Evaluation of a Prediction Protocol to Identify Potential Targets of Epigenetic Reprogramming by the Cancer Associated Epstein Barr Virus

Kirsty Flower1,9, Elizabeth Hellen2, Melanie J. Newport2, Susan Jones1, Alison J. Sinclair1,*

1 School of Life Sciences, University of Sussex, Brighton, United Kingdom, 2 Brighton and Sussex Medical School, Brighton, United Kingdom

Abstract

Background: Epstein Barr virus (EBV) infects the majority of the human population, causing fatal diseases in a small proportion in conjunction with environmental factors. Following primary infection, EBV remains latent in the memory B cell population for life. Recurrent reactivation of the virus occurs, probably due to activation of the memory B-lymphocytes, resulting in viral replication and re-infection of B-lymphocytes. Methylation of the viral DNA at CpG motifs leads to silencing of viral gene expression during latency. Zta, the key viral protein that mediates the latency/reactivation balance, interacts with methylated DNA. Zta is a transcription factor for both viral and host genes. A sub-set of its DNA binding sites (ZREs) contains a CpG motif, which is recognised in its methylated form. Detailed analysis of the promoter of the viral gene BRLF1 revealed that interaction with a methylated CpG ZRE (RpZRE3) is key to overturning the epigenetic silencing of the gene.

Methodology and Principal Findings: Here we question whether we can use this information to identify which host genes contain promoters with similar response elements. A computational search of human gene promoters identified 274 targets containing the 7-nucleotide RpZRE3 core element. DNA binding analysis of Zta with 17 of these targets revealed that the flanking context of the core element does not have a profound effect on the ability of Zta to interact with the methylated sites. A second juxtaposed ZRE was observed for one promoter. Zta was able to interact with this site, although co-occupancy with the RpZRE3 core element was not observed.

Conclusions/Significance: This research demonstrates 274 human promoters have the potential to be regulated by Zta to overturn epigenetic silencing of gene expression during viral reactivation from latency.

EBV latency is disrupted following physiological activation of B-lymphocytes, through the expression of Zta (BZLF1, ZEBRA, EB1, Z); [13–15]. Zta is a sequence-specific DNA-binding protein, which resembles the bZIP family of transcription factors and plays a critical role in the reactivation of viral gene expression and replication of the genome. Through direct interaction with Zta response elements (ZREs) in promoters, Zta regulates the expression of viral and cellular genes. Many host and viral promoters that have been evaluated to date contain ZREs within the proximal five hundred nucleotides of 5’ sequence. Thus far, eight have experimentally verified binding sites for Zta in their proximal promoter regions: BSLF2+BMLF1 [16,17]; BRLF1 [18]; BZLF1 [17,19,20]; the joint promoter for BHLF1 and BHRF1 [19]; the lytic EBNA1 promoter Fp [21]; BRRF1 [22]; and BMRF1 [23]. Furthermore, 6 host genes are directly regulated by Zta through ZREs in their promoters DIHS9 [24]; EGR1 [25,26]; CITTA [27]; IL-8 [28]; IL-10 [29]; and IL-13 [30]. Zta interacts with a diverse range of ZREs
repressive methylation status and thus overturn the epigenetic
interaction with methylated CpG-containing ZREs allows Zta to
but are only recognized when methylated [35]. The ability of Zta
to interact with methylated CpG-containing ZREs allows Zta to
activate gene expression in the latent viral genome despite the
repressive methylation status and thus overturn the epigenetic
silencing of the viral genome [22,32–35].

To date, three genes with CpG-containing ZREs have been studied:
EBV BRLF1, EBV BRRF1 and human EGR1 [22,32–35]. Of these,
investigation of the regulation of the EBV gene BRLF1 provided compelling evidence that the interaction of Zta with a
methylated ZRE was instrumental in reactivating EBV into lytic cycle
in B-lymphocytes [32–35]. The viral BRLF1 gene includes three
ZREs in the promoter proximal region [18,32]. Two of the ZREs
contain CpG motifs; RpZRE2, is a class II ZRE and the other,
RpZRE3 is a class III ZRE [35]. The ability of a single point-
mutation in Zta to differentiate between the interaction of Zta with
methylated and non-methylated RpZRE3 allowed the relevance of
the interaction of Zta with this promoter to be established [34,36,37].
The viral BRRF1 gene contains two CpG-containing ZREs, which
Zta only interacts with in their methylated states [22]. The presence
of a CpG-containing ZRE in the promoter of the human EGR1 gene,
which is recognized by Zta in its methylated form, suggests that EBV
can overturn epigenetic silencing of host genes in order to modulate
the host cell environment [26].

