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Repression of CIITA by the Epstein–Barr virus transcription factor Zta is independent of its dimerization and DNA binding

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Repression of the cellular *CIITA* gene is part of the immune evasion strategy of the γherpes virus Epstein–Barr virus (EBV) during its lytic replication cycle in B-cells. In part, this is mediated through downregulation of MHC class II gene expression via the targeted repression of *CIITA*, the cellular master regulator of MHC class II gene expression. This repression is achieved through a reduction in *CIITA* promoter activity, initiated by the EBV transcription and replication factor, Zta (*BZLF1*, EB1, ZEBRA). Zta is the earliest gene expressed during the lytic replication cycle. Zta interacts with sequence-specific elements in promoters, enhancers and the replication origin (ZREs), and also modulates gene expression through interaction with cellular transcription factors and co-activators. Here, we explore the requirements for Zta-mediated repression of the *CIITA* promoter. We find that repression by Zta is specific for the *CIITA* promoter and can be achieved in the absence of other EBV genes. Surprisingly, we find that the dimerization region of Zta is not required to mediate repression. This contrasts with an obligate requirement of this region to correctly orientate the DNA contact regions of Zta to mediate activation of gene expression through ZREs. Additional support for the model that Zta represses the *CIITA* promoter without direct DNA binding comes from promoter mapping that shows that repression does not require the presence of a ZRE in the *CIITA* promoter.

INTRODUCTION

Epstein–Barr virus infects people and has a life-long association with them, occasionally causing diseases including infectious mononucleosis, Burkitt's lymphoma, Hodgkin's lymphoma and nasopharygeal carcinoma (Magrath, 2012; Molyneux *et al.*, 2012; Saha & Robertson, 2011). Epstein–Barr virus infects human B-lymphocytes and epithelial cells and establishes long-term latency in memory B-lymphocytes (Babcock *et al.*, 1998). These cells are largely protected from immune attack by the silencing of viral gene expression. The virus is sporadically reactivated following B-cell activation and differentiation into plasma cells (Crawford & Ando, 1986; Laichalk *et al.*, 2002; Laichalk & Thorley-Lawson, 2005). As EBV enters the lytic replication cycle, it expresses around 90 viral genes that are required for the regulation of viral gene expression, replication of the viral genome, assembly, packaging and egress of the virion (Farrell, 2005). Many viral genes expressed during viral lytic replication are excellent targets for immune recognition (Adhikary *et al.*, 2006; Long *et al.*, 2011). Attack by the immune system during viral replication would threaten cell survival and thus the successful generation of virions, but EBV has evolved several strategies to evade immune responses during viral lytic replication (Zuo & Rowe, 2012).

An important regulator of EBV lytic replication, termed Zta (BZLF1, ZEBRA, EB1), is a transcription factor, a replication factor and it disrupts several signal transduction pathways (Kenney, 2007). Routes by which Zta activates gene expression have been documented for both viral and host promoters. Many promoters are targeted by the interaction of the sequence-specific DNA-binding domain of Zta with sequence-specific 7 nt DNA elements, termed ZREs (Adamson & Kenney, 1999; Bergbauer *et al.*, 2010; Bhende *et al.*, 2004, 2005; Broderick *et al.*, 2009; Dickerson *et al.*, 2009; Flower *et al.*, 2011; Holley-Guthrie *et al.*, 1990; Kalla *et al.*, 2012, 2010; Karlsson *et al.*, 2008; Kenney *et al.*, 1989; Ramasubramanyan *et al.*, 2012a, b; Sinclair, 2003; Sinclair *et al.*, 1991; Woellmer *et al.*, 2012). At least 32 distinct ZRE sequence variants are specifically recognized by Zta (Flower *et al.*, 2011).

