Abstract:
Repression of the cellular CIITA gene is part of the immune evasion strategy of the herpes virus Epstein-Barr virus (EBV) during its lytic replication cycle in B-cells. In part this is mediated through down regulation of MHC class II gene expression via the targeted repression of CIITA, the cellular master regulator of MHC class II gene expression. The 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.
Repression of CIITA by the Epstein-Barr virus transcription factor Zta is independent of its dimerization and DNA binding

Nicolae Balan*, Kay Osborn and Alison J. Sinclair*

School of Life Sciences, University of Sussex, Brighton, BN1 9QG UK

running title: Gene repression by EBV transcription factor

*present address: Institute of Cancer Research, 123 Old Brompton Road, London SW7 3RP, UK.

NB and KO contributed equally to this work.

#address correspondence to Alison Sinclair aj.sinclair@sussex.ac.uk

(+44) 1273 678 194

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Abstract (229 words)

Repression of the cellular CIITA gene is part of the immune evasion strategy of the \( \gamma \) herpes virus Epstein-Barr virus (EBV) during its lytic replication cycle in B-cells. In part this is mediated through down regulation of MHC class II gene expression via the targeted repression of CIITA, the cellular master regulator of MHC class II gene expression. The 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 Nasopharyngeal 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 has 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-nucleotide DNA elements termed ZREs (for example (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; Kalla et al., 2010; Karlsson et al.),
2008; Kenney et al., 1989; Ramasubramanyan et al., 2012a; Ramasubramanyan et al., 2012b; 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).

Down regulation 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 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 down regulate 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 γ-herpesvirus 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. 1(a) and (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 provides 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 RNA polymerase II dependent transcription. To address whether this was the case, we undertook experiments to explicitly question 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. 1 (c,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 are down regulated at the RNA level following Zta expression in BL cells (Supplementary Fig. 1).
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 question whether Zta relies on additional viral components to repress CIITA expression, we introduced the CIITA promoter-reporter gene into an EBV-negative sub-clone 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 5-fold (Fig. 2). This clearly demonstrates that Zta-mediated repression of CIITA does not depend on additional EBV genes.

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 of these retain the nuclear localization signal (Mikaelian 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 3(a)). 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 an equivalent level
(Fig. 3(b)). 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.
3(c)). 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 show 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 the one, which was shown to be a Zta
binding site previously (Li et al., 2009). We confirm using Chromatin
precipitation coupled to next generation DNA sequencing that Zta binds to the
promoter region of CIITA (Supplementary Fig. 2), but note 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. 4(a)). Both of these promoters are expressed
at equivalent levels (Table 2). Both full length Zta and the dimerization-deficient
mutant Zta-H199ter repressed the promoter missing the ZRE (Fig. 4(b)). 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. 5(a)). 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. 5(b)).

**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 Supplementary Fig. 3). 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).

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 are also down regulated. 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 plays 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 (NB, AJS 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 (Mikaelian 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).

Materials and 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 Hind III site at the 3’ end of the sequence. The promoter was sub-cloned into the pGL3 enhancer plasmid, which contains a luciferase reporter construct down-stream 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 a BamHI restriction enzyme sites added at the 5’ end and Hind III sites at the 3’ end. The DNA sequence between co-ordinates 40472 and 40818, 45793 and 44746 and 76186 and 77231 of the EBV genome (Human herpesvirus 4 complete wild type genome Accession: NC_007605.1) were synthesized for the promoter regions for the BHLF1, BFLF2 and BLLF3 genes respectively. The promoters were sub-cloned into the pCpGL plasmid (Klug & Rehli, 2006), which is based on pGL3 basic and contains a luciferase reporter construct down stream 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 x 10^7 cells in 0.25ml of medium were incubated with 10µg of plasmid DNA and pulsed with 250V at a capacitance of 975µF in a Gene Pulser II electroporator (Bio-Rad).