The RpZRE3 core element (TCGCGAA), which was clearly shown to be required for overturning epigenetic silencing of
BRLF1 in B-lymphocytes [34,36,37], was chosen to identify all
human genes that contain an exact match to this CpG-containing
ZRE within the −500 to +1 region of the promoter and evaluate
whether they are recognised by Zta in their natural context.

Materials and Methods

Identification of Human Promoters Containing RpZRE3-
Core Elements: Initial Sampling

Promoter regions (defined as −500 to +1 base pairs from the
transcription start site) of a sample of human protein-coding genes in
Ensembl (49) [38] were extracted using the Biomart data
management system [39], The sample represented 40% of the
human genome. A search was made for all occurrences of an exact
(forwards and reverse) match to identify those promoters with the
TCGCGAA RpZRE3 core element.

Identification of Human Promoters Containing RpZRE3-
Core Elements: Whole Genome Scanning

An identical screen was undertaken on the entire human
genome from Ensembl (50) [40], resulting in the identification of
274 promoters which were designated the RpZRE3-promoter-
274 data-set. The 7 nucleotides of the RpZRE3 core element,
along with 10 flanking nucleotides on each side, were extracted from
each of the 5 genes from the initial sampling. The Transcription Factor Binding Site (TFBS) Perl modules [41]
were used to create a Position Weight Matrix (PWM) based on
the entire length of these 27-nucleotide sequences. TFBS Perl
modules were also used to search the promoter regions (~500 to +1)
of all human protein coding genes in Ensembl 50 [40] which
were extracted using Biomart [39]. Those promoters that
matched the PWM with a threshold value >80% and contained an
exact match to the RpZRE3-core element were additionally
filtered using 2 criteria (a) the presence of CpG islands and (b)
function (based on the over-representation of Gene Ontology
(GO) terms [42]). The location of CpG islands were predicted
using the EMBoss program CpGPlot [43] with default options.
Only genes with a CpG island present in the promoter were
retained. The GO term annotations for molecular function and
biological processes were extracted from Ensembl for each gene
[40]. The number of genes in the RpZRE3 promoter dataset with
each GO term annotation was compared with the total number of
genes in Ensembl with the same GO term. The GO terms that
occurred with a significantly higher frequency (p<0.05) in the
RpZRE3 dataset, compared to entire genome, were defined as
over-represented.

DNA Binding Analysis by EMSA on RpZRE3 Containing
Promoters

Double stranded labelled DNA probes were made by labelling
6 pmol of oligonucleotide (27 nt long) at the 5‘ end with
γ-32P[ATP (30 µCi) using polynucleotide kinase (Roche).
12 pmol of the complementary oligonucleotide strand was added
and incubated with the labelled single strand at 95°C for 2
minutes, 65°C for 10 minutes, and 37°C for 5 minutes to anneal
the strands. The concentration of the probe was 33.3 nM. The
oligonucleotides comprised the core 7-mer sequence surrounded
by 20 nucleotides corresponding to the cognate flanking sequence
for each promoter and were synthesised. Where indicated in
the figure, the central CpG motif was methylated on both cytosines
during synthesis (Sigma).

Zta protein was in vitro translated using wheatgerm extract
(Promega). This was incubated with the labelled probe (at a final
probe concentration of 3.3 nM) for 30 minutes at room
temperature, before the sample was fractionated on an 8% native
casein gel at 100 volts for 1 hour. Following detection of
the radioactive DNA on the gel, the sample was exposed to film
and developed to obtain the specific band(s) corresponding to
the EbV gene of interest. The autoradiography film was scanned
and quantitated using ImageQuant software (GE
Healthcare, UK).

Competition EMSA were carried out with a 100X excess of a
double stranded non-labelled oligonucleotide in addition to the
standard EMSA reaction. Equal quantities of each of the
complementary oligonucleotides were annealed in the same
manner as the labelled probes and diluted to yield a solution
concentration of 3.3 µM. This was added to the EMSA reaction
at a final concentration of 333 nM (i.e. 100X excess).

