Genome-Wide Analyses of Zta Binding to the Epstein-Barr Virus Genome Reveals Interactions in both Early and Late Lytic Cycles and an Epigenetic Switch Leading to an Altered Binding Profile

Sharada Ramasubramanyan,^a Aditi Kanhere,^{b,c} Kay Osborn,^a Kirsty Flower,^a Richard G. Jenner,^b and Alison J. Sinclair^a

School of Life Sciences, University of Sussex, Brighton, E. Sussex, United Kingdom^a; MRC Centre for Medical Molecular Virology, Division of Infection and Immunity, University College London, London, United Kingdom^b; and School of Biosciences, University of Birmingham, Birmingham, United Kingdom^c

The Epstein-Barr virus (EBV) genome sustains substantial epigenetic modification involving chromatin remodelling and DNA methylation during lytic replication. Zta (ZEBRA, BZLF1), a key regulator of the EBV lytic cycle, is a transcription and replication factor, binding to Zta response elements (ZREs) in target promoters and EBV lytic origins of replication. *In vitro*, Zta binding is modulated by DNA methylation; a subset of CpG-containing Zta binding sites (CpG ZREs) is bound only in a DNA methylation-dependent manner. The question of how the dynamic epigenetic environment impacts Zta interaction during the EBV lytic cycle is unknown. To address this, we used chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-Seq) to identify Zta binding sites across the EBV genome before and after viral DNA replication. Replication did not alter the association of Zta across many regions of the EBV genome, but a striking reduction in Zta binding occurred at some loci that contain CpG ZREs. Separating Zta-bound DNA into methylated and nonmethylated fractions, we found that promoters that contain CpG ZREs were enriched in the methylated fraction but that Zta binding to promoters lacking CpG ZREs was not reduced. We hypothesize that the loss of DNA methylation on the EBV genome during the lytic cycle causes the reduced binding to CpG ZREs; this may act as a lytic cycle epigenetic switch. However, the epigenetic changes associated with the replicated EBV genome do not affect the interaction of Zta with many loci that are rich in non-CpG ZREs; this leads to sustained binding at these regions.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that is associated with a number of diseases, including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, and posttransplant lymphoproliferative disease (6, 25, 33, 34, 40).

Primary EBV infection results in the establishment of viral latency within B lymphocytes (28, 39). Lytic replication occurs when latent virus is reactivated within B cells with physiological stimuli or chemical assault (27). The switch from latency to the lytic cycle involves global changes in the viral transcriptome, coordinated by the viral transcription and replication factor Zta (BZLF1, EB1, ZEBRA) (26).

Zta binds to Zta response elements (ZREs), which are characterized by 7-bp motifs resembling the AP1 and C/EBP α binding sites (17, 29, 36, 37). Investigations of individual EBV and host promoters revealed that Zta has the ability to interact with different classes of ZREs; class I ZREs do not contain a CpG motif, and the majority of these are bound in a methylation-independent manner. Class II and III ZREs contain an integral CpG motif, and *in vitro* assays show that binding by Zta is either dependent on (class III) or enhanced by (class II) DNA methylation (3, 4, 7, 15, 19–21). Therefore, CpG ZREs have the potential to encode epigenetic information. Attempts to identify Zta binding sites genome-wide have employed *in vitro* DNA binding and chromatin precipitation (ChIP) using either the DNA binding domain of Zta (3) or a motif identification approach (11). These studies identified further CpG ZREs in the EBV genome that display enhanced Zta binding when methylated *in vitro*.

The lytic promoters on the EBV genome are heavily methylated during latency (10, 19). In contrast, during the lytic cycle, the viral genome is hypomethylated (10). This is surprising, as the

methylation patterns on genomic DNA are normally inherited faithfully during DNA replication through the action of a DNA methyl transferase that recognizes hemimethylated DNA (5, 22); the inheritance of these epigenetic markers must be subverted during or possibly after the lytic replication of the EBV genome. DNA methylation is normally associated with repressive chromatin through the interaction with methylated CpG-dependent binding proteins which enforce a repressive chromatin environment in the region, resulting in a lack of gene expression (22). The extent of DNA methylation on the EBV genome in latency (10, 19) suggests that CpG ZREs will be recognized by Zta during the latency/lytic cycle transition. Indeed, several key viral lytic cycle promoters contain CpG ZREs, and furthermore, the reactivation of lytic cycle gene expression has been shown to require EBV genome methylation (3, 4, 7, 19).

In addition to its role in the latency/lytic cycle switch, Zta is also expressed transiently following B-cell infection (14, 19, 24, 35). During the subsequent immortalization process, a subset of lytic genes is expressed, but this does not lead to viral replication. The failure of Zta to trigger full lytic replication following *de novo*

Received 3 July 2012 Accepted 14 September 2012

Published ahead of print 26 September 2012

Address correspondence to Alison J. Sinclair, a.j.sinclair@sussex.ac.uk.

A.K. and K.O. contributed equally to this study.

Supplemental material for this article may be found at <http://jvi.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.01705-12

The authors have paid a fee to allow immediate free access to this article.

infection is considered to be linked to the lack of viral DNA methylation during the early stages of infection (10, 19, 41). The viral genome then becomes progressively methylated during the immortalization process, and data showing that cell transformation is aided by Zta (16, 18, 19, 24) suggest that the epigenetic status of the EBV genome may facilitate immortalization while preventing untimely lytic replication.