Downregulation of gene expression by Zta has been documented for the TNFR1 gene, through the cellular C/EBP genes (Bristol *et al.*, 2010). Additionally, post-translational

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modifications of Zta have been shown to reduce the ability of Zta to regulate gene expression, specifically phosphorylation at residue S209 (Asai *et al.*, 2009) and sumoylation through residue K12 (Hagemeier *et al.*, 2010; Murata *et al.*, 2010).

Zta has been shown to downregulate the expression of the master regulator of MHC class II gene expression, *CIITA*, in an EBV-positive B-cell line, with both protein and RNA levels decreasing following induction of EBV lytic cycle activation (Li *et al.*, 2009). The product of *CIITA* is a non-DNA-binding cellular transcriptional co-activator, which acts through interaction with DNA-bound proteins that lack integral activation domains. *CIITA* activates the expression of MHC class II genes (Chang *et al.*, 2002) and the reduced expression of *CIITA* observed in B-cells undergoing lytic cycle correlates with the reduced expression of MHC class II observed at the cell surface (Li *et al.*, 2009). Repression of *CIITA* gene expression is also driven by the related γherpesvirus, KSHV (Cai *et al.*, 2013). Here, we investigate the route by which Zta represses *CIITA* expression.

RESULTS

CIITA promoter is specifically repressed by the EBV Zta protein

The effect of Zta expression on the activity of the *CIITA* promoter and the viral *BHLF1* promoter were compared in EBV-positive Raji cells following co-transfection of reporter constructs with an expression vector for a polyhistidine-tagged version of Zta (Bailey *et al.*, 2009). The impact of Zta expression was expressed relative to the maximal activity for each promoter (Fig. 1a, b). Expression of Zta repressed the *CIITA* promoter, whilst in the same experiment it dramatically activated expression of a viral promoter containing ZREs (*BHLF1*). This provided confirmation of the results of Li *et al.* showing that a short region of the *CIITA* promoter is sufficient to mediate repression following Zta expression (Li *et al.*, 2009). The repression of *CIITA* promoter activity could result from the overexpression of a transactivator domain that non-specifically sequesters basal transcription factors or co-activators, thereby inhibiting all

