A Noncanonical Basic Motif of Epstein-Barr Virus ZEBRA Protein Facilitates Recognition of Methylated DNA, High-Affinity DNA Binding, and Lytic Activation

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ABSTRACT The pathogenesis of Epstein-Barr virus (EBV) infection, including development of lymphomas and carcinomas, is dependent on the ability of the virus to transit from latency to the lytic phase. This conversion, and ultimately disease development, depends on the molecular switch protein, ZEBRA, a viral bZIP transcription factor that initiates transcription from promoters of viral lytic genes. By binding to the origin of viral replication, ZEBRA is also an essential replication protein. Here, we identified a novel DNA-binding motif of ZEBRA, N terminal to the canonical bZIP domain. This RRTRK motif is important for high-affinity binding to DNA and is essential for recognizing the methylation state of viral promoters. Mutations in this motif lead to deficiencies in DNA binding, recognition of DNA methylation, lytic cycle DNA replication, and viral late gene expression. This work advances our understanding of ZEBRA-dependent activation of the viral lytic cascade.

IMPORTANCE The binding of ZEBRA to methylated and unmethylated viral DNA triggers activation of the EBV lytic cycle, leading to viral replication and, in some patients, cancer development. Our work thoroughly examines how ZEBRA uses a previously unrecognized basic motif to bind nonmethylated and methylated DNA targets, leading to viral lytic activation. Our findings show that two different positively charged motifs, including the canonical BZIP domain and a newly identified RRTRK motif, contribute to the mechanism of DNA recognition by a viral AP-1 protein. This work contributes to the assessment of ZEBRA as a potential therapeutic target for antiviral and oncolytic treatments.

KEYWORDS AP-1-like protein, BZLF1, DNA-binding domain, DNA methylation, Epstein-Barr virus, lytic activation, noncanonical DNA-binding domain, ZEBRA, bZIP protein, transcription factors

Epstein-Barr virus (EBV), the first virus linked to human cancer development, is highly prevalent in the adult population (1, 2). The virus is typically present in the latent state, but it can sporadically convert to its lytic form (3–5). This conversion is dependent on the lytic transcriptional activator ZEBRA, also known as Z, Zta, and BZLF1 (6). As a viral lytic cycle activator, ZEBRA binds to promoters of viral lytic cycle genes and to the origin of lytic replication, oriLyt (7). Upon DNA binding, ZEBRA coordinates transcription and replication through physical contact with viral and host cellular machinery to alter the transcriptional and replication landscape of the virus and the cell (8). This ZEBRA-dependent coordination is essential to EBV function. Deletion of ZEBRA from the viral genome renders the virus unable to activate lytic gene expression and DNA replication (9).

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ZEBRA triggers the conversion of EBV from its latent to lytic state by binding to virus-specific heptad DNA sequences known as ZEBRA responsive elements (ZREs). There are three classes of ZREs (10–13), at least one of which is found in the promoters of 94% of EBV genes (14). Approximately half of the ZEBRA viral promoter-binding events are methylation dependent. For example, *Rp*, the promoter of the EBV *BRFL1* gene, an initial target of ZEBRA in the earliest stages of lytic activation, contains three ZREs: unmethylated ZRE1 and methylated ZRE2 and ZRE3. Methylation of the CpG site in ZRE2 causes a 4-fold enhancement in ZEBRA binding affinity (10). Interestingly, ZEBRA is only able to bind ZRE3 when the promoter is methylated (10, 11, 15). The mechanism by which ZEBRA recognizes and selectively binds to both methylated and unmethylated DNA is central to the understanding of how it converts the virus from its latent to lytic state. In addition to binding methylated promoters, ZEBRA also binds to nonmethylated canonical AP-1 DNA sequences and to seven nonmethylated ZREs in the origin of lytic replication (*oriLyt*) to facilitate the assembly of the replication complex (16).

As a homologue of the cellular activator protein 1 (AP-1) proteins c-Jun and c-Fos, ZEBRA recognizes and interacts with DNA through its basic leucine zipper (bZIP) domain (12, 17). ZEBRA has a modular structure similar to that of other members of the AP-1 transcriptional activator family. ZEBRA's N-terminal transactivation domain (Fig. 1A, blue) binds to cellular machinery, including RNA polymerase II and other associated proteins, to coordinate transcription (18). ZEBRA contains a casein kinase 2 regulatory domain located between the transactivation domain and the bZIP domain that includes two phosphorylation sites. Phosphorylation of ZEBRA at these sites is not necessary for the capacity of ZEBRA to activate transcription of early genes but is indispensable for the role of ZEBRA in genome replication (19, 20). The rest of the ZEBRA domains, such as the bZIP DNA binding (Fig. 1A, gray) and dimerization domain, are similar to those found in other AP-1 transcription factors.