Therefore, the methylation status of EBV regulatory regions has been proposed to be important for immortalization and for facilitating the activation of lytic cycle genes during lytic cycle reactivation. Here, we ask whether the DNA methylation changes observed on the EBV genome affect the interaction of the Zta protein during the lytic cycle. We have used chromatin immunoprecipitation coupled with massively parallel sequencing (ChIP-Seq) to map the interaction between the endogenous Zta protein and the EBV genome during the lytic cycle, before and after viral genome replication. This identified many novel sites of interaction on the genome.

MATERIALS AND METHODS

Cell culture and induction of EBV lytic replication. Group I EBV-positive Akata Burkitt's lymphoma cells (38) were maintained in RPMI medium supplemented with 10% (vol/vol) fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Invitrogen) at 37°C with 5% CO₂. For EBV lytic induction, cells were seeded in log-phase growth medium at 5×10^5 cells/ml. After 24 h, the cells were concentrated to 2×10^6 cells/ml and treated with 0.125% rabbit anti-human IgG (Dako) or Dulbecco's phosphate-buffered saline (DPBS). The cells were harvested 12, 24, or 48 h postinduction. To prevent EBV genome replication, cells induced with anti-IgG were treated with 100 µM acyclovir.

Antibodies. Goat polyclonal antibody sc-17503 to Zta (Santa Cruz Biotechnology) was used for ChIP assays, and BZ1 mouse monoclonal antibody to Zta was used to detect the protein by Western blotting (44); control goat and rabbit immunoglobulin G (IgG) was obtained from Santa Cruz Biotechnology for ChIP assays. β-Actin (Sigma) was used to detect proteins by Western blotting.

Western blotting. Proteins from total cell lysates were fractionated on a 12% NuPage gel (Invitrogen) using SDS-PAGE and transferred to nitrocellulose. Zta was detected using the BZ1 mouse monoclonal antibody (44) and β-actin, followed by species-specific secondary horseradish peroxidase (HRP)-conjugated antibody and enhanced chemiluminescence (ECL) (GE Healthcare).

Viral load. A total of 2×10^6 Akata cells were harvested, and DNA was extracted using the Wizard genomic DNA purification kit (Promega). DNA was subjected to quantitative PCR (qPCR) using primers specific to the EBV and human genomes (EBV DNA polymerase gene and the human β-globin gene). The relative amount of EBV genome copies to human genome copies was analyzed as described previously (12).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed as described previously (2, 31). Briefly, the lysates were sonicated on ice ($10 \times$ with 10-s pulses; 30% amplitude output on a Branson model 250 Microtip at setting 5 [Sonics Vibracell]) to obtain 200- to 600-bp DNA fragments. For standard ChIP, 0.5 to 10 µg of antibodies was used with chromatin from 5×10^6 cells. The immune complexes were collected with preblocked 50% protein A/G-Sepharose bead slurry. Following stringent washes and protein digestion, the eluted DNA was purified using a gel extraction kit (Qiagen).

PCR primers for ChIP and cDNA are given in Table 1.

ChIP-Seq. Chromatin was isolated from cells exposed to both acyclovir and IgG, and ChIP was performed as described above with a few modifications. Briefly, the 50% protein A/G-Sepharose bead slurry was preblocked in 0.5% (wt/vol) bovine serum albumin (BSA) in DPBS. Chromatin from 1×10^8 cells was precleared with protein A/G bead

TABLE 1 Primers used for qPCR

EBV gene target	qPCR primers for ChIP (5' to 3')
BNRF1-F (31)	TGTGACACCAACAGGTGTTGCCTTG
BNRF1-R (31)	ACCCCAAAGAGGGCAAAGCCTAC
BCRF1-F (31)	GGGAGGTACATGTCCCCAGCATT
BCRF1-R (31)	CTGTGGACTGCAACACAACATTGCC
BFLF2-F	ATCTGCAGCCAGGCCCTTAGCC
BFLF2-R	CAAAACACGCTGTGGATGTGCC
BLLF3-F	TCCATCTGGGCACTTCTGACGCT
BLLF3-R	CCGTCAGCAGCGTGTTCACAA
BLLF2-F	CTCAGTGACATGGAAGAGGTTG
BLLF2-R	CCAACCACACCTTAGGAGGA
BZLF1-F	AGCCAAGGCACCAGCCTCCT
BZLF1-R	TGCATGAGCCACAGGCATTGCT
BRRF1-F (31)	CCTGTTGTTTCGAGAATGG
BRRF1-R (31)	AATTACAGCCGGGAGTGTG
BRLF1-F (31)	GGGTGACATGGATTACTGGTC
BRLF1-R (31)	TGATGCAGAGTCGCCTAATG
BKRF4-F (11)	CATTGCTCTCTGAGCGGTTA
BKRF4-R (11)	ACCAGATGCTTCTTGAGATTG
BTRF1-F (11)	AGCTACGCAATCGGAGTCA
BTRF1-R (11)	GGAGGCTCAGTCTAGCAG
OriLyt R-fl (ORI-F) (31)	CCGCATGTCCAACCACCACG
OriLyt R-fl (ORI-R) (31)	ATGCTACCTAGGCCTGCGTCC
OriLyt R (OL5-F) (31)	CAGCTGACCGATGCTCGCCA
OriLyt R (OL5-R) (31)	ATGGTGAGGCAGGCAAGGCG
BNLF2A-F	GGCCGTGGGGTTCGTCATCA
BNLF2A-R	ACGCTGCTTTTGGGTTCTTCTGGT