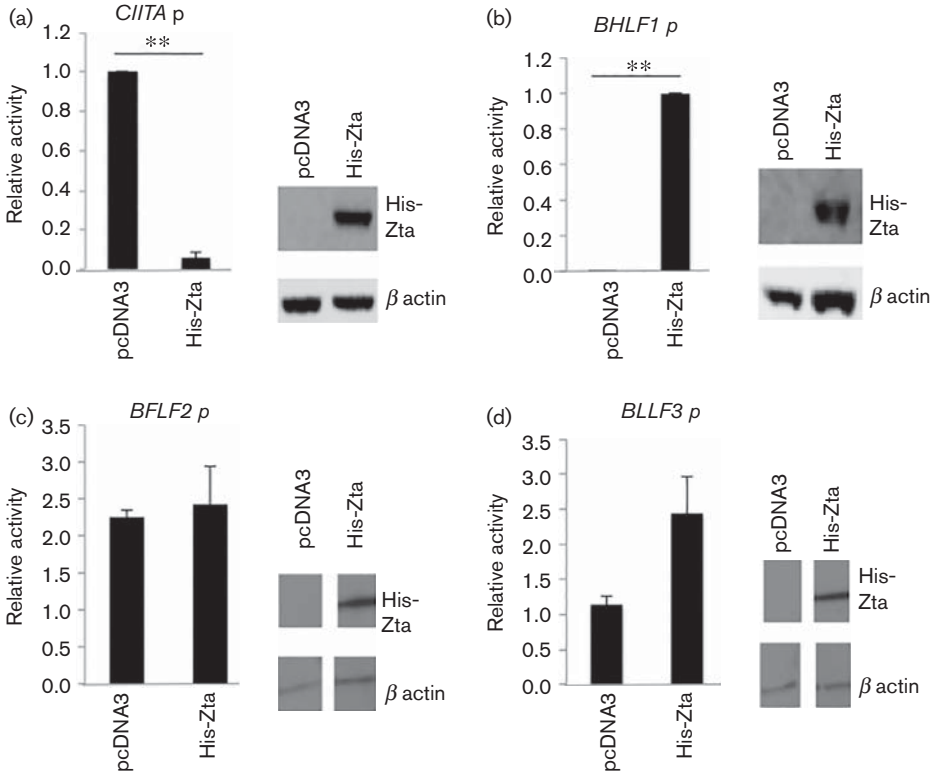

Fig. 1. Repression of *CIITA* promoter by Zta is specific. (a–d) The *CIITA* (−286 to +54) (a), *BHLF1* (b), *BFLF2* (c) and *BLLF3* (d) promoter-luciferase plasmids and the indicated expression vectors were introduced into cells by electroporation, and 48 h later cells were harvested and the luciferase activity determined. Promoter activity in Raji cells relative to the maximal activity of *CIITA* promoter (transfected with control plasmid) with the standard deviation from six assays (three replicate samples from each of two separate experiments) is shown. For comparisons +/− Zta, ** represents p of significant difference <0.01. The expression of His-Zta and endogenous protein were analysed by Western blot of proteins from the transfected cells.

RNA polymerase II-dependent transcription. To address whether this was the case, we undertook experiments to explicitly determine whether Zta repressed other promoters. We generated promoter-reporter gene constructs for two viral promoters, *BFLF2* and *BLLF3*. The impact of His-Zta expression on each promoter was assessed in Raji cells (Fig. 1c, d). This showed that neither *BLLF3* nor *BFLF2* promoters were repressed by Zta expression. We further investigated the repression of *CIITA* in BL cells by following two downstream targets of CIITA expression, HLA-DOA and HLA-DBM. Both were downregulated at the RNA level following Zta expression in BL cells (Fig. S1, available in the online Supplementary Material).

As Raji cells contain an EBV genome, changes in viral gene expression may occur as a consequence of activating a partial lytic replication cycle through the expression of Zta (Kallin & Klein, 1983). In order to determine whether Zta relies on additional viral components to repress *CIITA* expression, we introduced the *CIITA* promoter-reporter gene into an EBV-negative subclone of Akata Burkitt's lymphoma cells (AK31) (Jenkins *et al.*, 2000). In this cell background we saw that co-expression of Zta drove repression of the *CIITA* promoter-reporter gene around fivefold (Fig. 2). This clearly demonstrates that Zta-mediated repression of *CIITA* does not depend on additional EBV genes.

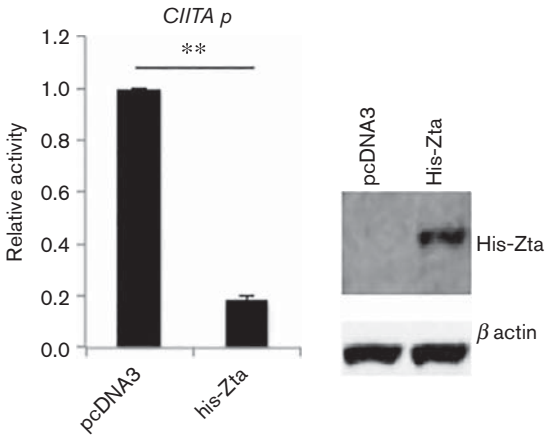

Fig. 2. Repression of *CIITA* promoter by Zta is independent of other viral proteins. The *CIITA* (−286 to +54) promoter-luciferase plasmid and the indicated expression vectors were introduced into an EBV-negative subclone of the Akata BL cells (AK31) by electroporation, and 48 h later cells were harvested and the luciferase activity determined. Promoter activity is expressed relative to the maximal activity of *CIITA* promoter (transfected with control plasmid) with the standard deviation from six assays (three replicate samples from each of two separate experiments). For comparisons +/− Zta, ** represents p of significant difference <0.01. The expression of His-Zta and endogenous protein were analysed by Western blot of proteins from the transfected cells.

