Epigenetic Control of Viral Life-Cycle by a DNA-Methylation Dependent Transcription Factor

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Abstract

Epstein-Barr virus (EBV) encoded transcription factor Zta (BZLF1, ZEBRA, EB1) is the prototype of a class of transcription factor (including C/EBPalpha) that interact with CpG-containing DNA response elements in a methylation-dependent manner. The EBV genome undergoes a biphasic methylation cycle; it is extensively methylated during viral latency but is reset to an unmethylated state following viral lytic replication. Zta is expressed transiently following infection and again during the switch between latency and lytic replication. The requirement for CpG-methylation at critical Zta response elements (ZREs) has been proposed to regulate EBV replication, specifically it could aid the activation of viral lytic gene expression from silenced promoters on the methylated genome during latency in addition to preventing full lytic reactivation from the non-methylated EBV genome immediately following infection. We developed a computational approach to predict the location of ZREs which we experimentally assessed using in vitro and in vivo DNA association assays. A remarkably different binding motif is apparent for the CpG and non-CpG ZREs. Computational prediction of the location of these binding motifs in EBV revealed that the majority of lytic cycle genes have at least one and many have multiple copies of methylation-dependent CpG ZREs within their promoters. This suggests that the abundance of Zta protein coupled with the methylation status of the EBV genome act together to co-ordinate the expression of lytic cycle genes at the majority of EBV promoters.

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Introduction

Infection of human B-lymphocytes by Epstein-Barr virus results in the establishment of a latent state in which a highly restricted set of viral genes are expressed [1]. This is accompanied by extensive methylation of CpG motifs in non-expressed viral genes [2,3,4]. In response to physiological stimuli, such as engagement of the B-cell receptor, epigenetic silencing of the viral genome is overturned, resulting in widespread activation of viral gene expression and lytic replication [4,5]. The expression of a subset of host genes is also altered during this period [6,7,9,10,11,12,13,14].

The switch between latency and the lytic cycle is orchestrated by the viral gene BZLF1, which encodes the protein Zta (also known as ZEBRA, BZLF1, EB1, or Z) [15,16,17]. Zta resembles the AP1 family of bZIP transcription factors but has a unique dimerisation domain and does not form heterodimers with cellular bZIP proteins [19]. Three classes of Zta DNA binding sites (Zta response elements (ZREs)) have been defined for Zta [19]. Class I ZREs include classical AP1-like recognition elements. However, some Zta binding sites contain a CpG motif and Zta has the unusual property of binding preferentially to these ZREs when they are methylated [20,21], defining class II ZREs [19]. Remarkably, some CpG-containing ZREs are only recognized in their methylated form (class III ZREs) [19,20,21,22,23,24,25]. Methylation of the viral genome occurs during latency and has recently been shown to be required for EBV replication [3]. The ability of Zta to bind to methylated ZREs suggests that Zta may have a direct role in overriding the epigenetic silencing of the viral genome to activate expression of viral genes required for lytic replication.

The requirement for methylation at critical ZREs may also contribute to the establishment of latency during the immortalization of infected cells. The EBV genome is not methylated when it enters cells but the genome gradually becomes methylated during immortalization and the establishment of viral latency [3,4]. Zta is transiently expressed during the early period immediately after infection and is required for efficient immortalization [3]. It is therefore essential that Zta should not activate the full lytic replication cycle at this stage. A plausible hypothesis to explain this is that expression of key lytic cycle genes are controlled by class III ZREs that do not function in their unmethylated form.

We developed a computational approach to identify candidate ZREs and applied it to a genome-wide analysis of the EBV genome that revealed many novel target loci. The implications of these data for the ability of EBV to evade epigenetic silencing of the host viral genome is discussed.
Results

Prediction of ZREs core sequences bound by Zta using PROMO

In order to predict novel ZRE core sequences, we started by searching three well-characterized Zta-responsive promoters from the EBV genome (BZLF1 promoter (Zp) [26,27,28,29], BRLF1 promoter (Rp) [27,29,30] and BMRF1 promoter [31] using the PROMO algorithm [33,34] and the position frequency matrix (PFM) for Tranfe 8.3 Zta transcription factor entry T00923 [32,33]. These 3 promoters are known to contain eight previously verified sites: in Zp (ZREIIIA and ZREIIIB); in Rp (ZRE1, ZRE2 and ZRE3) and in BMRF1 promoter (AP1, ZRE(-44) and ZRE(-107)) (Figure 1 and Table S1) however, the PROMO algorithm only predicted one of these sites (RpZRE1) using the PFM T00923. In addition, 6 novel sites were predicted (Table S2), The ability of Zta to interact with each predicted site was assessed using electrophoretic mobility shift assays (EMSA) (Figure 1, Table S2), although three novel sites were identified, eight known sites were missed and three false positives were predicted indicating that the PFM used had a low sensitivity.