slurry. A total of 2% (vol/vol) of the precleared extract was retained as the input control sample, while the remainder was incubated with 10 µg of Zta-specific antibody or control goat IgG. The input control sample and the precipitated DNA were sequentially treated with 0.2 µg/ml RNase A and 0.2 µg/ml proteinase K and DNA purified. The sequencing libraries were prepared using 10 ng of the input and ChIP DNA using the ChIP-Seq sample preparation kit (Illumina) following the manufacturer's protocol, except that the library (150- to 350-bp fragments) was purified from the gel after PCR amplification. The DNA fragments were separated on a 2% agarose gel in Tris-acetate-EDTA (TAE) buffer and visualized using SYBR Safe stain, and 150- to 300-bp fragments were excised and purified using a gel extraction kit (Qiagen). The library was sequenced using an Illumina Genome Analyzer IIx.

ChIP-Seq data analysis: image analysis and sequence mapping. Initial processing of sequencing images generated by the Illumina Genome Analyzer IIx was carried out using the Cassava pipeline. Polony identification, base calling, and quality control (QC) statistics were performed using GOAT and Bustard modules. Short reads of 36 bp were aligned to the EBV genome sequence (accession number NC_007605) using Bowtie software (23). Only reads with zero to two mismatches were aligned, and only uniquely aligned reads passing the quality threshold were retained. The viral genome copy number in cells with replicating EBV is much higher than that in cells with nonreplicating EBV. To correct for this bias, we selected a random subset of the sequence reads of the same size as those in the nonreplicating EBV data set. For all data sets, when multiple reads matched to the same position, only a single read was considered. Sequence reads were extended to 150 bp, and Wig files were generated by calculating tag density in 10-bp windows and normalized to reads per million total reads using in-house R scripts. Data for ChIP sequencing runs were then background corrected using input DNA from cells with replicating and nonreplicating EBV. The data are shown in Table S4 in the supplemental material.

ChIP-Seq data analysis: Zta binding at promoters. Due to the small size of the viral genome and very closely spaced peaks, we were not able to

FIG 1 Zta association with the EBV genome during genome replication. (A) Akata cells were exposed to acyclovir and anti-immunoglobulin (α -IgG) as indicated for 48 h. Total protein lysates were fractionated using SDS-PAGE, and the expression of Zta was determined by Western blot analysis. Expression of β -actin was determined as a control. (B) Amount of EBV genome in cells treated with anti-immunoglobulin alone or anti-immunoglobulin and acyclovir, measured using quantitative PCR. Data are expressed relative to cells which were not exposed to either anti-immunoglobulin or acyclovir. (C) Chromatin was prepared from Akata BL cells induced in response to exposure with anti-IgG for 48 h. Chromatin was precipitated with either 0.5 μ g (black) or 2.0 μ g (gray) of anti-Zta antibody. The associated DNA was purified and amplified with primers specific for OriLyt flank (fl) or OriLyt. Zta binding is represented relative to the input chromatin. (D) Zta binding across the EBV genome during early (red; treated with anti-immunoglobulin and acyclovir) or late (green; treated with anti-immunoglobulin only) lytic replication in Akata cells. The numbers of sequencing reads from Zta ChIP-enriched DNA are plotted per million background-subtracted total reads, and the sequences are aligned with the EBV genome (RefSeq accession number NC_007605). The position of known repetitive elements (for which reads cannot be aligned) and the position and orientation of transcription start sites (TSS) for EBV genes are indicated, with rightward transcripts shown as diamonds and leftward transcripts shown as triangles.

use the standard peak calling programs for this analysis. Instead, a region between positions -500 and 200 with respect to the transcription start site (TSS) of the EBV genes was defined as the promoter region (11). The binding strength of Zta in the promoter region was measured as the number of reads in this region per million background subtracted total reads.

ChIP-Seq data analysis: metagene analysis. We extracted sequences for 10-kb regions centered on previously published class III and class I ZREs (with and without CpG, respectively) (11). For each data set, the average number of reads/million for positions relative to the binding site were calculated.

Enrichment for methylated and nonmethylated DNA. Sonicated genomic DNA from Akata cells (induced with IgG for 48 h) and reverse-

cross-linked DNA eluted from ChIP were separated into DNA methylated and nonmethylated fractions using a MethylCollector kit (Active Motif).

RESULTS

Genome-wide identification of Zta binding sites during the EBV lytic cycle. We sought to identify the full range of Zta binding sites across the EBV genome during the lytic cycle. The lytic cycle was triggered by treatment of the Burkitt's lymphoma cell line Akata with anti-IgG antibody, and cells were harvested for analysis of both the early and late phases. For analysis of the early lytic cycle, the EBV DNA polymerase inhibitor acyclovir was used to block

FIG 2 Zta association with the known sites of Zta binding during the lytic cycle. (A) Zta binding (reads/million) across a 2-kb area around the left origin of lytic replication is plotted relative to genome position. Known transcriptional start sites (arrows) and predicted ZREs (triangles) are marked. (B) Zta binding (reads/million) across a 2-kb area around the right origin of lytic replication is plotted relative to genome position. Details are as for panel A. (C) Zta binding (reads/million) across a 5-kb area around the immediate early promoters is plotted relative to genome position. Details are as for panel A.