Table 1. Impact of mutation of K12 and S209 on Zta-mediated repression

	His-Zta	His-Zta K12R	His-Zta S209A	His-Zta S209D
CIITA promoter activity	1.00	1.00	1.00	1.00
Relative promoter activity following His-Zta expression	0.16	0.07	0.07	0.04
Standard deviation	0.15	0.08	0.01	0.00

To explore the relevance of post-translational modifications of Zta to the Zta-mediated repression of the *CIITA* promoter, we generated mutants of Zta at amino acid residues K12 and S209 to prevent either sumoylation or phosphorylation. Following transfection, we found that neither post-translational modification was required for Zta to repress the *CIITA* promoter (Table 1).

Domains of Zta mediating repression of CIITA promoter

We then explored which domains of Zta protein mediate the repression of *CIITA*. Two versions of Zta were generated, both retaining the nuclear localization signal (Mikaélian *et al.*, 1993). One mutant omits the N-terminal transactivation domain (ZtaΔTA); this protein was previously shown to be able to bind to DNA but not to transactivate a reporter construct (Packham *et al.*, 1990). The second omits the dimerization and C-terminal region (ZtaH199ter) and has been shown previously to be unable to bind DNA (Hicks *et al.*, 2003) or to form dimers (Schelcher *et al.*, 2005) (Fig. 3a). Following transfection into Raji cells, we find that deletion of the transactivation domain ablates the ability of Zta to repress the *CIITA* promoter, despite the proteins being expressed at equivalent levels (Fig. 3b). In contrast, deletion of the dimerization and C-terminal regions of Zta only resulted in a small reduction in the repression of the *CIITA* promoter (Fig. 3c). The slightly lower level of repression observed with ZtaH199ter might result from the reduced abundance of this form of the protein. Taken together, these data show that a major component of Zta-mediated repression of the *CIITA* promoter occurs independently of a need for Zta to form dimers.

It has been shown previously that a Zta binding site within the *CIITA* promoter allows repression by Zta (Li *et al.*, 2009). Our data showed that dimerization is not an obligate requirement for repression, implying that DNA binding is not required. To explore this further, we assessed the promoter for potential ZREs (Flower *et al.*, 2011) and found only one, which was shown previously to be a Zta binding site (Li *et al.*, 2009). Using chromatin precipitation