Application of a novel ZRE PFM to predict CpG containing ZREs

A new PFM was generated using the core sequences of five CpG-containing ZREs (denoted PFM_{CpG5}) from the promoters described above and the BRRF1 promoter [22] (Figure 2). The accuracy of the PFM was evaluated by searching for ZREs in the well-characterized viral promoters (Rp, Zp and BMRF1p). PFM_{CpG5} identified all 5 verified CpG containing sites and predicted two novel sites; one located in Rp, centered on -114, and one located in the BMRF1 promoter, centered on -148. DNA binding experiments demonstrate that Zta interacts with both sites in a methylation-dependent manner, characteristic of class III ZREs (Figure 3), thus the new PFM (ZRECpG5) has a high level of sensitivity. The PFM_{CpG5} was then used to predict core ZREs in the complete EBV genome. Within the EBV genome a total of 16 novel sequence variants of CpG ZREs were predicted (A–P) (Figure 3). EMSAs were undertaken with each of the novel ZRE core sequences (both non-methylated and methylated) to evaluate Zta binding (Figure 3). All but two of the predicted ZRE sequences bound in the methylated form. Only one sequence bound significantly in the unmethylated form. Therefore 13 out of 16 predictions are classified as Class III ZREs, 1 is classified as Class II and 2 did not interact with Zta significantly. Combined with previously published ZREs, this resulted in a total set of 32 distinct sequence variants of ZREs (ZRE_{32}) (Table 1).

Identification of ZRE core binding sequences in the EBV genome

Global analysis of the EBV genome was then undertaken using an exact pattern match with the 32 validated variants of the ZRE core sequence (Figure 4). This revealed 469 locations within the EBV genome that matched one of the ZRE core sequences (Table S3 and http://bioinf.biochem.sussex.ac.uk/EBV).
The occurrence of ZREs throughout the EBV genome appears to be widespread; with 81 out of 86 (94%) EBV promoters containing at least 1 ZRE core sequence (Table S4). This suggests that Zta has the potential to regulate the expression of the majority of EBV genes. Furthermore, 58 EBV promoters contained at least 1 CpG containing ZRE. These regions are methylated during latency [3,4], suggesting that methylation-dependent Zta interaction with ZREs could influence the expression of a broad range of EBV genes once Zta is synthesized at the onset of lytic cycle.

Of particular relevance to the control of EBV gene expression immediately after infection are 22 EBV genes that contained CpG ZREs but have no methylation independent ZREs in their promoters (Table 2). These genes are prime contenders to be regulated in a strictly methyl-dependent manner by Zta. These were originally classified as displaying early lytic, late lytic, or immediate-early expression during viral latency yet unmethylated following replication and immediately after infection [3,4]. Several are required for EBV replication and include components of the helicase/primase complex (BBLF1, BRLF1, and BBLF4) and DNA polymerase (BALF4). In addition, the promoters for BBLF3 and BBLF2/BBLF3 have been validated as being targets for Zta that are completely dependent on methylation for Zta activation [3]. Our discovery that one in five EBV promoters contain CpG ZREs but have no methylation independent ZREs strongly supports the hypothesis that the unmethylated status of the EBV genome guards against the expression of the full range of lytic genes and therefore lytic replication during the establishment of latency.

Zta is the prototypic member of a family of transcription factors that interact with DNA in a methylation-dependent manner. C/EBP alpha has recently been shown to share the same characteristics [36]. It has been suggested that the interaction between C/EBP alpha and methylated sequence elements are needed to activate tissue specific genes during differentiation [36].

The biphasic methylation cycle is observed for several different classes of viruses that establish latency [4]. Yet even KSHV, which is closely related to EBV, does not contain a functional Zta homologue. The question arises as to how the methylated genomes of these viruses can be reactivated. We suggest that the recent discovery that a cellular transcription factor also has methylation dependent DNA binding properties [36] implies that other viruses may rely on host methylation dependent transcription factors to differentially control the expression of their genomes during the establishment of latency or replication.