Oligonucleotides

The oligonucleotides used were double strand versions of the
following sequences (5’-3’):

XPC GGTGGCGTCACCTCGCGAAGTTTGGAATTGG
ZC3H8 GCCTTCGCCGCTCGCGGAAAGGGAGGACC
HDAC2 TCCCCAACCGTCGCCGAAAGCTCAGCCGC
MNT CTGGCGCGGTCTCGCGGAAAGGGAGGACC
Cyclin L2 GGCGGGCGTCCTCGCGGAAAGCTCAAGCC
RpZRE3 GATTTATAGCATCGCGAATTTTGAGTGC
CAPN2 CGGCGAGGGGCCTCGCGGAATTTTGAGTGC
CDO1 GTGGCGCGGATATCGCGGGGATCGCGGG
KIF1B GCTTCCCGGCTCGCGGAAACCCAGGCC
FALZ GGGCGCCGACATCGCGGGGATCGGGG
LLGL1 TCGGCCGGGCCTGGCGAAAGGGAGGGCC

500 to

1) of all human protein coding genes in Ensembl 50 [40] which

were extracted using Biomart [39]. Those promoters that
matched the PWM with a threshold value >80% and contained an
exact match to the RpZRE3-core element were additionally
filtered using 2 criteria (a) the presence of CpG islands and (b)
function (based on the over-representation of Gene Ontology
(GO) terms [42]). The location of CpG islands were predicted
using the EMBoss program CpGPlot [43] with default options.
Only genes with a CpG island present in the promoter were
retained. The GO term annotations for molecular function and
biological processes were extracted from Ensembl for each gene
[40]. The number of genes in the RpZRE3 promoter dataset with
each GO term annotation was compared with the total number of
genes in Ensembl with the same GO term. The GO terms that
occurred with a significantly higher frequency (p<0.05) in the
RpZRE3 dataset, compared to entire genome, were defined as
over-represented.

DNA Binding Analysis by EMSA on RpZRE3 Containing
Promoters

Double stranded labelled DNA probes were made by labelling
6 pmol of oligonucleotide (27 nt long) at the 5‘ end with
γ-32P[ATP (30 µCi) using polynucleotide kinase (Roche).
12 pmol of the complementary oligonucleotide strand was added
and incubated with the labelled single strand at 95°C for 2
minutes, 65°C for 10 minutes, and 37°C for 30 minutes to anneal
the strands. The concentration of the probe was 33.3 nM. The
oligonucleotides comprised the core 7-mer sequence surrounded
by 20 nucleotides corresponding to the cognate flanking sequence
for each promoter and were synthesised. Where indicated in
the figure, the central CpG motif was methylated on both cytosines
during synthesis (Sigma).

Zta protein was in vitro translated using wheatgerm extract
(Promega). This was incubated with the labelled probe (at a final
probe concentration of 3.3 nM) for 30 minutes at room
temperature, before the sample was fractionated on an 8% native
polyacrylamide gel at 100 volts for 1 hour. Following detection of
the radioactive DNA on the gel, the sample was exposed to film
and developed to obtain the specific band(s) corresponding to
the EbV gene of interest. The autoradiography film was scanned
and quantitated using ImageQuant software (GE
Healthcare, UK).

Competition EMSA were carried out with a 100X excess of a
double stranded non-labelled oligonucleotide in addition to the
standard EMSA reaction. Equal quantities of each of the
complementary oligonucleotides were annealed in the same
manner as the labelled probes and diluted to yield a solution
concentration of 3.3 µM. This was added to the EMSA reaction
at a final concentration of 333 nM (i.e. 100X excess).

