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Induction of the Early Growth Response 1 Gene by Epstein-Barr Virus Lytic Transactivator Zta

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Early growth response 1 (Egr-1) is a cellular transcription factor involved in diverse biologic functions. Egr-1 has been associated with Epstein-Barr virus (EBV) infection, but it is still unknown whether any EBV protein regulates Egr-1 expression. In this study, we first showed that EBV reactivation is involved in upregulation of Egr-1 and that Egr-1 can be induced by Zta, an EBV lytic transactivator. Zta not only binds to the Egr-1 promoter but also activates the ERK signaling pathway to trigger binding of Elk-1 to the Egr-1 promoter. In addition, knockdown of Egr-1 significantly reduces the spontaneous expression of Zta and Rta in EBV-infected 293 cells, suggesting that a positive-feedback network involving Egr-1 is required for EBV reactivation. This study also implies that Zta has the potential to affect expression of certain genes through Egr-1.

Early growth response 1 (Egr-1), also designated zif268, NGFI-A, and Krox24, is a cellular transcription factor belonging to a family of zinc finger DNA-binding proteins (49). According to its diverse target genes, Egr-1 has been associated with a broad range of biologic functions such as cell proliferation (3, 4, 41), apoptosis (39, 51, 54), and differentiation (28, 50) in a cell-type-dependent manner. Encoded by an immediate-early gene, Egr-1 can be rapidly induced by many stimuli, including growth factors, cytokines, and various stresses (6, 31, 44, 46). Most of the stimuli trigger cellular signaling converged to mitogen-activated protein kinase (MAPK) pathways, which lead to phosphorylation and activation of a ternary complex factor, Elk-1 (26, 31, 46). Activated Elk-1 interacts with the serum response factor (SRF) to form a ternary complex that binds to a DNA element composed of a core serum response element (SRE) and the adjacent Ets motif (25, 52). There are five such binding sites for the ternary complex in the promoter of the Egr-1-encoding gene, and activation of signal transduction from MAPKs to the ternary complex is essential for Egr-1 expression induced by various stimuli (26, 31, 45, 46).

Several clues indicate that Egr-1 is also linked to infection with Epstein-Barr virus (EBV), a human gammaherpesvirus closely associated with several lymphoid and epithelial malignancies (43). First, Egr-1 is upregulated when EBV interacts with B lymphocytes at the initial infection stage, and constitutive expression of Egr-1 correlates with certain types of EBV latency in B-lymphoid cell lines (5). Notably, Egr-1 has also been associated with the lytic state of EBV infection (56). EBV reactivation into the lytic cycle is initiated from activation of two immediate-early viral promoters, Zp and Rp, which are driven to express the BZLF1 (Zta) and BRLF1 (Rta) proteins, respectively (22). Both Zta and Rta are key lytic transactivators which autostimulate their own expression, reciprocally induce each other, and cooperatively direct the downstream expression cascade of EBV lytic genes (18, 20, 42, 48). A previous study showed that Egr-1 can activate Rp and identified two Egr-1-binding sites in the promoter (56). These two Egr-1-binding sites are required for activation of Rp by several EBV reactivation-inducing agents, such as phorbol ester, gemcitabine, and doxorubicin, further suggesting that Egr-1 may play an important role in lytic switching (19, 56).

In this study, we explore how Egr-1 expression is regulated and involved in the EBV lytic cycle. Although Egr-1 may be upregulated by certain EBV reactivation-triggering stimuli (37, 56), it is still unknown whether any EBV lytic protein contributes to the induction of Egr-1. We first observed that EBV reactivation is required for upregulation of Egr-1 in some cell lines and that Egr-1 can be induced by ectopic expression of Zta. Further examination revealed that Zta utilizes at least two ways to upregulate Egr-1 expression: Zta not only binds to the Egr-1 promoter but also activates ERK, one of the MAPKs, to trigger binding of Elk-1 to the Egr-1 promoter. In addition, small interfering RNA (siRNA)-directed knockdown of Egr-1 significantly reduces the spontaneous expression of Zta and Rta in EBV-infected 293 cells, suggesting that a positive-feedback network involving Egr-1 is required for EBV reactivation. This study also implies that Zta has the potential to affect the expression of certain genes through Egr-1.

