase inhibitor, as a major cellular target of the tumor suppressor protein p53 (6). In our study, using differential hybridization screening procedures, we have characterized abundant cellular targets of Zta. This substantiates the idea that in addition to activating viral gene expression, Zta may also play a role in altering cellular gene expression. Moreover, the activation of such genes might be fundamentally linked to the viral life cycle. However, it should be pointed out that while HeLa cells have epitheloid characteristics, they are highly transformed. Therefore, to extrapolate our findings to normal epithelium or B lymphocytes, it will be necessary to repeat these studies with other epithelial and B-cell lines. In addition, future experiments, using more sensitive screening procedures (e.g., subtractive hybridization or differential display) should identify other, less abundant cellular targets of Zta that could also have important functions in EBV-host interactions.

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