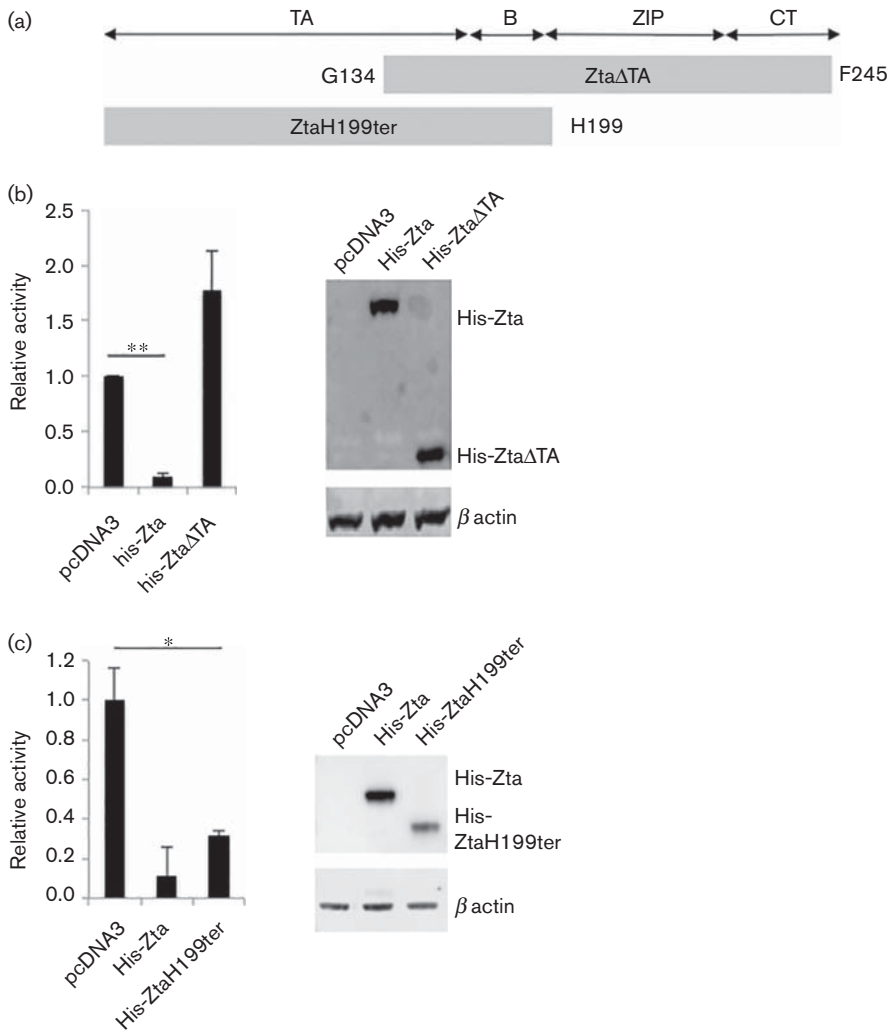

Fig. 3. Zta repression of CIITA promoter requires the transactivation domain. (a) Schematic of the Zta protein and the two mutant versions that were evaluated. TA, transactivation domain; B, basic DNA contact region; ZIP, dimerization bZIP domain; CT, carboxy terminal region (required for dimerization and replication). (b, c) The CIITA promoter-luciferase plasmids (−286 to +54) and either control, His-Zta or His-Zta mutant expression vectors were introduced into Raji BL cells by electroporation. 48 h later, cells were harvested and the luciferase activity and protein concentrations determined. For comparisons +/− Zta, ** represents p of significant difference <0.01; * represents p of significant difference <0.05. (b) Promoter activity of −286 to +54 promoter with His-ZtaΔTA with the standard deviation from six assays (three replicate samples from each of two separate experiments), together with a Western blot. (c) Promoter activity of −286 to +54 promoter with His-Zta199ter with the standard deviation from six assays (three replicate samples from each of two separate experiments), together with a Western blot.

coupled with next-generation DNA sequencing we confirmed that Zta bound to the promoter region of CIITA (Fig. S2), but noted that this does not distinguish between direct and indirect binding. To evaluate the relevance of the potential ZRE, we generated a promoter reporter construct in which the region containing a ZRE was deleted (Fig. 4a). Both of these promoters were expressed at equivalent levels (Table 2). Both full-length Zta and the dimerization-deficient mutant Zta-H199ter repressed the promoter missing the ZRE (Fig. 4b). This supports our contention that the ability of Zta to repress the expression of *CIITA* does not rely on direct DNA binding.

From these data we devised a model to account for Zta-mediated activation and repression of gene expression. In cells expressing MHC class II, CIITA expression is driven by the interaction of cellular factors (RNA polymerase II and cellular co-activators) (Fig. 5a). Once Zta is expressed it interferes with the activation machinery operating at the CIITA promoter, without the need to dimerize or bind to the promoter (Fig. 5b).