EBV reactivation is involved in upregulation of Egr-1. Recently we have established stable clones of EBV-infected 293 cells (293A) and found that some cell clones (designated lytic clones) exhibit spontaneous expression of EBV lytic genes such as those for Zta, Rta, and BMRF1 (10, 11). Compared with uninfected and latently infected counterpart cell clones, the 293A lytic clones showed significant upregulation of Egr-1 (Fig. 1A). To clarify the association between EBV reactivation and Egr-1 induction, we used a Zta-targeted siRNA, siZ1, to block the expression cascade of viral lytic genes in a lytic clone,

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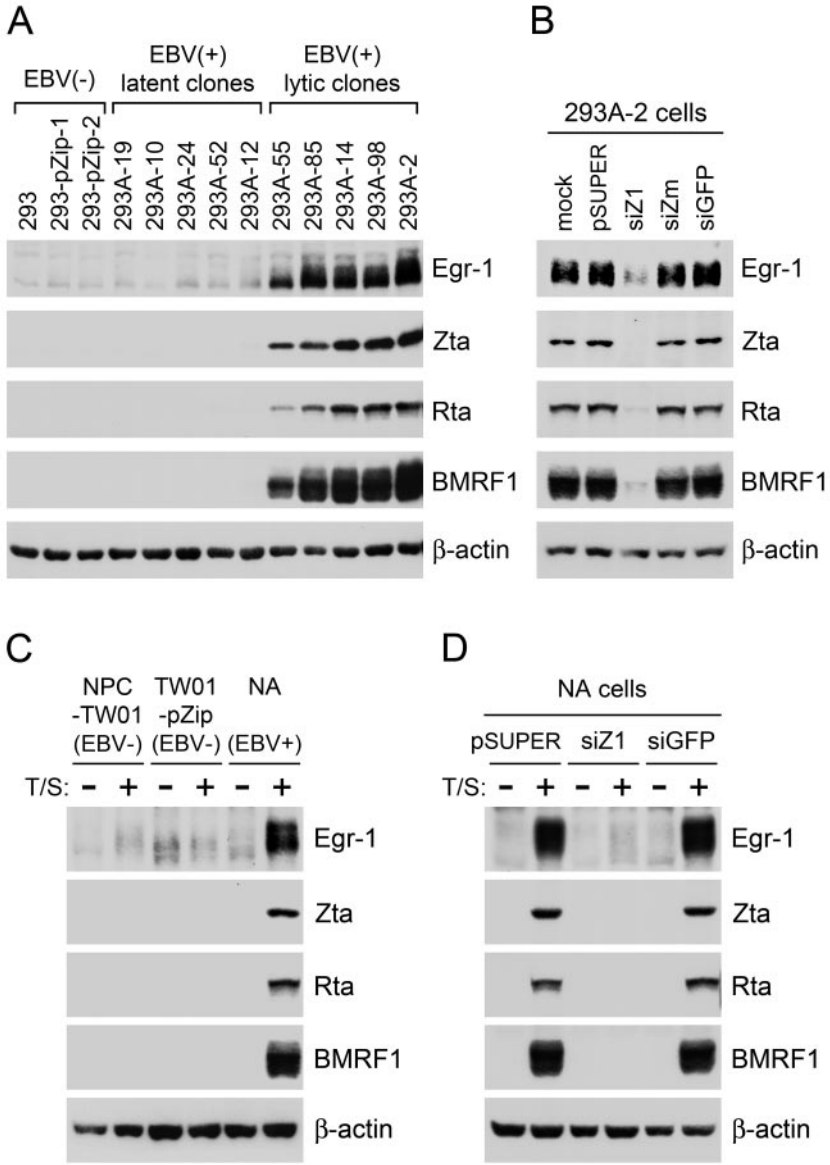

FIG. 1. Upregulation of Egr-1 is a downstream event of EBV reactivation. (A) Induction of Egr-1 is associated with expression of EBV lytic genes in 293A cells. Ten subclones of EBV-infected 293A cells, as well as parental 293 cells and two G418-resistant transfectants (293-pZip-1 and -2), were examined for protein expression with an immunoblotting assay described previously (10, 11). Primary antibodies used in this assay included C-19 (anti-Egr-1; Santa Cruz Biotechnology), 4F10 (anti-Zta), 467 (anti-Rta), 88A9 (anti-BMRF1), and AC-15 (anti-β-actin; Sigma). Egr-1 proteins were detected as a broad band, which has been reported previously and is likely to be attributed to posttranslational modifications (5). (B) Zta-targeted siRNA siZ1 inhibits both spontaneous EBV reactivation and expression of Egr-1 in 293A-2 cells. Vector plasmid pSUPER or a derivative expressing siZ1, mutated siZ1 (siZm), or an irrelevant siRNA (siGFP) was transfected into 293A-2 cells as described previously (10, 11), and protein expression was examined with an immunoblotting assay. (C) Induction of Egr-1 is associated with the EBV lytic cycle induced by chemicals. EBV-positive NA cells and their EBV-negative counterparts, i.e., parental cell line NPC-TW01 and its G418-resistant transfectant TW01-pZip, were treated with (+) or without (−) 40 ng/ml TPA plus 3 mM sodium *n*-butyrate (T/S) for 24 h and then subjected to an immunoblotting assay. (D) Inhibition of EBV lytic expression by siZ1 blocks the induction of Egr-1 in NA cells. This experiment was carried out as described previously (10). NA cells were pretransfected with vector plasmid pSUPER or siRNA-expressing plasmids for 48 h, followed by treatment with (+) or without (−) TPA plus sodium *n*-butyrate (T/S) for 24 h and then subjected to an immunoblotting assay.