Recognizing methylated ZREs is essential for proper initiation of the lytic cascade by ZEBRA. However, methylation of cytosines at CpG sites in cellular DNA is typically affiliated with silencing of transcription (21). In the case of latent EBV infection, viral hijacking of the host cellular machinery leading to cellular proliferation results in a heavily methylated viral genome during the maintenance stage of infection. The preference of ZEBRA for binding methylated DNA is thought to prevent lytic infection from occurring prior to establishment of latent infection in the host. Thus, it is important to understand how ZEBRA reads the methylation state of viral promoters. In this study, we identified a positively charged segment of ZEBRA, previously misannotated as part of the transactivation domain, that is essential for the recognition of the DNA methylation state, increased DNA-binding affinity, and lytic cycle activation. Delineating the regulation of ZEBRA DNA-binding interactions and methylation selectivity advances our understanding of the mechanism of ZEBRA-dependent activation of the viral lytic cascade. This understanding may inform the development of novel antiviral and oncolytic treatments.

RESULTS

Defining ZEBRA's DNA recognition domain for high-affinity DNA binding.

Previous research showed that the N terminus (Fig. 1A, blue) of ZEBRA is involved in recruitment of host cellular factors but is not involved in DNA substrate specificity (18). To date, the minimal DNA-binding domain of ZEBRA has been defined based on its sequence similarities to canonical AP-1 proteins (22). Based on this sequence homology, the minimal DNA-binding domain includes residues 166 to 245 of ZEBRA (Fig. 1A, gray). These amino acids span the canonical basic domain homologous to AP-1 and the subsequent dimerization domain. After a close inspection of the ZEBRA protein sequence, we noticed a unique basic region (¹⁵⁷RRTRK¹⁶¹) upstream of the canonical minimal DNA-binding domain of ZEBRA (Fig. 1A, blue). The charge composition of this previously unrecognized motif suggested that this region also is involved in DNA binding.

FIG 1 Noncanonical basic motif in ZEBRA is important for high-affinity binding to DNA. (A) Schematic of full-length ZEBRA domain architecture and its truncations. (B) Sequence of the ZpZIIIB 21-mer DNA substrate. (C) Electrophoretic mobility shift assays with full-length and N-terminal truncations of FLAG-tagged ZEBRA expressed in eukaryote HKB5-B5 cells binding to radiolabeled ZIIIB 21-mer. Ten μ g of cell extract was loaded in each lane. The numbers at the bottom of the gel indicate the percentage of the DNA shifted by ZEBRA. The letter B marks a background band. (D to F) ITC measurement for the binding of ZEBRA full length (D), Z(155-245) (E), or Z(166-245) (F) to ZpZIIIB 14-mer DNA. All measurements were completed in triplicate.

To test our hypothesis, we designed three ZEBRA constructs for DNA-binding assays: full-length ZEBRA [Z(1-245)], a truncation retaining the RRTRK basic motif upstream of the bZIP domain [Z(155-245)], and a shorter truncation that includes only the previously defined, minimal DNA-binding domain [Z(166-245)] (Fig. 1A). All the constructs are fused to either a maltose-binding protein (MBP) or FLAG tag on the N terminus. We used electromobility shift assays (EMSAs) and isothermal titration calorimetry (ITC) to measure the DNA-binding affinity of the different ZEBRA constructs to the ZpZIIIB DNA sequence (Fig. 1B), a high-affinity nonmethylated ZRE heptad that is naturally found in the promoter of the EBV BZLF1 gene encoding ZEBRA.

EMSA results clearly revealed the importance of amino acids 155 to 166 of ZEBRA, containing the newly identified RRTRK basic motif, for DNA binding. We mixed radiolabeled 21-mer ZpZIIIB with lysates of HKB5-B5 mammalian cells expressing FLAG-

TABLE 1 Summary of binding affinities of different purified ZEBRA proteins with different DNA substrates^a

ZEBRA construct	Binding affinity (nM) for:						
	ZpZIIIB 14-mer	RpZRE3 14-mer unmethylated	RpZRE3 14-mer methylated site 1	RpZRE3 14-mer methylated site 2	RpZRE3 14-mer methylated sites 1 and 2	RpZRE3 11-mer methylated sites 1 and 2	RpZRE3 9-mer methylated sites 1 and 2
Z(1-245)	140 ± 40						
Z(155-245)	140 ± 30, 140 ± 10*	1,500 ± 300	400 ± 100	50 ± 10	60 ± 30, 180 ± 40*	150 ± 50	1,200 ± 400
Z(166-245)	1,800 ± 800	9,700 ± 5,000			1,800 ± 600		
Z(155-245) AATAA	750 ± 200	3,400 ± 700			980 ± 100	1,900 ± 300	3,100 ± 400
Z(175-245)	3,500 ± 700						

^aAll measurements were done by ITC except for those indicated with an asterisk, which were measured by fluorescence polarization.

tagged ZEBRA constructs (Fig. 1C). The best DNA binding was observed with Z(155-245), which caused a 30% shift of the radiolabeled ZpZIIIB; full-length ZEBRA shifted 15% of the labeled DNA. In contrast, Z(166-245), lacking the RRTRK motif, was deficient in DNA binding; only 2% of the DNA was supershifted, similar to the control cytomegalovirus (CMV) FLAG alone (Fig. 1C). These results indicate that the presence of a second basic motif, RRTRK, is essential for ZEBRA to bind efficiently to the ZpZIIIB site. The commonly used minimal construct of Z(166-245) is deficient in binding to ZpZIIIB DNA.