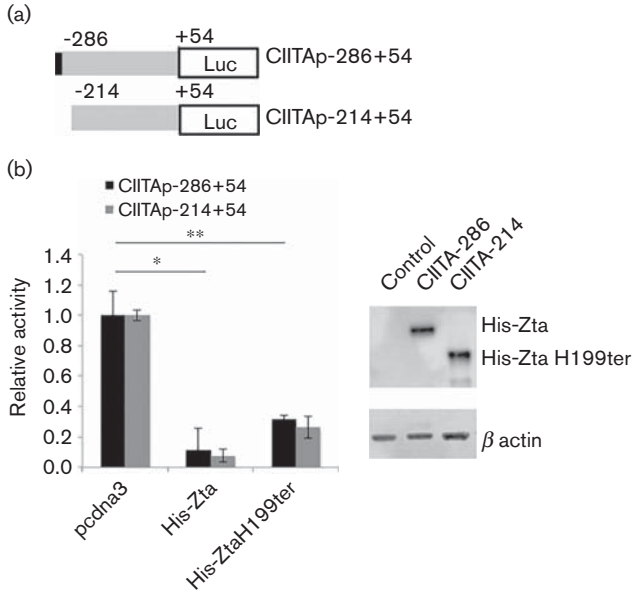

Fig. 4. Zta repression of *CIITA* promoter occurs without binding to the ZRE. (a) Schematic of the *CIITA* mutant promoters used in these experiments. The location of the ZRE is indicated by a filled box. (b) The *CIITA* promoter-luciferase plasmids (either −286 to +54 or −214 to +54) and either control, His-Zta or His-Zta mutant expression vectors were introduced into Raji BL cells by electroporation. 48 h later, cells were harvested and the luciferase activity and protein concentrations determined. The *CIITA* basal promoter activity is shown (grey bars) together with the His-Zta mediated activity (black bars), with the standard deviation from six assays (three replicate samples from each of two separate experiments). For comparisons +/− Zta, ** represents p of significant difference <0.01; * represents p of significant difference <0.05. For both His-Zta and His-ZtaH199ter, the significance is equal for each of the different promoters. Western blot analysis of protein expression in the transfected cells is also shown.

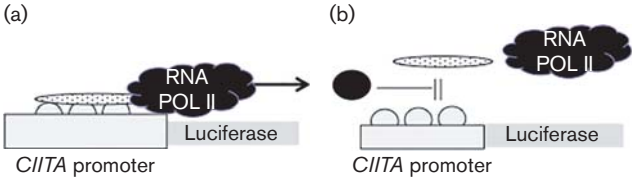

Fig. 5. Proposed model to explain Zta-mediated gene repression of the *CIITA* promoter. (a) The active *CIITA* promoter is shown. Basal transcription factors are represented as white spheres and co-activators by the stippled oval. RNA polymerase II is represented by the black cloud, with transcription indicated by an arrow. (b) The ability of a non-DNA-binding form of Zta (filled oval) to repress expression of the *CIITA* promoter suggests that some repression can occur without direct DNA contact. The simplest model to account for this has the N-terminal part of Zta blocking the interaction of the basal transcription machinery.

DISCUSSION

The EBV protein Zta is often described as the master regulator of EBV lytic cycle replication. Indeed, the ability of Zta to regulate viral gene expression is crucial to the success of viral lytic replication, as mutation of the *BZLF1* gene in recombinant EBV demonstrates (Feederle *et al.*, 2000). The activation of viral gene expression is considered to occur through the interaction of Zta with sequence-specific ZREs in the promoters of viral genes, and the attraction of co-activator proteins such as p300, TFIID and other RNA polymerase II components to the promoters (Lieberman & Berk, 1991, 1994) (Fig. 5 and Fig. S3). Recent genome-wide analyses have shown that Zta has extensive interactions across the EBV genome and a specific role in the transcriptional activation of many viral promoters (Bergbauer *et al.*, 2010; Ramasubramanyan *et al.*, 2012a).