Oligonucleotides

The oligonucleotides used were double strand versions of the
following sequences (5’-3’):

XPC GGTGGCGTCACCTCGCGAAGTTTGGAATTGG
ZC3H8 GCCTTCGCCGCTCGCGGAAAGGGAGGACC
HDAC2 TCCCCAACCGTCGCCGAAAGCTCAGCCGC
MNT CTGGCGCGGTCTCGCGGAAAGGGAGGACC
Cyclin L2 GGCGGGCGTCCTCGCGGAAAGCTCAAGCC
RpZRE3 GATTTATAGCATCGCGAATTTTGAGTGC
CAPN2 CGGCGAGGGGCCTCGCGGAATTTTGAGTGC
CDO1 GTGGCGCGGATATCGCGGGGATCGGGG
KIF1B GCTTCCCGGCTCGCGGAAACCCAGGCC
FALZ GGGCGCCGACATCGCGGGGATCGGGG
LLGL1 TCGGCCGGGCCTGGCGAAAGGGAGGGCC


LMO4 GGATCCCGGTTCCGAAGGGAGCCAGCCAC MBD4 CCTCCTGCTCTCTGGAACCCGCGCCGC PLEKH1 AGCGCGCTCCCTCCGAAGGTTGGCCCC PKD1 CTCTCCGATGCTCTGGAACCTTCCCGGCC SEC14L CGCGCGACTCTCGGAAGGCGACGCCGC TADA1 GCTGGCCCTCTTCTGGGAAAGGGCGAGCGA TOP2B CCGGCGCCCATCGGAAGAGATCCGGAGC XPCLMNTR GGTTCGCGTACTCGGAAGGAGGAGGC MNTLXPCR CCGGGCGCTCCTCGGAAGTGGAAATTTG XPC Mcore GGTGGCGTACACCCTCCTAGTGAAATTTG XPCLM3More GTTGGCGCTCCTCCCTTATGGAATTTG API mut GATCGAACCTTTAGGAAAACATACG

Prediction of Transcription Factor Binding Sites Using Promo

The transcription factor binding site prediction program PROMO [44] was used with the default setting of 15% dissimilarity value, to identify potential bZIP transcription factor binding sites within the XPC oligonucleotide.

Results

Identification of Human Promoters Containing the RpZRE3-Core Element: Initial Sampling

The initial search for RpZRE3-core elements in a sample of human promoters revealed 67 genes with an RpZRE3-core element, 5 of these that are involved in gene regulation were selected for DNA binding analysis. These 5 genes were ZC3H8 (ENSG00000116148), HDAC2 (ENSG00000196591), XPC (ENSG00000154767), MNT (ENSG00000070444) and CyclinL2 (ENSG00000116148) and together with the RpZRE3 element from the viral BRLF1 promoter (Rp), formed the RpZRE3-promoter-6 data-set.

DNA-binding assays were undertaken with the methylated forms of each of the 5 human promoters using 27mer double strand oligonucleotides encompassing the 7-nucleotide core element and 10-nucleotide flanking region on each side together with RpZRE3 from Rp. Electrophoretic mobility shift assays (EMSA) were undertaken with in vitro translated Zta protein. Zta protein/DNA complexes formed readily with the oligonucleotides from all six promoters (Figure 1). The specificity of the assay is shown by the lack of complex formation with control protein. In addition, we undertook competition experiments with an excess of unlabelled oligonucleotides and included a version with a mutant ZRE to further probe the specificity of the interaction. This confirms that Zta interacts with all six RpZRE3 core elements specifically.

Identification of Human Promoters Containing RpZRE3-Core Elements: Whole Genome Scanning

The complete human genome was scanned for additional RpZRE3-core elements. This resulted in a data-set of 274 genes, denoted the RpZRE3-promoter-274 data-set (see Table S1). To assess whether there was an influence of flanking sequence on the interaction of Zta with these ZREs we undertook a systematic filtering process. The RpZRE3-promoter-274 dataset was first filtered by a Position Weight Matrix (PWM), which was generated from the RpZRE3-promoter-6 data-set. Matches were further filtered for genes with at least one CpG island in the promoter and an over-represented GO term. This resulted in 12 previously unidentified promoters and a further one that had been identified in the initial screen (Figure 2). Together with the promoters from the initial screen and the viral BRLF1 promoter (RpZRE3-promoter-6 data-set), these form the RpZRE3-promoter-18 data-set.

Oligonucleotides were designed using the same principles with 10 nucleotides of flanking sequence on each side of the RpZRE3-core element, and the ability of Zta to interact with each site in its methylated form was assessed. The EMSA analysis revealed that all twelve of the newly identified methylated promoters were recognised by Zta (Figure 3).