EBV genome replication. As expected, we were able to confirm Zta expression in both samples at equivalent levels, while EBV genome levels were elevated 40-fold in the absence of acyclovir but reduced markedly in its presence (Fig. 1). Zta-associated chromatin was isolated using ChIP, in antibody excess (Fig. 1), and subjected to massively parallel DNA sequencing (ChIP-Seq). The resulting sequence reads were aligned with the EBV wild-type genome (GenBank accession number NC_007605) and mapped

relative to the start sites of EBV genes (Fig. 1D). The profiles of Zta interaction with the EBV genome during both early (plus acyclovir) and full (minus acyclovir) lytic cycle revealed that Zta associates with extensive areas of the EBV genome both prior to and during the period of EBV genome replication.

To verify our genome-wide data set, we compared Zta binding with previously identified Zta binding sites. It has been shown that Zta interacts with the origins of lytic replication *in vivo* (3, 31) and the region including the immediate early promoters, *BZLF1* and *BRLF1* (3, 20, 31, 43), and the early promoter *BRRF1* (7). The ChIP-Seq analysis revealed high-level association of Zta with both of the origins of lytic replication (OriLyt L 40301 to 41293; OriLyt R 143207 to 144444), *BZLF1*, *BRLF1*, and *BRRF1* (Fig. 2A to C). We also found that Zta bound to previously identified ZRE motifs (27). Together, these data confirm that the ChIP-Seq analysis detects previously known regions of Zta binding that are functionally relevant for the expression of viral gene expression regulators and viral genome replication.

Identification of Zta-interacting promoters. To systematically identify Zta-bound EBV promoters across the genome during the early lytic cycle, we measured sequence tag density in a large window covering each promoter region (−500 to +200 from the transcription start sites) (11). The promoters associated with the highest number of background-subtracted sequence reads are shown in Table 2, and the data for all EBV promoters are provided in Table S1 in the supplemental material. All of these regions contained at least one Zta binding peak (see Tables S2 and S3 in the supplemental material). Several of the promoters with the highest numbers of sequence reads are known targets of Zta, including *BRLF1*, *BZLF1* (Fig. 2C), *BSLF2/BMLF1* (Fig. 3E), *BALF2* (Fig. 3J), and *BMRF1* (Fig. 3D) (3, 20, 31, 43). Other promoters contain novel interaction sites for Zta in cells (*BLRF1*, *BNRF1*, *BCRF1*, *BFLF2*, *BNLF2a*, *BLLF3*, *BLRF2*, *BNLF2b*, *BKRF3*, *BKRF4*, *BKRF2*, *BILF2/RPMS1*, and *BGLF3.5*) (Table 2).

We next sought to determine whether Zta binding to these

TABLE 2 EBV promoters with the highest density of background-subtracted sequence tags mapping between −500 and +200 from the gene start

Promoter name	Promoter sequence tags per million in the early lytic cycle	Previously identified as a Zta binding site by ChIP	Gene function ^a	Class of lytic gene (8)
BNRF1	61.45	No	Tegument protein	Late
RPMS1	58.17	No	BART	Unclassified
BILF2	52.27	No	Glycoprotein	Late
BLRF2	51.21	No	Virion protein	Late
BLLF3	50.43	No	dUTPase	Early
BFLF2	49.81	No	Virus egress	Early
BNLF2a	48.11	No	Immune evasion	Early
BRLF1	46.35	Yes (21)	Transcription factor	Immediate early
BRRF2	45.54	No	Tegument protein	Late
BSLF2/BMLF1	45.11	Yes (3)	RNA export	Early
BZLF1	41.1	Yes (43)	Transcription and replication factor	Immediate early
BALF2	39.19	Yes (3)	EBV ssDNA binding protein	Early
BMRF1	38.63	Yes (3)	EBV DNA polymerase accessory protein	Early
BNLF2b	37	No	Unknown	Early
BKRF3	36.37	No	Uracil DNA glycosylase	Early
BGLF3.5	32.69	No	Tegument	Unclassified
BLRF1	30.28	No	Glycoprotein	Late
BCRF1	29.97	No	IL-10 homologue	Late
BKRF2	29.56	No	Glycoprotein	Late

^a BART, BamHI-associated rightward transcript; ssDNA, single-stranded DNA; IL-10, interleukin 10.

FIG 3 Zta association with novel sites of binding at EBV promoters in early lytic cycle. Zta binding (reads/million) across a 2-kb area around each of the indicated promoters is plotted relative to genome position. Known transcriptional start sites (arrows) and predicted ZREs (triangles) are marked.

genes was accompanied by the presence of a ZRE (Fig. 3). Comparison of binding with our previous ZRE motif prediction (11) revealed that each of the promoters contained at least one ZRE consensus sequence, with some containing multiple ZREs. The densely packed nature of Zta binding to the EBV genome during the early lytic cycle is apparent from these detailed maps, with examples being the *BRLF1/BRRF2* (Fig. 2C), *BLLF3/BRLF1/BRLF2* (Fig. 3F), *BILF2/RPMS1* (Fig. 3I), and *BKRPF2/BKRPF3* (Fig. 3H) regions shown in Fig. 3.