293A-2 (10). As has been proven in our previous studies (10, 11), transfection with a plasmid expressing siZ1, but not a mutated or irrelevant siRNA, efficiently inhibited the expression of all of the EBV lytic genes examined (Fig. 1B). Notably, the siZ1-directed reduction of EBV lytic expression was accompanied by a decrease in Egr-1 (Fig. 1B), suggesting that expression of EBV lytic genes is required for upregulation of Egr-1.

Supporting evidence was further obtained from the EBV reactivation induced by chemicals in an EBV-infected nasopharyngeal carcinoma cell line, NA (12). Treatment of NA cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and

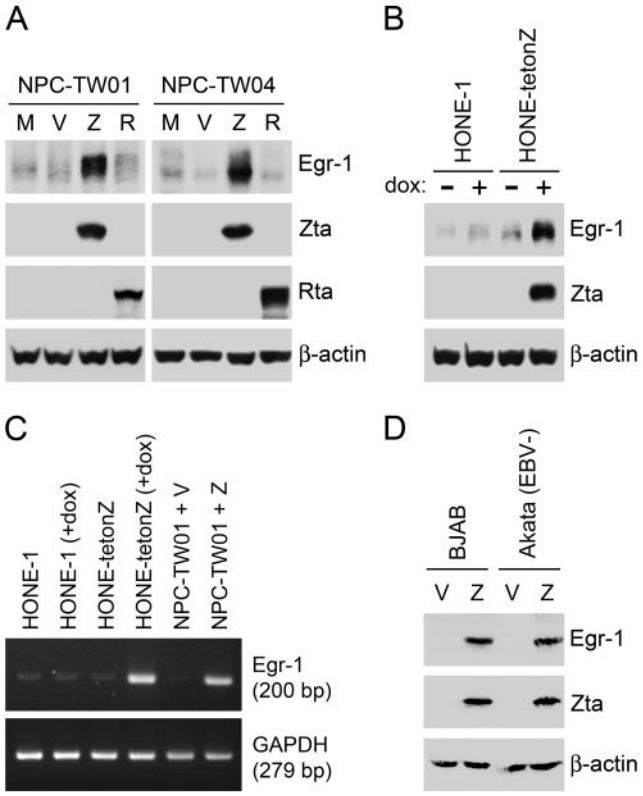

FIG. 2. Zta induces Egr-1 expression. (A) EBV-negative NPC-TW01 and NPC-TW04 cells were mock transfected (M) or transfected with vector plasmid pSG5 (V) or a pSG5-derived plasmid expressing Zta (Z) or Rta (R) by using Lipofectamine 2000 reagent (Invitrogen). Protein expression was examined with an immunoblotting assay. (B) A tetracycline-regulated, Zta-expressing cell line, HONE-tetonZ, and control cell line HONE-1 were treated with (+) or without (−) 1 μg/ml doxycycline (dox) for 24 h and then subjected to an immunoblotting assay. (C) Reverse transcription-PCR assay for Egr-1 transcripts. Cells were treated as described for panels A and B and then subjected to an assay to detect expression of Egr-1 and the internal control GAPDH glyc(glyceraldehyde-3-phosphate dehydrogenase). The details of the primers and PCR conditions used were described previously (47). (D) EBV-negative B-lymphoid cell lines BJAB and Akata were transfected with the vector plasmid (V) or the Zta-expressing plasmid (Z) by electroporation following our previously described protocol (11). Protein expression was examined with an immunoblotting assay.

sodium *n*-butyrate stimulated expression of EBV lytic genes, and meanwhile, Egr-1 was also significantly increased (Fig. 1C). The same treatment did not upregulate Egr-1 in EBV-negative counterparts of NA cells, i.e., parental cell line NPC-TW01 and its G418-resistant transfectant TW01-pZip, implying that the EBV lytic cycle contributes to the induction of Egr-1 (Fig. 1C). In agreement with this hypothesis, siZ1-mediated inhibition of EBV lytic expression blocked upregulation of Egr-1 in chemically activated NA cells (Fig. 1D), indicating that induction of Egr-1 is under the control of at least one lytic gene product of EBV.

Zta induces Egr-1 expression. In search of a lytic protein to induce Egr-1, we transfected plasmids expressing individual lytic genes into two EBV-negative epithelial cell lines, NPC-TW01 and NPC-TW04 (32), and examined Egr-1 expression with an immunoblotting assay. Figure 2A shows that ectopic expression of Zta, but not Rta, increased the expression of Egr-1. In a tetracycline-regulated, Zta-expressing epithelial cell line, HONE-tetonZ (34), doxycycline-induced Zta expression also triggered the upregulation of Egr-1 (Fig. 2B). Some other EBV proteins, such as BMRF1, BGLF4, and LMP1, were not found to induce Egr-1 (data not shown). In a reverse transcription-PCR analysis, Zta increased Egr-1 transcripts in both NPC-TW01 and HONE-tetonZ cells, suggesting that Zta-mediated induction of Egr-1 occurs at a transcriptional level (Fig. 2C). Confirming this induction in another cell type, we demonstrated that ectopic expression of Zta also upregulated Egr-1 in two EBV-negative B-lymphoid cell lines, BJAB and Akata (Fig. 2D).