This analysis demonstrated that for the RpZRE3-promoter-18 data-set, the flanking sequence surrounding the methylated core 7-mer element did not have a profound effect on the ability of Zta to interact with promoters and therefore the core element was sufficient to facilitate binding. This was illustrated by the generation of a PWM using sequence from the RpZRE3-promoter-18 data-set (Figure 4), which revealed negligible sequence conservation outside of the core element.

Recognition of the Non-Methylated Promoters

Zta is able to recognise many response elements which do not contain a CpG motif and therefore do not have a methylated core element (Class I ZREs) [15,35]. In addition, Zta recognises one CpG containing ZRE, RpZRE2, even in the absence of methylation (Class II ZREs) [33]. The ability of Zta to recognise the promoters in their non-methylated forms may impact on the ability of EBV to alter their gene expression, so we investigated the classification of ZRE for each of the 17 human promoters.

A series of DNA binding experiments with the oligonucleotides representing the human promoters in the RpZRE3-promoter-18 data-set, were undertaken comparing non-methylated with methylated RpZRE3-core elements. The interaction of Zta with the sites was quantitatively, as demonstrated by the reduction in complex formation as the Zta protein concentration was titrated on each of the methylated promoters (Figure 5). All bar one display negligible binding to the non-methylated oligonucleotides (at least 10-fold lower than to the methylated sites) and can therefore be classified as class III ZREs.

Zta displayed a reproducible interaction with the non-methylated XPC oligonucleotide; the interaction reached 50% of the binding observed with the methylated oligonucleotide (Figure 5). This raises the possibility that the XPC core RpZRE3 element is influenced by the flanking sequence to behave as a class II ZRE.

Additional ZRE Juxtaposed with an RpZRE3-Core Element in Flanking Region

To identify whether sequences conferring methylation independent recognition was confined to a specific flank of XPC, a series of mutant oligonucleotide probes that exchanged flanking sequences between XPC and a class III site (MNT), that is not recognised when non-methylated, were designed. Analysis of the interaction with Zta by EMSA revealed that the ability to confer Zta binding resided within the 5' sequence of XPC; the hybrid XPCLMNTR was able to bind but MNTLXPCR was not (Figure 6). This suggested that the 5' XPC flanking sequence of the RpZRE3 element influenced binding. However, it was surprising to discover that mutation of the RpZRE3 element within XPC did not prevent interaction with Zta (Figure 6).

A further explanation for the interaction of Zta with the non-methylated XPC promoter is that an obscure ZRE is present in the 5' flank. To address this further we attempted to identify putative ZREs in the XPC promoter using the transcription factor binding site prediction program PROMO [44,45]. Although this program contains a PWM for Zta binding sites, none were identified in this sequence. However, PROMO predicted the
Figure 1. Interaction of Zta with methylated RpZRE3 core element from 5 human promoters. (a) The nucleotide sequence of one strand of each oligonucleotide spanning the indicated ZREs is shown, with the conserved RpZRE3 core element (TCGCGAA) aligned. Double strand versions of these sequences were used as probes in EMSA, with the 2 cytosines within the CpG core motif methylated. The RpZRE3 from EBV BRLF1 promoter is included. (b) DNA binding between in vitro translated Zta with each probe was undertaken by EMSA. The ability of Zta (Z) to interact with the probe was compared with an unprogrammed translation lysate (C) as a negative control. The complex was separated on an 8% gel by electrophoresis and visualised by phosphoimaging. The excess probe can be seen at the bottom of the gel. The probe used in each experiment is indicated below the gel. (c) Competition of each ZRE sequence (at 100X excess) against labelled RpZRE3 was determined by competition EMSAs. Data from at least 2 experiments was taken to calculate competition i.e. the percentage of labelled probe displaced by unlabelled competitor. Methylated ZREs were used for both probe and competition. AP1 mut, an oligonucleotide previously shown not to interact with Zta [36], was used as a negative control to define the level of non-specific binding. Error bars indicate standard error.