Gene-specific ChIP experiments were undertaken to verify the interaction of Zta with the selection of these promoters in chromatin from early and late lytic cycle (Fig. 4). We compared cells induced into lytic cycle for 12 h with cells induced for 48 h in the presence or absence of acyclovir. The binding of Zta to a region flanking OriLyt (OriLyt R-fl) is minimal and set the background for this assay, and the interaction with OriLyt (OriLyt R) is the strongest that we have observed on the EBV genome and served as a positive control (31). It is clear from the analysis of four promoter regions chosen from Table 2 that Zta reproducibly associated with the promoters for *BNRF1*, *BCRF1*, *BFLF2*, and *BLLF3* from early stages of the lytic cycle. We conclude that our ChIP-Seq analysis does identify genuine Zta binding sites across the EBV genome.

Differential interaction of Zta with the EBV genome in the early and late stages of the lytic cycle. Given that Zta is important for regulating multiple stages of the lytic cycle, we addressed the possibility that Zta binding patterns change as the lytic cycle progresses. In the first instance, we identified promoter regions

(−500 to +200 from the transcription start site) with a >2-fold difference in average sequence read density between early and full lytic cycle from the ChIP-Seq data sets (Fig. 1). Although 700 nucleotides is quite a large window to consider, the analysis highlighted some regions as differing substantially. Specifically, nine promoter regions displayed at least a 2-fold loss of Zta binding in

FIG 4 Zta association with EBV lytic promoters validated by chromatin immunoprecipitation. Zta binding using chromatin immunoprecipitation with control antibody (white bars) and Zta antibody from Akata cells induced to enter into the lytic cycle by treatment with anti-immunoglobulins for 12 h (shaded bars), 48 h in the presence of acyclovir (gray bars), and 48 h in the absence of acyclovir (black bars). Regions analyzed are shown on the x axis. Data are shown from 2 experiments, with the standard deviation shown.

TABLE 3 EBV promoters displaying 2-fold or greater reduction in Zta ChIP-enriched sequence tags averaged across 700 nucleotides of the promoter in the full lytic cycle

Gene	Promoter sequence tags per million for:		Ratio (early/full)
	Early lytic cycle	Full lytic cycle	
BBRF1	11.14	1.22	0.11
BALF4	6.82	1.31	0.19
BARF0	6.58	1.59	0.24
LF2	4.35	1.49	0.34
BBLF4	28.5	10.47	0.37
BKRF4	22.28	8.25	0.37
BTRF1	23.57	8.93	0.38
BFRF3	4.84	2.01	0.42
BPLF1	5.37	2.24	0.42

late lytic replication (Table 3). Data are shown for three regions that are constant (*OriLyt L*, *BFLF2*, and *BLLF3*) (Fig. 5A to C) and also for three regions that differ (*BKRF4*, *BBLF4*/*BBRF1*, and *BTRF1*) (Fig. 5D to F). For these, Zta interacted in the early stage of the lytic cycle but was substantially reduced when full replication was permitted.

Genome-wide analysis of Zta binding to CpG and non-CpG ZREs. We noticed that the promoters for which binding by Zta was reduced during full lytic cycle all contain CpG ZREs (11). This led us to question whether differential Zta binding correlates with the presence of CpG ZREs. To address this, we undertook a genome-wide analysis of Zta interaction as a function of ZRE class using the ChIP-Seq data (Fig. 1). We averaged the Zta binding profile for a 1-kb region spanning all 203 predicted class I (non-CpG) ZREs and all 237 predicted class III (CpG) ZREs (11). This demonstrated that the global interaction with non-CpG ZREs is similar in the presence and absence of acyclovir, with a slight increase associated with full lytic replication (Fig. 6A). In contrast, the interaction of Zta with CpG ZREs differs markedly (Fig. 6B). Clear association with Zta is observed in early lytic cycle, but the

association with Zta decreases when the lytic cycle is allowed to run its full course.

We used published EBV genome mapping data to identify sets of promoters that contained exclusively CpG or non-CpG ZREs (11). We then performed quantitative PCR to measure the enrichment of these promoter sequences in Zta ChIP samples from two further experiments comparing early and full lytic cycle. We found that the interaction of Zta with promoters that do not contain CpG ZREs was not appreciably altered (Fig. 6C). In contrast, when the lytic cycle was allowed to run its full course, the interaction with promoters containing exclusively CpG ZREs was significantly reduced (Fig. 6D). We conclude that Zta binding is selectively lost from CpG ZREs during EBV lytic replication.

Zta association with CpG ZRE-containing EBV promoters is dependent on methylation. We hypothesized that the differential DNA binding observed in the presence and absence of acyclovir may be due to differential recognition of methylation-dependent CpG ZREs by Zta. To test this, we investigated Akata cells undergoing the full lytic cycle. Methylation-dependent DNA precipitation was analyzed for two EBV loci, *OriLyt* and *BKRF4*; this showed that both methylated and nonmethylated EBV genomes are present at this stage, although the majority are nonmethylated (Fig. 7A). Zta-bound chromatin was then isolated using ChIP; this showed a clear association of Zta with *OriLyt*, with the background set by the control antibody and the flanking region (Fig. 7B). DNA was isolated from the Zta-associated chromatin and subsequently separated into methylated and nonmethylated fractions using a methyl binding domain protein coupled to paramagnetic beads. Promoter regions that contain non-CpG ZREs showed equivalent Zta binding in the presence and absence of methylation (*BFLF2* and *BLLF3*).

In contrast, for three promoter regions that contain at least one CpG ZRE (*BKRF4*, *BLLF2*, and *BRRF1*), Zta showed a distinct preference for DNA methylation (Fig. 7C).