The Egr-1 promoter contains two responsive regions for Zta-mediated activation. Next, reporter gene assays were performed to study whether and how Zta activates the promoter of the gene for Egr-1. As illustrated in Fig. 3A, a series of Egr-1 promoter regions were cloned into the pGL2-basic plasmid (Clontech) to drive the firefly luciferase gene. Zta induced luciferase activities more than fourfold if the reporter construct covered positions −457 to +9 of the Egr-1 promoter (Fig. 3B). Deletion of the promoter region from position −457 to position −439 reduced half of the Zta-mediated transactivation, and further deletion of positions −439 to −395 abolished the residual responsiveness to Zta (Fig. 3B). This result suggests that there are at least two Zta-responsive regions in the Egr-1 promoter; one is between positions −457 and −439, while the other is between positions −439 and −395.

Within the promoter region from position −457 to position −439, we found two adjacent copies of elements imperfectly matching (with one nucleotide aberration) the consensus sequence of the Zta response element (ZRE), T-G/T-A/T-G-C/T-G/A/C-A (Fig. 3A) (13, 29). It is well documented that Zta can activate its target promoters through direct binding to DNA sequences which match or slightly deviate from the ZRE consensus (20, 21, 35, 48). The second Zta-responsive region, between positions −439 and −395, of the Egr-1 promoter contains the most upstream SRE-Ets site targeted by the SRF-Elk-1 ternary complex (Fig. 3A). It has been reported that the upstream SRE-Ets is the major responsive element for activation of the Egr-1 promoter triggered by various stimuli (15, 46). As shown in Fig. 3C, site-directed mutation of either the putative ZREs or the indicated Ets (Elk-1-binding) elements diminished the Zta-mediated transactivation of the Egr-1 promoter, and dual mutation of both elements completely removed the responsiveness to Zta, further supporting the idea that both the ZREs and the Ets elements are involved in the Zta-induced activation of the Egr-1 promoter.

Zta and Elk-1 bind to their corresponding elements in the Egr-1 promoter. To demonstrate that Zta contributes to DNA binding of Zta itself and Elk-1 to the identified Zta-responsive regions of the Egr-1 promoter, an electrophoretic mobility shift assay (EMSA) and a DNA affinity precipitation assay (DAPA) were carried out. Figure 4B shows that Zta formed a complex with the EMSA probe which covers the ZREs of the Egr-1 promoter. The DAPA experiment further confirmed that the ZRE-containing probe precipitated Zta proteins while the probe with mutated ZREs did not (Fig. 4C). Zta also induced an Elk-1-containing complex that bound to the DNA probe covering the most upstream SRE-Ets region of the Egr-1