doi:10.1371/journal.pone.0009443.g001
Figure 2. Identification of human promoters containing RpZRE3 core elements in the human genome. (a) The bioinformatics analysis undertaken for the genome wide scan is represented as a flow diagram. Genetic input or output is represented by ovals, and filters by trapeziums. (b) A Position Weight Matrix (PWM) was created using the aligned sequences of the RpZRE3-promoter-6 data-set. These sequences are shown in Fig. 1(a). This was used to filter the genomic data from the human promoter sequences (−500 to +1). (c) Overrepresented GO terms found for the 274 genes were identified. (d) The 13 genes identified as candidate genes, 12 previously unknown genes and 1 from the initial screen, and their associated Ensembl accession codes.

doi:10.1371/journal.pone.0009443.g002
presence of AP1, c-fos and c-jun binding sites within the 5’ flank of XPC (5’TGCGTCA) (Figure 7). c-fos and c-jun proteins are both members of the bZIP transcription factor family; they form AP1 DNA binding activity as either homodimers or heterodimers. It is relevant that fos/jun dimers share some DNA recognition motifs with Zta [15–17,31,46–49]. This led to the hypothesis that the predicted AP1 site in the 5’ flanking sequence is an additional ZRE that is responsible for binding to the non-methylated XPC oligonucleotide. To test the hypothesis, further mutations were introduced into the 5’ XPC flanking sequence in the predicted AP1 site (5’TCCGGCT). The ability of Zta to interact with the double AP1/core mutant site (XPCLM3Mcore) was compared with the core-only mutant. Dual mutation of the predicted AP1 site and the core abrogated the ability of Zta to interact with the XPC oligonucleotide, demonstrating that binding of Zta to the non-methylated XPC site resided with this AP1 site (Figure 7).

This analysis showed that the RpZRE3 core element is not recognised in its non-methylated state in the context of any of the viral or cellular promoters in the RpZRE3-promoter-18 data-set, and that this ZRE is specifically recognised when methylated.

Discussion

A computational search revealed a set of 274 human genes with cellular promoters containing the 7-mer RpZRE3 core element. Flanking sequence can influence the interaction of some transcription factors with DNA, for example, the interaction of the E2F family with DNA is influenced by a region of at least 8 nucleotides 5’ and 11 nucleotides 3’ to the central nucleotide [50]. Before assigning all 274 genes as candidates for regulation by Zta, it was important to assess whether the cognate flanking sequence had a profound effect on binding by Zta. Consideration of the sequences represented in the flanking sequence of this set of genes revealed it to be diverse; all four nucleotides were represented in 55% of positions and three of the four nucleotides were represented at the remaining positions. Indeed, the immediate flank of the RpZRE3 core element, consisting of four nucleotides both 5’ and 3’, had 100% representation of all four nucleotides. It can therefore be concluded that the flanking sequence does not prevent binding to the methylated RpZRE3 core, and a PWM generated from the RpZRE3-promoter-18 data-set showed little conservation outside the core motif. In contrast, the interaction with the non-methylated core element initially appeared to be influenced by flanking sequence in only 1 of the 18 promoters. However, further analysis revealed this to be due to the presence of a second ZRE in the flanking sequence.

These results show that the approach of undertaking a computational pattern match search for the 7-mer RpZRE3 core element is a fast and reliable method to identify promoters containing ZREs that are recognized by Zta only when they are methylated (class III ZREs).

The identification of two adjacent juxtaposed ZREs in the XPC promoter presents an interesting problem; can both sites be occupied at once? The natural juxtaposition of ZREs has been previously described for a viral promoter; BZLF1 and a cellular promoter EGR1. In the BZLF1 promoter, the elements are situated with 12 base pairs between the central nucleotides; both can be occupied simultaneously and both are functionally relevant [17,19–20]. For the EGR1 promoter, the elements are immediately adjacent with just 8 nucleotides between the central nucleotides [25,26]. There is no evidence for simultaneous occupation by Zta and only one element appears to be functional in vivo [26]. The arrangement of the XPC promoter places the elements 8 nucleotides apart, identical to EGR1, and there is no evidence from the DNA-binding experiments for simultaneous occupation of the sites. As one ZRE is recognised in a methylation-dependent manner and the other is recognised when non-methylated, it is possible that the arrangement may provide a fail-safe mechanism to ensure that the gene is regulated in both its methylated and non-methylated states.
The simple computational approach taken to identify the location of this methylation-dependent and novel DNA-binding motif in human gene promoters identified 274 genes potentially regulated by overcoming epigenetic silencing during the viral replicative cycle. Given the known functions of Zta in reprogramming viral gene expression, disrupting cell cycle control and replicating viral DNA, it is interesting to note that the Gene Ontology terms that are over-represented in the RpZRE3-promoter-274 gene-set are largely involved in transcription, chromatin re-modelling and mitosis. This strongly suggests that Zta may activate this set of cellular genes in order to accomplish these functions. Testing whether these genes are activated during latency disruption in memory B-lymphocytes in vivo is technically challenging given both the scarcity of memory B-lymphocytes in peripheral circulation and the infrequency of latency disruption in vivo.