Taken together, these data suggest that the disassociation of Zta from CpG ZRE-containing promoters that we observe during

FIG 5 Promoter regions enriched for Zta binding in early and late lytic cycle. Zta binding (reads/million) at selected genes that show a 2-fold or greater difference in Zta-enriched sequence reads is shown relative to genome position. The plus-acyclovir signal is shown in red, and the minus-acyclovir signal is in green. (A) Region flanking *OriLyt L*; (B) *BFLF2* region; (C) *BLLF3* region; (D) *BBRF1*/*BBLF4* region; (E) *BTRF1* region; (F) *BKRF4* region.

FIG 6 Genome-wide association of Zta with CpG and non-CpG ZREs in the EBV genome during early and full lytic cycle. (A and B) Average number of sequence reads (per million) for Zta ChIP-enriched DNA at non-CpG ZREs (A) and CpG ZREs (B) in the EBV genome in early (red) versus full (green) lytic cycle. (C) Zta binding from ChIP analysis expressed relative to binding at OriLyt in early (black; presence of acyclovir) and full (gray; absence of acyclovir) lytic cycle from Akata cells treated with anti-immunoglobulins for 48 h. Regions analyzed are shown, with OR1 as a representative nonbinding region and *BFLF2* and *BLLF3* representing promoters that contain only non-CpG ZREs (within the region of -500 to $+200$ from the gene start). (D) Zta binding from ChIP analysis expressed relative to binding at OriLyt in early (black) and full (gray) lytic cycle. Regions analyzed are shown, with *BKRF4*, *BLLF2*, and *BRRF1* representing promoters that contain exclusively CpG ZREs (within the region of -500 to $+200$ from the gene start).

the EBV lytic cycle is the result of a change in the proportion of methylated genomes.

DISCUSSION

The identification of Zta binding sites across the Epstein-Barr virus genome revealed extensive interactions that advance our understanding of the breadth of the Zta association with the EBV genome. Our analysis reveals that Zta interacts with many more EBV promoters than have been described previously. EBV lytic gene expression occurs in three temporal waves, immediate early genes, early genes, and late genes (8, 17); we show that Zta interacts with promoters in all three sets. A number of the promoters bound by Zta direct the expression of early lytic cycle genes, reinforcing the model whereby the product of the immediate early gene, Zta, directly activates the expression of early genes. Where gene-specific ChIP studies had previously been undertaken on individual EBV promoters, our ChIP-Seq data generally exhibited good concordance, for example, in comparing the interaction of Zta with the *BRLF1* and *BZLF1* promoter regions and at OriLyt (Fig. 2) (31). Furthermore, Zta association with *BRRF1* is clearly apparent from our ChIP-Seq data, as also identified previously (7). From our analysis of the ChIP-Seq data for the promoter regions with the highest Zta association, it is clear that many, but not all, predicted ZREs are recognized by Zta. This implies that there is an element of selectivity in the interaction of Zta with ZREs. Some Zta binding regions are broader than others, independent from the number of predicted ZREs in the region. This suggests that other factors, such as coassociating host or viral proteins or chromatin structure, may influence the interaction of Zta with DNA in cells.

It is not known whether Zta interacts with EBV DNA after replication of the genome during the lytic cycle. An interesting observation from these data was that in spite of a 40-fold increase in EBV genome copy number in the absence of acyclovir, the

FIG 7 Impact of DNA methylation on Zta interaction with chromatin during the lytic cycle. (A) Genomic DNA was isolated from Akata cells undergoing the full lytic cycle, and the methyl and nonmethyl fractions were collected. The relative amount of DNA in both fractions was assessed at each of the indicated loci for methylated DNA (black bars) and nonmethylated DNA (gray bars). (B) ChIP showing the amount of DNA precipitated by the Zta antibody (black) compared to that by the control antibody (open), with each expressed relative to Zta binding at OriLyt. OriLyt fl represents a region that Zta does not interact with. (C) DNA isolated from the ChIP in panel B was fractionated into nonmethylated and methylated DNA. Zta binding to methyl (black) and nonmethyl (gray) DNA was determined relative to binding at OriLyt. *BKRF4*, *BLLF2*, and *BRRF1* contain CpG ZREs, and *BFLF2* and *BLLF3* contain only non-CpG ZREs. (D) Model of the ability of Zta to interact with CpG (circles) and non-CpG (squares) ZREs in response to differential DNA methylation. During early lytic cycle, the EBV genome is predominantly methylated, so both types of ZRE are recognized. During the full lytic cycle, the EBV genome becomes predominantly nonmethylated, leading to a differential association of Zta with CpG ZREs.

amount of Zta bound per EBV genome remained the same at many loci in the presence or absence of acyclovir (Fig. 1). This suggests that the extensive interactions of Zta with the EBV genome occur on both replicated and nonreplicated genomes.