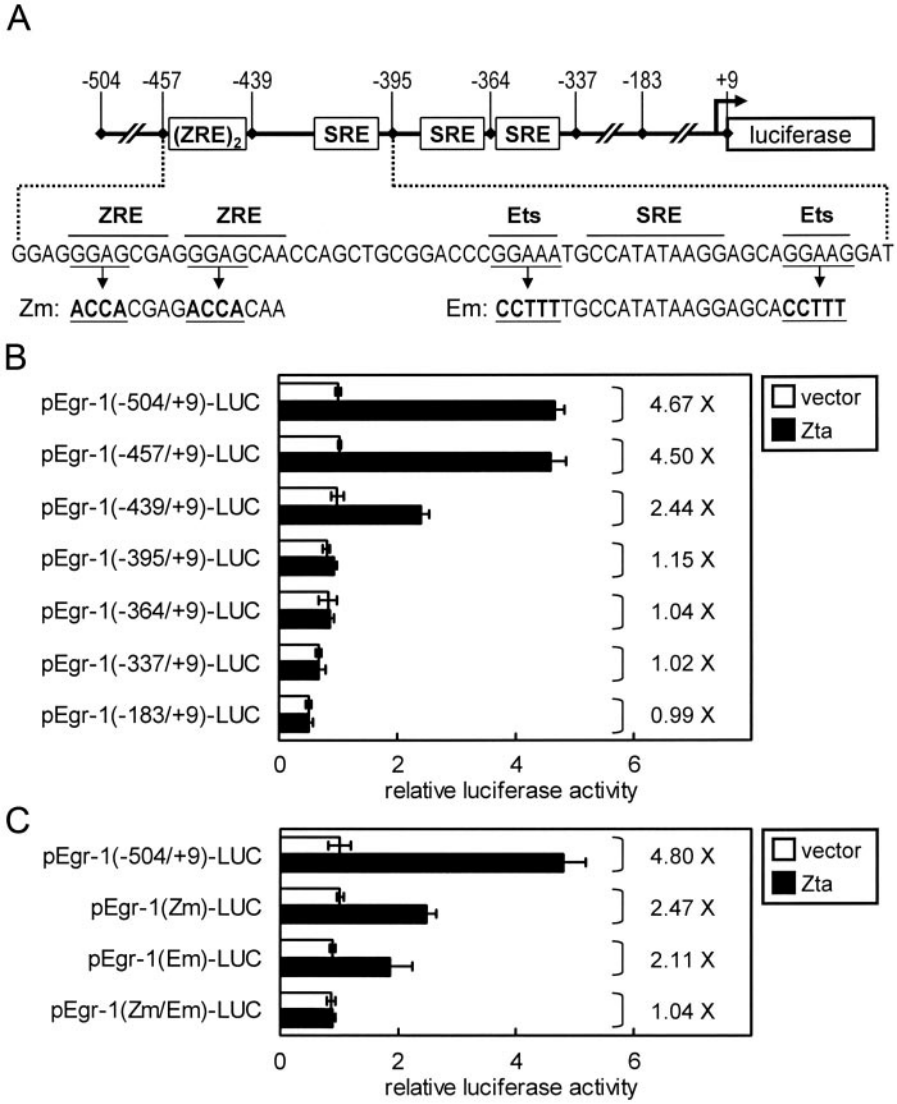

FIG. 3. The Egr-1 promoter contains two responsive regions for Zta-mediated activation. (A) Schematic illustration of the Egr-1 promoter that drives the luciferase gene in the reporter plasmid. The promoter sequence between positions −457 and −395 containing the putative ZREs and well-documented SRE-Ets is shown. Mutated sequences introduced into ZRE (Zm) and Ets (Em) are also indicated. (B) Identification of Zta-responsive regions in the Egr-1 promoter. EBV-negative NPC-TW01 cells were transfected with the indicated reporter plasmids in conjunction with either a control vector or a Zta-expressing plasmid. Detailed conditions of the reporter gene assay were described previously (11). Shown is a representative result from three repeated experiments. The Zta-induced fold activation ($n \times$) for each reporter is provided. (C) Mutagenesis study of the Zta-responsive regions in the Egr-1 promoter. Single or dual mutations of the ZREs (Zm) and Ets (Em) elements were introduced into reporter plasmid pEgr-1(−504/+9)-LUC. This experiment was performed and results are presented in the same way as that in panel B.

promoter, and the complex did not form if the Ets sequences were mutated (Fig. 4D). Since expression of Zta did not affect the amount of Elk-1 proteins in the nuclear extract (Fig. 4E), it is likely that Zta activates the preexisting Elk-1 proteins to form the complex binding to the Egr-1 promoter.

The ERK signaling pathway is activated by Zta and is important for Zta-mediated induction of Egr-1. As has been mentioned, formation of the SRF-Elk-1 ternary complex binding to SRE-Ets is attributed to activation of MAPK signaling pathways (25, 31). Since Zta induced a similar Elk-1-containing complex binding to the Egr-1 promoter (Fig. 4D), we speculated that Zta may also trigger a similar signaling pathway(s). To test which MAPK is activated by Zta, we examined the phosphorylation statuses of three MAPKs (ERK, p38, and JNK) in the absence or presence of Zta expression. Figure 5A shows that phosphorylated (i.e., activated) forms of p44/p42 ERKs were prominently increased by Zta, while Zta had little effect on the phosphorylation of p38 MAPK and p54/p46 JNKs. Treatment with an inhibitor of ERK activation, U0126 or PD98059, not only repressed the basal level of Egr-1 but also reduced the Zta-mediated upregulation of Egr-1 (Fig. 5B). On the other hand, inhibitors of p38 or JNK did not affect Egr-1 expression (data not shown). Treatment with U0126 blocked Zta-induced phosphorylation of p44/p42 ERKs and impaired Zta-triggered binding of Elk-1 to the Egr-1 promoter, while the amount of Elk-1 was not affected (Fig. 5C). There-