The co-location of the RpZRE3 core element in 274 human promoters is unlikely to have been driven by an evolutionary advantage to the virus, but may reflect the involvement of a cellular transcription factor interacting with the same element. This would suggest that this set of genes share a common mode of regulation during human development or differentiation. Further

Figure 5. Comparison of Zta interaction with non-methylated and methylated RpZRE3s from human promoters. DNA binding analysis of in vitro translated Zta protein (Z) or an unprogrammed translation lysate (C) with both non-methylated and methylated versions of the probes indicated to the right of each gel was carried out by EMSA as described in Fig. 1. A titration of Zta protein (1:2, 1:4, 1:8) was used to compare non-methylated binding to that of the methylated equivalent. Quantitation of the complexes formed between Zta and the non-methylated and methylated probes, from at least 2 experiments, is represented as a histogram to the right of the corresponding gel. Complex formation is shown relative to the maximum binding for each probe. Error bars indicate standard error.

doi:10.1371/journal.pone.0009443.g005
more, it will be interesting to question whether co-occurrence of the RpZRE3-core element with other transcription factor binding sites forming a co-operative cis-regulator module which may illuminate the regulation of these host and viral genes.

Supporting Information

Table S1  RpZRE3-promoter-274 data-set: A table of genes which contain a RpZRE3 core element in the 500 bp promoter region.

Found at: doi:10.1371/journal.pone.0009443.s001 (0.05 MB XLS)

Acknowledgments

We thank Queensta Miller and James Heather for discussion.

Author Contributions

Conceived and designed the experiments: KF EH MJN SJ AJS. Performed the experiments: KF EH. Analyzed the data: KF EH. Contributed reagents/materials/analysis tools: KF EH. Wrote the paper: KF EH MJN SJ AJS.

References

1. Murray PG, Young LS (2001) Epstein-Barr virus infection: basis of malignancy and potential for therapy. Expert Rev Mol Med. pp 1–20.
2. Talbot SJ, Crawford DH (2004) Viruses and tumours–an update. Eur J Cancer 40: 1996–2005.
3. Young LS, Rickinson AB (2004) Epstein-Barr virus: 40 years on. Nat Rev Cancer 4: 757–768.
4. Thorley-Lawson DA, Allday MJ (2008) The curious case of the tumour virus: 50 years of Burkitt’s lymphoma. Nat Rev Microbiol 6: 913–924.
5. Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA (1998) EBV persistence in memory B cells in vivo. Immunity 9: 395–404.

6. Niller HH, Wolf H, Minarovits J (2009) Epigenetic dysregulation of the host cell genome in Epstein-Barr virus-associated neoplasia. Semin Cancer Biol 19: 150–164.

7. Minarovits J (2006) Epigenotypes of latent herpesvirus genomes. Curr Top Microbiol Immunol 310: 61–80.

8. Emberg I, Falk K, Minarovits J, Basson P, Tursz T, et al. (1989) The role of methylation in the phenotype-dependent modulation of Epstein-Barr nuclear antigen 2 and latent membrane protein genes in cells latently infected with Epstein-Barr virus. J Gen Virol 70 (Pt 1): 2989–3002.

9. Massucci MG, Contreras-Salazar B, Nagar E, Falk K, Minarovits J, et al. (1989) 5-Azacytidine up regulates the expression of Epstein-Barr virus nuclear antigen 2 (EBNA-2) through EBNA-6 and latent membrane protein in the Burkitt’s lymphoma line Ra J. Virology 63: 3135–3141.

10. Tao Q, Robertson KD (2003) Stealth technology: how Epstein-Barr virus utilizes DNA methylation to cloak itself from immune detection. Clin Immunol 109: 53–63.