Previously, Zta had only been considered to activate early lytic genes and to contribute to genome replication. In this study, we reveal that Zta is also associated with promoters for late lytic cycle genes. Late lytic genes were originally defined on the basis of their dependence on viral genome replication for expression. The classification of late lytic genes was based on the inhibition of their expression by an EBV polymerase inhibitor, phosphonoacetic acid (PAA) (8). Although genome-wide analyses agree that the expression of late lytic genes lags behind that of early lytic cycle genes (45), it has also been shown that the requirement for EBV DNA replication for their expression is not universal. While the promoters for some late genes are dependent on EBV genome replication for their expression (1), several late lytic cycle genes can be activated in the absence of viral genome synthesis (9, 30) and some are expressed in the presence of acyclovir in Akata cells undergoing lytic cycle (42). Furthermore, the viral *BcRF1* gene product acts as a TATA binding protein for some late genes (13), which may affect the timing of their expression. Whether Zta binding has functional consequences either before or after EBV genome replication remains to be determined.

We also found that Zta associates with regions of the EBV genome devoid of promoters (such as around genome positions 37000, 45000, and 79000) (Fig. 1). This raises the possibility that Zta may have an additional function in the EBV lytic cycle, such as replication compartment localization or genome packaging.

A major discovery from this study is the identification of a novel epigenetic switch during the lytic cycle which leads to the redistribution of Zta at certain EBV promoters. Our genome-wide analysis of Zta interactions with CpG ZREs shows marked reduction in late lytic cycle. The separation of Zta-bound DNA into methylated and nonmethylated fractions further pinpoints this to differences in DNA methylation of the EBV genome in late lytic cycle. Together, this supports the model that the observed epigenetic switch in Zta binding to CpG ZREs results from the inability of Zta to recognize CpG ZREs in the unmethylated state (13) and the generation of nonmethylated EBV genomes during the lytic cycle (20).

Other gamma herpesviruses which establish latency, such as Kaposi's sarcoma-associated herpesvirus, have a biphasic methylation cycle and may also exploit methylation-dependent DNA binding or encounter an epigenetic switch during the lytic cycle. Although Zta does not have a direct homologue in KSHV, a host transcription factor, such as C/EBP α , may mediate a similar switch, since c/EBP α has recently been shown to display a similar methyl DNA-dependent binding phenotype for a subset of binding sites that contain a CpG motif (32). This suggests that other viral genomes that display biphasic methylation patterns during the lytic cycle have the potential to trigger a similar lytic cycle epigenetic switch in transcription factor binding to limit the expression of viral genes.

ACKNOWLEDGMENTS

We thank Alana Durant for primer design, M. Rowe for providing BZ1 antibody, and K. Takada for providing Akata cells.

The research was funded by a Wellcome Trust grant to A.J.S., an MRC

Career Development Award to R.G.J., and the MRC Centre for Medical Molecular Virology.

REFERENCES

1. Amon W, et al. 2004. Lytic cycle gene regulation of Epstein-Barr virus. *J. Virol.* 78:13460–13469.
2. Bark-Jones SJ, Webb HM, West MJ. 2006. EBV EBNA 2 stimulates CDK9-dependent transcription and RNA polymerase II phosphorylation on serine 5. *Oncogene* 25:1775–1785.
3. Bergbauer M, et al. 2010. CpG-methylation regulates a class of Epstein-Barr virus promoters. *PLoS Pathog.* 6:e1001114. doi:10.1371/journal.ppat.1001114.
4. Bhende PM, Seaman WT, Delecluse HJ, Kenney SC. 2004. The EBV lytic switch protein, Z, preferentially binds to and activates the methylated viral genome. *Nat. Genet.* 36:1099–1104.
5. Bird AP, Wolffe AP. 1999. Methylation-induced repression—belts, braces, and chromatin. *Cell* 99:451–454.
6. Cohen JI. 2000. Epstein-Barr virus infection. *N. Engl. J. Med.* 343:481–492.
7. Dickerson SJ, et al. 2009. Methylation-dependent binding of the Epstein-Barr virus BZLF1 protein to viral promoters. *PLoS Pathog.* 5:e1000356. doi:10.1371/journal.ppat.1000356.
8. Farrell PJ. 2005. Epstein-Barr virus genome, p 263–287. In Robertson ES (ed), *Epstein-Barr virus*. Caister Academic Press, Wymondham, United Kingdom.
9. Feederle R, et al. 2000. The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators. *EMBO J.* 19:3080–3089.
10. Fernandez AF, et al. 2009. The dynamic DNA methylomes of double-stranded DNA viruses associated with human cancer. *Genome Res.* 19:438–451.
11. Flower K, et al. 2011. Epigenetic control of viral life-cycle by a DNA-methylation dependent transcription factor. *PLoS One* 6:e25922. doi:10.1371/journal.pone.0025922.
12. Gallagher A, et al. 1999. Detection of Epstein-Barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease. *Int. J. Cancer* 84:442–448.
13. Gruffat H, Kadjouf F, Mariame B, Manet E. 2012. The Epstein-Barr virus BcRF1 gene product is a TBP-like protein with an essential role in late gene expression. *J. Virol.* 86:6023–6032.
14. Halder S, et al. 2009. Early events associated with infection of Epstein-Barr virus infection of primary B-cells. *PLoS One* 4:e7214. doi:10.1371/journal.pone.0007214.
15. Heather J, Flower K, Isaac S, Sinclair AJ. 2009. The Epstein-Barr virus lytic cycle activator Zta interacts with methylated ZRE in the promoter of host target gene *egr1*. *J. Gen. Virol.* 90:1450–1454.
16. Hong GK, et al. 2005. Epstein-Barr virus lytic infection is required for efficient production of the angiogenesis factor vascular endothelial growth factor in lymphoblastoid cell lines. *J. Virol.* 79:13984–13992.
17. Israel B, Kenney SC. 2005. EBV lytic infection, p 571–611. In Robertson ES (ed), *Epstein-Barr virus*. Caister Academic Press, Wymondham, United Kingdom.
18. Jones RJ, et al. 2007. Roles of lytic viral infection and IL-6 in early versus late passage lymphoblastoid cell lines and EBV-associated lymphoproliferative disease. *Int. J. Cancer* 121:1274–1281.
19. Kalla M, Schmeink A, Bergbauer M, Pich D, Hammerschmidt W. 2010. AP-1 homolog BZLF1 of Epstein-Barr virus has two essential functions dependent on the epigenetic state of the viral genome. *Proc. Natl. Acad. Sci. U. S. A.* 107:850–855.
20. Karlsson QH, Schelcher C, Verrall E, Petosa C, Sinclair AJ. 2008. Methylated DNA recognition during the reversal of epigenetic silencing is regulated by cysteine and serine residues in the Epstein-Barr virus lytic switch protein. *PLoS Pathog.* 4:e1000005. doi:10.1371/journal.ppat.1000005.
21. Karlsson QH, Schelcher C, Verrall E, Petosa C, Sinclair AJ. 2008. The reversal of epigenetic silencing of the EBV genome is regulated by viral bZIP protein. *Biochem. Soc. Trans.* 36:637–639.
22. Klose RJ, Bird AP. 2006. Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.* 31:89–97.
23. Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25.