Discussion

Following several iterations of a predictive and evaluative approach, we identified a set of 32 distinct sequence variants in the core 7-nucleotide sequence to which Zta can bind. This includes 20 variants containing a CpG motif, the majority of which (90%) are only recognized by Zta when they are methylated.

The consensus binding sites identified for non-CpG ZREs are similar to the binding sites originally described for Zta (Figure 8). In contrast, the binding sites for CpG containing ZREs are remarkably different. This sequence is dominated by an almost invariant G 5’ to the absolute prerequisite for mc-CpG at positions 1’ and 2’ in the right-half of the core sequence.

The identification of 58 EBV promoters that harbor methylation dependent CpG ZREs, combined with the knowledge that the EBV genome is heavily methylated during latency [3,4], suggests that Zta plays an important role in overturning epigenetic silencing of over half of the EBV genes during lytic replication. Indeed, all three of the promoters tested displayed a strong interaction with Zta in vivo in ChIP analyses. A genome-wide DNA binding analysis was recently published identifying sequences to which a mutant form of Zta, that is replication and transactivation dead, can interact [3]. This report highlighted the strength of the interaction between Zta and methylation dependent binding of Zta to CpG ZREs in the EBV genome.

The EBV genes that contain only methylation-dependent ZREs are of particular interest. All of these genes are heavily methylated during viral latency yet unmethylated following replication and immediately after infection [3,4]. Several are required for EBV replication and include components of the helicase/primase complex (BBLF1, BRLF1, and BBLF4), the viral protein kinase (BGLF4), and DNA polymerase (BALF4). In addition, the promoters for BBLF3 and BBLF2/BBLF3 have been validated as being targets for Zta that are completely dependent on methylation for Zta activation [3]. Our discovery that one in five EBV promoters contain CpG ZREs but have no methylation independent ZREs strongly supports the hypothesis that the unmethylated status of the EBV genome guards against the expression of the full range of lytic genes and therefore lytic replication during the establishment of latency.

### Methods

**Computational prediction of ZREs core sequences bound by Zta**

The starting point for the computational approach was the Zta transcription factor entry T00923 in Transfac 8.3 that includes 6 experimentally verified ZRE binding sites [32,33]. The Promo algorithm [37,38] generated a position weight matrix (PWM)
based on the T00923 transcription factor entry, and we used it to search 3 well-characterized Zta-responsive promoters from the EBV genome (BZLF1 promoter (Zp) 500 bp upstream of the published transcription start sites were included. A positive match was taken as one with an 85% similarity rate.

DNA binding assays
Electrophoretic mobility shift assays (EMSA) were undertaken using Zta protein generated in a wheat germ in vitro translation system (Promega) and [32P]-radio labeled double strand oligonucleotides, as described previously [39].

Figure 3. Zta recognition and methylation dependence of PFMCPG5 predicted CpG containing ZREs. A. Flow diagram illustrating the information flow from the PFM to the predictions of novel ZREs in the EBV genome and their subsequent evaluation. B. Core heptamer sequences, in both forward and reverse complement, of PFMCPG5 predicted CpG containing ZREs within the EBV genome. C. PFMCPG5 was used to predict the potential for further ZREs in the EBV genome. Double strand oligonucleotides were generated. Following radio labeling, these were subject to in vitro methylation with SssI methyl transferase (+), or a mock reaction (−). Subsequently, they were incubated with in vitro translated Zta and subject to EMSA. The reactions contained control lysate, C, or Zta, Z.

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Where indicated in the figure, the central CpG motif was methylated on both cytosine residues during synthesis (Sigma) or methylated probes were synthesized or methylated in vitro using the CpG methyltransferase M.sssI (NEB) [23]. Zta protein (B95-8 strain) was in vitro translated using wheatgerm extract (Promega).