11. Israel BF, Kenney SC (2003) Virally targeted therapies for EBV-associated malignancies. Oncogene 22: 5122–5130.

12. Feng WH, Hong G, Delecluse HJ, Kenney SC (2004) Lytic induction therapy for Epstein-Barr virus-positive B-cell lymphomas. J Virol 78: 1893–1902.

13. Miller G (1989) The switch between EBV latency and replication. Yale J Biol Med 62: 205–213.

14. Speck SH, Chatila T, Flemington E (1997) Reactivation of Epstein-Barr virus: regulation and function of the BLZF1 gene. Trends in Microbiology 5: 399–405.

15. Sinclair AJ (2003) BZIP proteins of human Gamma Herpesviruses. Journal of General Virology 84: 1941–1949.

16. Farrell P, Rowe D, Rooney CM, Kouzarides T (1989) Epstein-Barr virus BLZF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos.

17. EMBO J 8: 127–132.

18. Uriel G, M B, Chambard P, Sergeant A (1989) The Epstein-Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites. EMBO J 8: 1447–1453.

19. Sinclair AJ, Brilliant M, Shanahan F, Farrell DJ (1991) Pathways of activation of the Epstein-Barr virus productive cycle. J Virol 65: 2237–2244.

20. Lieberman PM, Hardwick JM, Sample J, Hayward GS, Hayward SD (1990) The zta transactivator involved in induction of lytic cycle gene expression in Epstein-Barr virus-infected lymphoblasts binds to both AP-1 and ZRE sites in target promoter and enhancer regions. J Virol 64: 1145–1155.

21. Lieberman PM, Speck SH (1999) Autoregulation of Epstein-Barr Virus putative lytic switch gene BLZF1. Journal of Virology 64: 1277–1232.

22. Zetterberg H, Jansson A, Ryno L, Chen F, Karlsson A, et al. (2002) The Epstein-Barr virus ZEBRA protein activates transcription from the early lytic F promoter by binding to a promoter- proximal AP-1-like site. Journal of General Virology 83: 2007–2014.

23. Dickenson SJ, Xing Y, Robinson AR, Seaman WT, Graffit H, et al. (2009) Methylation-dependent binding of the Epstein-barr virus BLZF1 protein to viral promoters. PLoS Pathog 5: e1000356.

24. Quinnlan EB, Holley-Guthrie EA, Norris M, Gutch D, Bacherheimer SL, et al. (1993) Direct BRLF1 binding is required for cooperative BZLF1/BRLF1 interaction on Epstein-Barr virus promoters. PLoS Pathog 5: e1000356.

25. Quinnlan EB, Holley-Guthrie EA, Norris M, Gutch D, Bacherheimer SL, et al. (1993) Direct BRLF1 binding is required for cooperative BZLF1/BRLF1 interaction on Epstein-Barr virus promoters. PLoS Pathog 5: e1000356.

26. Ho Y, Qin S, Zhao J, Zhang X, Chen X, et al. (2012) DNA methylation to cloak itself from immune detection. Clin Immunol 109: 53–63.

27. Chen Y, Lee HH, Chen YT, Lu J, Wu SY, et al. (2006) Induction of the early growth response 1 gene by Epstein-Barr virus lytic transactivator Zta. J Virol 80: 7748–7755.

28. Huang Y, Lee HH, Chen YT, Lu J, Wu SY, et al. (2006) Induction of the early growth response 1 gene by Epstein-Barr virus lytic transactivator Zta. J Virol 80: 7748–7755.

29. Huang Y, Lee HH, Chen YT, Lu J, Wu SY, et al. (2006) Induction of the early growth response 1 gene by Epstein-Barr virus lytic transactivator Zta. J Virol 80: 7748–7755.

30. Huang Y, Lee HH, Chen YT, Lu J, Wu SY, et al. (2006) Induction of the early growth response 1 gene by Epstein-Barr virus lytic transactivator Zta. J Virol 80: 7748–7755.

31. Huang Y, Lee HH, Chen YT, Lu J, Wu SY, et al. (2006) Induction of the early growth response 1 gene by Epstein-Barr virus lytic transactivator Zta. J Virol 80: 7748–7755.