24. Ma SD, et al. 2011. A new model of Epstein-Barr virus infection reveals an important role for early lytic viral protein expression in the development of lymphomas. *J. Virol.* 85:165–177.
25. Macsween KF, Crawford DH. 2003. Epstein-Barr virus—recent advances. *Lancet Infect. Dis.* 3:131–140.
26. Miller G. 1989. The switch between EBV latency and replication. *Yale J. Biol. Med.* 62:205–213.
27. Miller IG, Jr, El-Guindy A. 2002. Regulation of Epstein-Barr virus lytic cycle activation in malignant and nonmalignant disease. *J. Natl. Cancer Inst.* 94:1733–1735.
28. Murray PG, Young LS. 2001. Epstein-Barr virus infection: basis of malignancy and potential for therapy. *Expert Rev. Mol. Med.* 3(28):1–20.
29. Petosa C, et al. 2006. Structural basis of lytic cycle activation by the Epstein-Barr virus ZEBRA protein. *Mol. Cell* 21:565–572.
30. Ragoczy T, Miller G. 1999. Role of the Epstein-Barr virus Rta protein in activation of distinct classes of viral lytic cycle genes. *J. Virol.* 73:9858–9866.
31. Ramasubramanyan S, Osborn K, Flower K, Sinclair AJ. 2012. Dynamic chromatin environment of key lytic cycle regulatory regions of the Epstein-Barr virus genome. *J. Virol.* 86:1809–1819.
32. Rishi V, et al. 2010. CpG methylation of half-CRE sequences creates C/EBP α binding sites that activate some tissue-specific genes. *Proc. Natl. Acad. Sci. U. S. A.* 107:20311–20316.
33. Rowe M, Kelly GL, Bell AI, Rickinson AB. 2009. Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology. *Semin. Cancer Biol.* 19:377–388.
34. Saha A, Robertson ES. 2011. Epstein-Barr virus-associated B-cell lymphomas: pathogenesis and clinical outcomes. *Clin. Cancer Res.* 17:3056–3063.
35. Shannon-Lowe C, et al. 2009. Features distinguishing Epstein-Barr virus infections of epithelial cells and B cells: viral genome expression, genome maintenance, and genome amplification. *J. Virol.* 83:7749–7760.
36. Sinclair AJ. 2006. Unexpected structure of Epstein-Barr virus lytic cycle activator Zta. *Trends Microbiol.* 14:289–291.
37. Sinclair AJ, Farrell PJ. 1992. Epstein-Barr virus transcription factors. *Cell Growth Differ.* 3:557–563.
38. Takada K. 1984. Cross-linking of surface immunoglobulins induces Epstein-Barr virus in Burkitt's lymphoma cell lines. *Int. J. Cancer* 33:27–32.
39. Thorley-Lawson DA. 2001. Epstein-Barr virus: exploiting the immune system. *Nat. Rev. Immunol.* 1:75–82.
40. 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.
41. Tierney RJ, et al. 2000. Methylation of transcription factor binding sites in the Epstein-Barr virus latent cycle promoter Wp coincides with promoter down-regulation during virus-induced B-cell transformation. *J. Virol.* 74:10468–10479.
42. Wang FZ, et al. 2009. Maribavir inhibits Epstein-Barr virus transcription in addition to viral DNA replication. *J. Virol.* 83:12108–12117.
43. Yin Q, Jupiter K, Flemington EK. 2004. The Epstein-Barr virus transactivator Zta binds to its own promoter and is required for full promoter activity during anti-Ig and TGF- β 1 mediated reactivation. *Virology* 327: 134–143.
44. Young LS, et al. 1991. Differentiation-associated expression of the Epstein-Barr virus BZLF1 transactivator protein in oral hairy leukoplakia. *J. Virol.* 65:2868–2874.
45. Yuan J, Cahir-McFarland E, Zhao B, Kieff E. 2006. Virus and cell RNAs expressed during Epstein-Barr virus replication. *J. Virol.* 80:2548–2565.