Table 2. Impact of Zta expression on −286 and −214 *CIITA* promoters

	Average luciferase units *CIITA* (−286/+54)	Average luciferase units *CIITA* (−214/+54)
Control	440050 +/− 7113	472832 +/− 15031
His-Zta	18448 +/− 464	66785 +/− 1917

Li discovered that Zta-mediated repression of CIITA expression occurs in EBV-positive Raji cells, but did not investigate whether other viral genes are required for the repression (Li *et al.*, 2009). We confirm this and furthermore we show that the expression of two CIITA-dependent genes is also downregulated. As Zta expression in EBV-positive BL cells is sufficient to initiate the viral lytic replication cycle, many downstream changes in gene expression are expected, and it is important to determine whether repression requires Zta action alone or whether it acts in concert with additional viral proteins. Our demonstration that Zta is able to repress the *CIITA* promoter in an EBV-negative BL cell line unequivocally demonstrates that Zta-mediated repression does not require other viral gene products.

The relevance of two forms of post-translational modification of Zta that have been described as transcriptionally repressive was explored. The involvement of phosphorylation at S209 by the viral protein kinase *BGLF4* (Asai *et al.*, 2009) was investigated using the phospho-mimetic mutant version of Zta, S209D, and the phosphorylation dead mutant version, Zta S209A. Covalent addition of SUMO at K12 (Hagemeier *et al.*, 2010; Murata *et al.*, 2010) was assessed using the non-sumoylatable mutant version, Zta K12R. Both of these post-translational modifications have been described as transcriptionally repressive (Asai *et al.*, 2009; Hagemeier *et al.*, 2010; Murata *et al.*, 2010). As none of these Zta mutants compromised the

ability of Zta to repress the *CIITA* promoter, we conclude that neither post-translational modification is likely to be responsible for the observed repression of the *CIITA* promoter by Zta.

Zta also regulates gene expression by disrupting transcriptional activation by NFκB and p53 (Morrison & Kenney, 2004; Zhang *et al.*, 1994). This occurs through physical interactions between Zta and the p65 component of NFκB and between Zta and p53 protein (Morrison & Kenney, 2004; Zhang *et al.*, 1994). However, it is unlikely that either NFκB or p53 play a role in Zta-mediated *CIITA* repression, as both require the dimerization region of Zta, which is not necessary for repression of *CIITA*. In addition, mutation of the NFκB interaction site in the *CIITA* promoter does not alter either basal expression or Zta mediated repression (N. Balan & A. J. Sinclair, unpublished data). It is intriguing that Zta has been shown previously to modulate expression of a viral promoter (Zp) without the need to bind directly to DNA (Flemington *et al.*, 1994).

A previous study suggests that Zta repression of the CIITA promoter is driven through the interaction of Zta with a single ZRE within the promoter (Li *et al.*, 2009). This is supported by the impact of mutations of the ZRE within the promoter and by the inability of Zta to repress the *CIITA* promoter when the basic region is lost. This study places emphasis on a need of Zta to bind directly to DNA to effect repression. Our experiments support a different conclusion in which Zta represses CIITA expression without binding directly to DNA. We rationalize the need for the basic region of the Zta protein based on a requirement for the nuclear localization domain, which is contained therein (Mikaélian *et al.*, 1993). Without entry to the nucleus, Zta would not be able to repress the CIITA promoter through either direct or indirect DNA binding.

In summary, we show that Zta-mediated repression of the *CIITA* promoter can occur without Zta contacting DNA directly; this is supported by the retention of repression when (i) the ZRE is deleted and (ii) by a version of Zta that is defective for dimerization and therefore defective for DNA binding. This discovery leads us to propose a mechanism to describe gene repression by Zta. In this model the amino terminal region of Zta is able to impede the function of an essential component of the transcriptionally active CIITA promoter, for example a DNA-bound transcription factor or a transcription factor-associated co-activator, thereby preventing its productive association with RNA Pol II and its accessory proteins (Fig. 5).

METHODS

Plasmid constructs. The *CIITA* promoter (−286 to +54) was cloned with a *Kpn*I restriction enzyme site included at the 5′ end and a *Hin*dIII site at the 3′ end of the sequence. The promoter was subcloned into the pGL3 enhancer plasmid, which contains a luciferase reporter construct downstream from a multi-cloning site and which includes a distal SV40 enhancer (Promega). A 5′ deletion version of the promoter was generated (−214 to +54); the location of the 5′ end of this promoter is immediately 3′ from the ZRE.