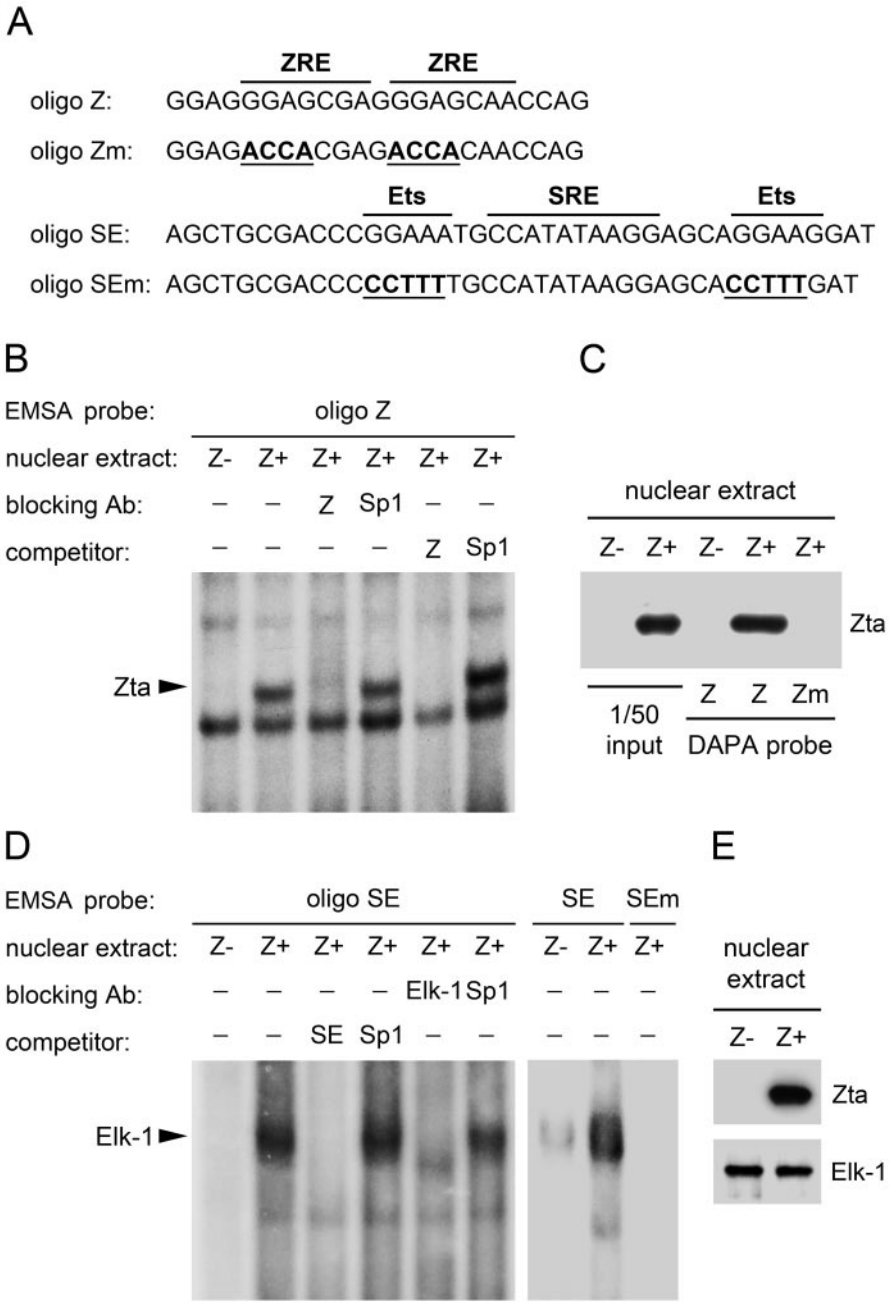

FIG. 4. Zta contributes to binding of Zta and Elk-1 to their corresponding elements in the Egr-1 promoter. (A) DNA sequences of the probes used in EMSA and DAPA experiments. Oligo Z represents the Egr-1 promoter region from position −457 to position −435, which contains two ZREs, and oligo SE represents the region from position −436 to position −395, which covers the most upstream SRE-Ets of the Egr-1 promoter. Shown are the sequences of one strand of the double-stranded DNA probes. (B) Binding of Zta to ZREs. Nuclear extracts with or without Zta expression (Z+ or Z−) were examined by EMSA. Oligonucleotides used as competitors included oligo Z and oligo Sp1 (Santa Cruz Biotechnology), and antibodies (Ab) used to identify specific complexes included 1B4 (anti-Zta) and PEP 2 (anti-Sp1; Santa Cruz Biotechnology). The shifted bands representing DNA binding of Zta are indicated by arrowheads. (C) Identification of the DNA-binding specificity of Zta. A DAPA experiment was performed as described previously (9). A biotin-labeled DNA probe (oligo Z or Zm) was used to bind proteins in the nuclear extracts with or without Zta expression (Z+ or Z−). The DNA-bound proteins were precipitated by magnetic beads (Promega) and then subjected to an immunoblotting assay with an anti-Zta antibody, 4F10. (D) Zta-induced binding of Elk-1 to the Egr-1 promoter. An EMSA was carried out as for panel B. Antibodies used to identify specific complexes included I-20 (anti-Elk-1; Santa Cruz Biotechnology) and PEP 2 (anti-Sp1). The shifted bands representing DNA binding of Elk-1 are indicated by arrowheads. (E) Immunoblotting assay to detect Zta and Elk-1 in nuclear extracts. The antibodies used were 4F10 (anti-Zta) and I-20 (anti-Elk-1).