Table 1. List of core ZREs included within ZRE32.

| ZRE Core Sequence | Class | Names |
|-------------------|-------|-------|
| Forward | Reverse |  |
| Zp (−96): TAAATTTAGGTGTGTCATGAGGTGA | I | Zp ZREIIIA |
| Zp (−365): ACAGATGGACCTGAGCCACCCGCC | I | Rp ZRE1 |
| Zp (−662): CCTCTTTGGCTGACACCTCTCGCCC | I | BMRF1 ZRE(−44) |
| Zp (−107): CTGCACTCACAT | I | BMRF1 ZRE(−107) |
| DMR59 ZRE1 | I | BMRF1 AP-1 |
| DMR59 ZRE2 | I | BMRF1 AP-2 |
| DMR6 ZRE3 | I | BMRF1 AP-3 |
| DMR6 ZRE4 | I | BMRF1 AP-4 |
| DMR6 ZRE5 | I | BMRF1 AP-5 |
| DMR6 ZRE6 | I | BMRF1 AP-6 |

Zta protein (B95-8 strain) was in vitro translated using wheatgerm extract (Promega).

Where indicated in the figure, the central CpG motif was methylated on both cytosine residues during synthesis (Sigma) or methylated probes were synthesized or methylated in vitro using the CpG methyltransferase M.sssI (NEB) [23]. Zta protein (B95-8 strain) was in vitro translated using wheatgerm extract (Promega).
Table 2. EBV genes that contain CpG ZREs but have no methylation independent ZREs in their regulatory regions, with the kinetics and extent of any change in their expression in Akata cells undergoing lytic cycle [6].

| Cycle       | Promoter/Gene | Class | Sequence       | Offset From Gene Start | Peak expression time (hr) | fold change expression |
|-------------|---------------|-------|----------------|-------------------------|--------------------------|------------------------|
| early lytic | BALF1         | II    | TCGCTCA        | 74                      | 12                       | 8.2                    |
|            |               | II    | TCGCTCA        | 138                     |                          |                        |
|            |               | III   | TCGCCCA        | 383                     |                          |                        |
|            |               | III   | ACGCTCA        | 416                     |                          |                        |
| late lytic | BALF4         | III   | TCGCGCA        | 164                     | 24                       | 15.2                   |
|            |               | III   | TCGCTCG        | 254                     |                          |                        |
|            |               | III   | ACGCMCA        | 414                     |                          |                        |
| unknown    | BARF0         | III   | CGGCCTCA       | 45                      | 36                       | 7.3                    |
|            |               | III   | TGGCCGCA       | 45                      |                          |                        |
| early lytic| BARF1.2       | II    | TGAGCGCA       | 114                     | 24                       | 9.2                    |
|            |               | II    | TGGCTCG        | 50                      |                          |                        |
|            |               | III   | TGGCTCG        | 221                     | 12                       | 7.9                    |
|            |               | III   | TGGCTCA        | 235                     |                          |                        |
|            |               | III   | ACGCTCA        | 357                     |                          |                        |
| late lytic | BBLF4         | III   | AGAGCGA        | 31                      | 24                       | 7                      |
|            |               | III   | TTCCGCGA       | 414                     |                          |                        |
| late lytic | BBRF0         | III   | TGGCCGCA       | 34                      | 24                       | 20.5                   |
|            |               | III   | TGGCGGA        | 177                     |                          |                        |
| late lytic | BDLF1         | III   | TGGCCCG        | 390                     | 24                       | 21.5                   |
|            |               | II    | TGGCTCA        | 133                     | 24                       | 14.3                   |
| early lytic| BGLF4         | II    | GCCGCTCA       | 260                     | 8                        | 12.1                   |
|            |               | III   | TGGCTCG        | 316                     |                          |                        |
| late lytic | BGRF1/BDRF1   | III   | CGGGCGA        | 49                      | 24                       | 8.3                    |
| late lytic | BKRF2         | II    | TGAGCGCA       | 155                     | 24                       | 14.1                   |
|            |               | III   | TGGCCGGA       | 185                     |                          |                        |
| early lytic| BKRF4         | III   | TGAGCGGA       | 246                     |                          |                        |
|            |               | III   | CGTCCGGA       | 302                     |                          |                        |
| early lytic| BLLF2         | III   | CCGCTCA        | 18                      | 24                       | 12.4                   |
|            |               | III   | TGGCCCG        | 175                     | 24                       | 4.2                    |
| late lytic | BORF1         | III   | CGGCCTC        | 65                      |                          |                        |
|            |               | III   | TGGCCGGA       | 130                     | 24                       | 12.4                   |
|            |               | III   | TGGCCGGA       | 10                      |                          |                        |
| early lytic| BRRF1         | III   | CGGCCTG        | 25                      | 24                       | 7                      |
|            |               | III   | TGGCGCT        | 53                      |                          |                        |
| late lytic | BTRF1         | III   | TGGCCGGA       | 292                     | 24                       | 8.5                    |
| unknown    | BWRF1 repeats | III   | CGGCCTG        | 72                      | ~                        | ~                      |
|            |               | III   | GGAGCGA        | 46                      |                          |                        |
|            |               | III   | GGAGCGA        | 422                     |                          |                        |
| latent     | Cp EBNAs      | III   | TGGCGCGA       | 70                      | 24–48                    | 6–8.2                  |
|            |               | III   | TTTGCGA        | 204                     |                          |                        |
|            |               | III   | GGAGCGA        | 248                     |                          |                        |
| latent     | Wp EBNAs      | III   | TCAAGCGA       | 180                     | 48                       | 6.1                    |