The *BHLF1, BFLF2* and *BLLF3* promoters were cloned with *Bam*HI restriction enzyme sites added at the 5′ end and *Hin*dIII sites at the 3′ end. The DNA sequence between co-ordinates 40 472 and 40 818, 45 793 and 44 746, and 76 186 and 77 231 of the EBV genome (Human herpesvirus 4 complete wild-type genome Accession: NC_007605.1) was synthesized for the promoter regions for the *BHLF1, BFLF2* and *BLLF3* genes, respectively. The promoters were subcloned into the pCpGL plasmid (Klug & Rehli, 2006), which is based on pGL3 basic and contains a luciferase reporter construct downstream from a multi-cloning site.

A plasmid driving the expression of hexa-histidine tagged Zta (His-Zta) (Bailey *et al.*, 2009) was used to express His-Zta, compared to the vector control pcDNA3 (Invitrogen).

Expression vectors for His-Zta K12R, His-Zta S209A and His-Zta S209D were generated by site-directed mutagenesis of His-Zta using the primers shown in Table 3. An expression vector for His Zta-199ter, which introduces a termination codon at the amino acid 199 of the Zta coding sequence, and His Zta-ΔTA, which deletes amino acids 1–133 of Zta, were generated by gene synthesis (Invitrogen).

Cell culture. Plasmids were introduced into EBV-positive Raji cells (Pulvertaft, 1965) or EBV-negative Akata cells (Jenkins *et al.*, 2000) by electroporation. 1×10^7 cells in 0.25 ml medium were incubated with 10 μg plasmid DNA and pulsed with 250V at a capacitance of 975 μF in a Gene Pulser II electroporator (Bio-Rad).

Luciferase assays. post-transfection, cells were harvested into 250 μl of Passive Lysis Buffer (Promega) and incubated at room temperature for 15 min. The lysed cells were then centrifuged for 10 min at 13 300 ***g*** in a Thermo Scientific Heraeus Fresco centrifuge and the supernatant was used to determine luciferase activity. 10 μl aliquots of each lysate sample were pipetted into a 96-well white luminescence plate and analysed using luciferase detection kit reagents with a Glomax detection system (Promega). A protein concentration assay was undertaken (Bio-Rad) and promoter activity was expressed as luciferase RLU μg^{-1} protein. Significance of different

Table 3. Oligonucleotides used to generate mutations

His-Zta S209A amino acid serine 209 mutated to alanine	GGCTGCTGCCAAATCAGCTGAAAATGACAGGCTGCGCC; GGCGCAGCCTGTCATTTTCAGCTGATTTGGCAGCAGCC
His-Zta S209D amino acid serine 209 mutated to glutamic acid	GGCTGCTGCCAAATCAGATGAAAATGACAGGCTGCGCC; GGCGCAGCCTGTCATTTTCATCTGATTTGGCAGCAGCC
His-Zta K12R amino acid lysine 12 mutated to arginine	CtCGACttCtGAAGAtGtAAgAtttACACCtGACCCAtACC; GGTATGGGTCAGGTGTAAATCTTACATCTTCAGAAGTCGAG

promoter activity was assessed using a Student's paired *t*-test with two-tail distribution.

Protein analysis. Proteins were extracted from cells by boiling in 2 × laemmli sample buffer and fractionated on Novex protein gels (Invitrogen). Following transfer to nitrocellulose membranes, the blots were incubated with the Zta-specific antibody sc-17503 (Santa Cruz), which recognizes the amino-terminal region of Zta, BZ1, which recognizes the basic and dimerization regions of Zta (Young *et al.*, 1991) or a rabbit polyclonal beta actin antibody (Sigma), followed by detection with HRP-linked secondary antibodies and ECL (Ramasubramanyan *et al.*, 2012b).

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