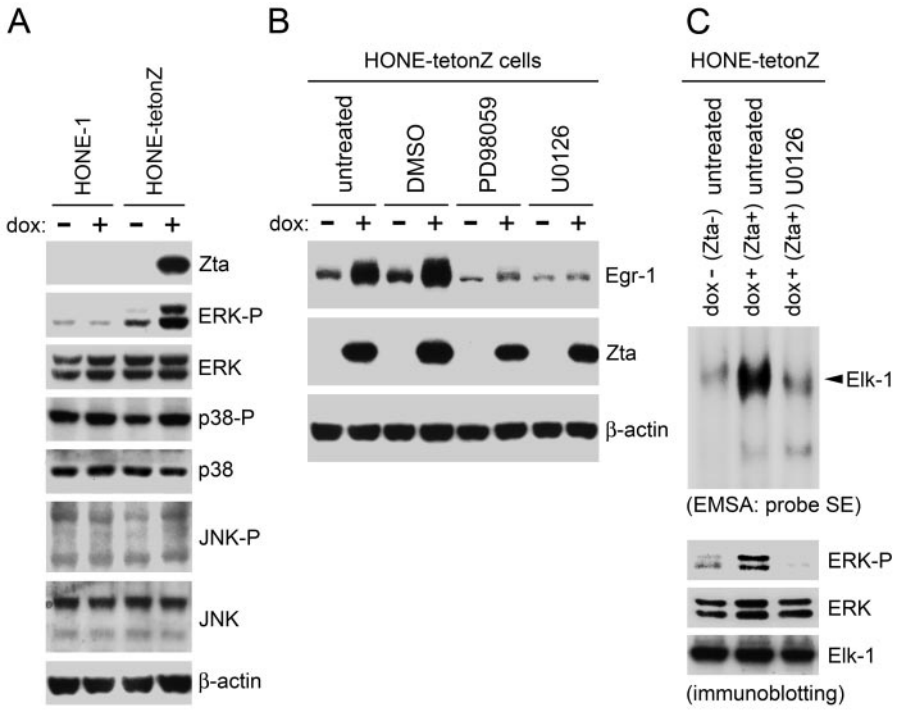

FIG. 5. The ERK signaling pathway is activated by Zta and is important for Zta-mediated induction of Egr-1. (A) Activation of the ERK pathway by Zta. HONE-tetonZ and control HONE-1 cells were treated with (+) or without (−) doxycycline (dox) for 24 h and then subjected to an immunoblotting assay. Antibodies used to detect phosphorylated and total forms of p44/p42 ERK, p38 MAPK, and p54/p46 JNK were purchased from Cell Signaling Technology. (B) Reduction of Zta-induced Egr-1 expression by inhibition of the ERK signaling pathway. HONE-tetonZ cells were left untreated or pretreated with solvent only (dimethyl sulfoxide) or inhibitors of ERK signaling (PD98059, 50 μM; U0126, 20 μM; Calbiochem) for 1 h and then subjected to treatment with doxycycline (dox) for 24 h. Protein expression was examined with an immunoblotting assay. (C) Inhibition of ERK activation impairs Elk-1 binding to the Egr-1 promoter. Nuclear extracts treated as indicated were subjected to EMSA with probe SE, which covers the most upstream SRE-Ets of the Egr-1 promoter (Fig. 4A and D). An immunoblotting assay was also performed to detect Elk-1 and phosphorylated and total p44/p42 ERKs in the nuclear extracts.

fore, the ERK signaling pathway activated by Zta contributes to both the DNA-binding activity of Elk-1 and the induction of Egr-1 expression.

Knockdown of Egr-1 expression reduces spontaneous EBV reactivation in 293 cells. It is known that (i) Zta activates Zp and Rp (20, 48), (ii) Rta activates Zp and Rp (1, 48), and (iii) Egr-1 activates Rp (56). This study further adds the finding that Zta can activate the Egr-1 promoter. It is likely that interstimulation among Zta, Rta, and Egr-1 forms a positive-feedback network that augments the expression of these proteins by one another during EBV reactivation. If Egr-1 is important for the positive regulatory network, inhibition of Egr-1 expression should impair the efficient accumulation of Zta and Rta in the EBV lytic cycle. We tested this hypothesis by using EBV-infected 293A lytic clones in which spontaneous expression of Zta, Rta, and Egr-1 is maintained at a moderate level and is readily detected (Fig. 1A). In 293A-2 and 293A-98 cells, transfection with Egr-1-targeted siRNA not only knocked down Egr-1 expression but also significantly reduced the levels of Zta, Rta, and a downstream lytic gene, that for BMRF1 (Fig. 6). Meanwhile, expression of an EBV latent gene, that for EBNA1, was not inhibited by the Egr-1-targeted siRNA, and a control siRNA did not affect the expression of the EBV genes examined (Fig. 6). This result indicates that Egr-1 is required