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Figure 5. ZREs in the EBV genome. The entire EBV genome was subjected to an exact pattern match search, using the ZRE32 set of core sequences. Each site was classed by binding behaviour and plotted by the first nucleotide of the site to form a genome wide map of ZREs. Class I sites are indicated by blue diamonds, Class II sites are indicated by red stars, Class III sites are indicated by green triangles, and gene starts and direction are indicated by arrows. The location of transcription start sites and their orientation are indicated by arrows.

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Figure 6. Chromatin precipitation (ChIP) analysis of Zta. Akata cells were induced or not to enter lytic cycle by the addition if anti-IgG as indicated. 48 hours later the cross-linking agent was added and chromatin and total proteins were harvested. A. The proteins were subject to western blot analysis for Zta expression. B. A chromatin precipitation experiment was undertaken with the Zta antibody and a control antibody. The precipitated chromatin and input chromatin were subject to western blot analysis to detect Zta protein. C. The location of ZREs and Q-PCR amplicons are illustrated for a region central to OriLyt left and for a region flanking OriLyt left. The genome co-ordinates are indicated. Transcription start sites and the direction of transcription are shown by arrows. Blue diamonds represents the class I ZREs. The amplicons used for Q-PCR are indicated as black horizontal bars. D. Association of Zta with OriLyt left was assessed using chromatin precipitation from Akata cells in lytic cycle, followed by Q-PCR. The binding is shown relative to maximal binding to OriLyt left.

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Figure 7. Chromatin precipitation (ChiP) analysis of Zta binding with the EBV genome. A. Five regions of the EBV genome are illustrated, together with gene names and sequence co-ordinates. Three contain core CpG ZREs and two do not. Transcription start sites and the direction of transcription are indicated with arrows. Green triangles represent class III CpG ZREs and the red star represents a class II ZRE. The amplicons used for Q-PCR are indicated as black horizontal bars. F. Association of Zta with the indicated regions of the EBV genome was assessed using chromatin precipitation from Akata cells in early lytic cycle (stalled prior to DNA replication with acyclovir), followed by Q-PCR. The binding is shown relative to maximal binding to OriLyt left (OL5).

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Chromatin was prepared from Akata cells [35], following induction with anti IgG, in the presence of 100 μM acyclovir essentially as described in [40], except that a mixture of Protein A and protein G were used to capture antibodies. Precipitation was undertaken using an amino-terminal Zta antibody from Santa Cruz.

Primers: absolute genomic position and sequence

**Chromatin Immunoprecipitation**

Chromatin was prepared from Akata cells [35], following induction with anti IgG, in the presence of 100 μM acyclovir essentially as described in [40], except that a mixture of Protein A and protein G were used to capture antibodies. Precipitation was undertaken using an amino-terminal Zta antibody from Santa Cruz.
Supporting Information

Table S1  The sequences of published ZREs used in this study are shown, together with their names and references. CpG motifs are shown in bold. (DOCX)

Table S2  ZREs predicted using PROMO, together with the results of their evaluation by DNA binding assays (EMSA) are shown. CpG motifs are shown in bold. (DOCX)

Table S3  All ZREs in the EBV genome are shown with the position of the central nucleotide using RefSeq NC_007605. (DOCX)

Table S4  The number of ZREs close to each EBV promoter (between −1000 and +200 from the annotated gene start) are shown using RefSeq NC_007605. (DOCX)

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Author Contributions

Conceived and designed the experiments: KF DT JH SJ AJ. Performed the experiments: KF DT JH SR. Analyzed the data: KF DT JH SR SJ AJ. Wrote the paper: KF AJ.

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