**Luciferase assays**

48 hours post-transfection cells were harvested into 250µl of Passive Lysis Buffer (Promega) and incubated at room temperature for 15 minutes. The lysed cells were then centrifuged for 10min at 8 krpm and the supernatant was used to determine luciferase activity. 10µl aliquots of each lysate sample were pipetted into a 96-well white luminescence plate and analyzed using luciferase detection kit reagents with a Glomax detection system (Promega). A protein concentration assay was undertaken (Biorad) and promoter activity was expressed as
luciferase RLU/µg protein. Significance of different promoter activity was assessed using a Student’s paired T-test with 2 tail distribution.

Protein analysis

Proteins were extracted from cells by boiling in 2X Laemeli 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, or 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).

Acknowledgements:

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We thank Professor Takada for Akata cells, Professor Farrell for AK31 cells and Professor Rowe for BZ1 monoclonal antibody.

References


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**Figure legends**

Figure 1. Repression of *CIITA* promoter by Zta is specific.

The *CIITA* (-286 to +54) (a), *BHLF1* (b), *BFLF2* (c) and *BLLF3* (d) promoter-luciferase plasmids and the indicated expression vectors were introduced cells by electroporation, 48 hours later cells were harvested and the luciferase activity determined. a-d. 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).

For comparisons +/- Zta ** represents p of significant difference <0.01; * represents p of significant difference <0.05. The expression of His-Zta and endogenous protein were analyzed by western blot of proteins from the transfected cells.
Figure 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 sub-clone of the Akata BL cells (AK31) by electroporation, 48 hours 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 analyzed by western blot of proteins from the transfected cells.

Figure 3. Zta repression of CIITA promoter requires the transactivation domain.

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 hours 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.

a. Schematic of the Zta protein and the two mutant versions that were evaluated.

TA is transactivation domain; B is basic DNA contact region; ZIP is dimerization bZIP domain; CT is the Carboxy terminal region (required for dimerisation and replication).

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.

Figure 4. Zta repression of CIITA promoter occurs without binding to the ZRE.

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 hours 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. For both His-Zta and His-ZtaH199ter, the significance is equal for each of the different promoters.

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 basal promoter activity is shown (open) together with the His-Zta mediated activity (black), with the standard deviation from six assays (three replicate samples from each of two separate experiments). Western blot analysis of protein expression in the transfected cells.

Figure 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.

### Tables

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

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

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<td>66785 +/- 1917</td>
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**Table 3. Oligonucleotides used to generate mutations.**
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Figure 4

(a) Diagram showing two constructs: CIITA-p-286+54 and CIITA-p-214+54.

(b) Bar graph showing relative activity of various constructs.

- pcDNA3
- His-Zta
- His-Zta H199ter
- Control
- CIITA-286
- CIITA-214

Graphs show relative activity levels compared to control.
Supp Figure 1. CIITA downstream targets.
The expression of targets of CIITA were investigated by RNA seq as described in Ramasubramanyan et al 2015).
The relative change in expression following Zta expression in Akata BL cells was determined and presented (FDR<0.05).
The red line indicates the maximum reduction expected as only 70% of cells express Zta.
Supp figure 2. Interaction of Zta with the CIITA promoter in BL cells.
The interaction of Zta with the CIITA locus was undertaken by ChIP-seq (as described in Ramasubramanyan et al 2015). The locus is shown chr16:10,923,269-11,066,626. A peak of binding is seen as a bar spanning the TSS of the gene in Akata BL cells undergoing lytic replication cycle.
Supp Figure 3. Activation of BHLF1 promoter depends on ZREs in BL cells.
The viral BHLF1 promoter-luciferase plasmid and a version that has a mutation of each ZRE within the promoter was introduced into BL cells by electroporation together with a his-Zta expression vector or control plasmid. 48 hours later cells were harvested and the luciferase activity and protein concentrations determined.
A. Relative activity of promoters (log10 scale).
B. Western blot showing his-Zta and actin expression.