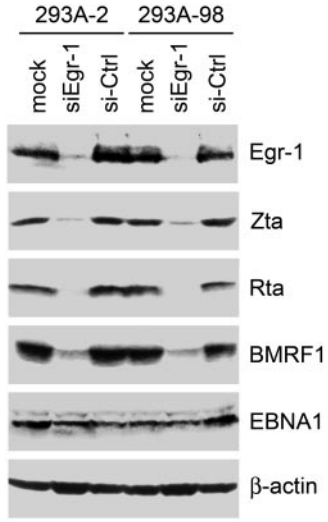

FIG. 6. Knockdown of Egr-1 expression reduces spontaneous EBV reactivation in 293 cells. Two 293A lytic clones were mock transfected or transfected with Egr-1-targeted siRNA (siEgr-1) or a control siRNA (si-Ctrl) twice within 1 week according to the manufacturer's (Invitrogen) instructions. An immunoblotting assay was carried out to detect the expression of the indicated proteins.

for efficient expression of Zta and Rta in spontaneous EBV reactivation.

Discussion. Upregulation of Egr-1 has been observed in cells infected with many viruses, such as human T-cell leukemia virus, human immunodeficiency virus, and vaccinia virus (2, 53, 55). The human T-cell leukemia virus type 1 Tax protein directly interacts with SRF to activate the target promoter (23), while vaccinia virus stimulates the ERK pathway to induce Egr-1 expression (2). Here we provide the first report of how Egr-1 is upregulated by EBV. The EBV lytic transactivator Zta induces Egr-1 expression in at least two ways. One is binding of Zta to the ZREs in the Egr-1 promoter, and the other is Zta-triggered activation of the ERK signaling pathway, which activates binding of the Elk-1-containing complex to the upstream SRE-Ets site in the Egr-1 promoter. According to the results of our reporter gene assays, the ZREs and the upstream Ets elements may contribute to Zta-induced activation of the Egr-1 promoter in an additive manner. In addition, our study with specific inhibitors supported the idea that ERK signaling is important for Zta-induced DNA binding of Elk-1 and for Zta-mediated upregulation of Egr-1.

Our data suggest a more complicated regulatory network involving Egr-1 for spontaneous EBV reactivation in 293 cells. siRNA-directed inhibition of Zta and Rta decreased Egr-1 expression (Fig. 1B), and knockdown of Egr-1 also reduced the spontaneous expression of Zta and Rta in 293A lytic clones (Fig. 6). It is likely that the constitutive expression of Zta, Rta, and Egr-1 in these cells is sustained by the interstimulation among the three transactivators; removal of one member in the positive-feedback network impairs the expression of the others. In this case, reciprocal induction between Zta and Rta is probably inefficient, so Egr-1 may act as an essential mediator to augment their expression. Interestingly, simultaneous activation of Zp, Rp, and the Egr-1 promoter has been observed after treatment with some EBV lytic inducers (16, 19, 37). The coexpression and interstimulation of the three transactivators are likely to promote a condition which efficiently drives the expression cascade of the EBV lytic cycle.

A previous study found that Zta can activate p38 and JNK (1). In our cell system, however, Zta has little effect on these two MAPKs and instead triggers the activation of another MAPK pathway, ERK. Possibly, Zta exerts differential regulation of the MAPKs in a cell-dependent manner. MAPK signaling pathways can also be activated by Rta and mediate Rta-triggered activation of Zp (1). Here we show that ERK activation contributes to Zta-induced Egr-1 expression, providing another possible mechanism by which MAPKs are involved in EBV reactivation. Indeed, inhibition of the p38, JNK, or ERK pathway has been shown to reduce EBV reactivation in previous studies, supporting the idea that cellular signaling pathways through MAPKs play multiple and important roles in the EBV lytic cycle (1, 17, 24).

This study also raises the possibility that Zta has the potential to affect the expression of certain genes through Egr-1. Several Zta-targeted genes have been reported to be regulated by Egr-1. For example, Zta can upregulate transforming growth factor β1 and p53 but the mechanisms remain unclear (7, 8, 36). Intriguingly, the genes for transforming growth factor β1 and p53 are two target genes induced by Egr-1 (30, 33, 39). Another clue comes from the observation that Zta suppresses an EBV latent promoter, Cp, during the lytic cycle (27). It has also been suggested that Egr-1 acts as a negative regulator of Cp activity (40). In addition, a previous study showed that Zta inhibits the transcriptional function of p65 NF-κB (38) and Egr-1 has been shown to repress p65 activity (14). It would be interesting to examine if Egr-1 is really involved in the Zta-mediated gene regulation mentioned above. Notably, Zta can also upregulate c-Fos, another cellular transcription factor (21). Through induction of immediate-early proteins like Egr-1 and c-Fos, Zta may efficiently expand the influences on gene regulation and affect a broad range of cellular activities.

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