We then used ITC to measure the binding affinities of recombinant MBP-fused ZEBRA constructs expressed and purified from *Escherichia coli* (see Fig. S1A in the supplemental material). As a control, we did not detect binding between the MBP fusion tag and DNA (Fig. S1B). ITC measurements showed that Z(155-245) and full-length ZEBRA bound DNA with a similar affinity at a dissociation constant (K_d) of ~140 nM (Fig. 1D and E and Table 1). The similarity in binding affinities of Z(1-245) and Z(155-245) suggests that the presence of the transactivation domain does not impact DNA binding. However, ITC revealed that Z(166-245) and Z(175-245), which both lack the RRTRK motif, are markedly impaired in high-affinity DNA binding to the ZpZIIIB DNA with an approximately 10-fold decrease in affinity (Fig. 1F, Table 1, and Fig. S1C). Thus, these commonly used minimal constructs of Z(166-245) are not sufficient for high-affinity DNA binding that requires the upstream RRTRK motif.

We used 293T cell extracts to compare the DNA-binding activity, as assessed by EMSA, of truncated ZEBRA proteins that did or did not contain the newly recognized basic motif with those containing the previously defined canonical basic domain present in Z(172-245) and Z(175-245) (Fig. S2). While Z(166-245) did not bind DNA, a result confirming the low affinity of DNA binding of this protein as measured by ITC (Fig. 1F), both Z(172-245) and Z(175-245) bound ZpZIIIB and RpZRE3, as detected by EMSA. This result suggests that there is a DNA-binding-inhibitory function present in Z(166-172).

ZEBRA's RRTRK basic motif is essential for methylation recognition. ZEBRA is unique among transcription factors for its ability to preferentially bind methylated DNA substrates. Since the amino acids 155 to 166 of ZEBRA containing the RRTRK motif are important for high-affinity binding to ZpZIIIB, we further examined whether this motif is also important for differentiating ZRE methylation states. We used three different methods (ITC, fluorescence polarization, and EMSA) to measure the affinity of different ZEBRA constructs to RpZRE3, a prototypical, doubly methylated DNA to which ZEBRA binds with high affinity (Fig. 2A).

First, we tested the binding efficiency of different ZEBRA constructs (Fig. 1A) to RpZRE3-methylated DNA using a fluorescence polarization (FP) assay (Fig. 2B). When the DNA is doubly methylated, Z(155-245) binds to the fluorescently tagged substrate with an affinity (K_d) of ~180 nM (Fig. 2B and Table 1). In contrast, no measurable DNA binding was observed using FP for either substrate by Z(166-245), which lacks the RRTRK basic region (data not shown). The lack of detectable binding using FP suggests that Z(166-245) has weak binding affinity for DNA, below the limit of detection using this method. Similarly, in the absence of methylation of the RpZRE3 substrate, binding

FIG 2 ZEBRA's noncanonical basic motif is important for binding methylated DNA. (A) Sequence of RpZRE3 20-mer DNA. Sites of methylation are highlighted with yellow stars. (B) FP affinity measurements of Z(155-245) binding to ZRE3 14-mer doubly methylated (red) versus unmethylated DNA (black). (C and D) ITC measurement for the binding of Z(155-245) to unmethylated (C) or to doubly methylated (D) RpZRE3 14-mer DNA. All measurements were completed in triplicate. (E and F) ITC measurement for the binding of Z(166-245) to unmethylated (E) or doubly methylated (F) RpZRE3 14-mer DNA. All measurements were completed in triplicate. (G) Electrophoretic mobility shift assays with 10 μ g of cell extract of HHKB5-B5 cells containing wild-type or N-terminal truncations of ZEBRA. Probes were unmethylated or methylated RpZRE3 20-mer. The letter B marks a background band.

was undetectable by FP even at high protein concentrations of Z(155-245) (Fig. 2B). These results support the notion that ZEBRA binding to RpZRE3 requires DNA methylation and the RRTRK motif of the protein.

We next used ITC and EMSA to confirm and further quantify the binding observations. Similar to the FP measurements, Z(155-245) binds the methylated RpZRE3 DNA as efficiently as full-length ZEBRA, and the Z(166-245) truncation is greatly impaired in binding RpZRE3 DNA (Fig. 2C and E and Fig. S1A, S2A, and S3A). The high-affinity binding is again observed to be methylation dependent. The binding affinity (K_d) of Z(155-245) for RpZRE3 with the two CpG sites methylated (Fig. 2A) is ~ 62 nM (Fig. 2D and Table 1), which is greatly reduced compared to $\sim 1,500$ nM when both sites are unmethylated (Fig. 2C and Table 1). ITC also allowed us to detect the low RpZRE3-binding affinities of Z(166-245), which binds unmethylated RpZRE3 DNA with a K_d of $\sim 9,700$ nM (Fig. 2E and Table 1) and doubly methylated DNA with a slightly enhanced affinity of $\sim 1,800$ nM (Fig. 2F and Table 1).

A close inspection of these data reveals that the RRTRK motif is required not only for high-affinity DNA binding but also for the recognition of the methylated state of DNA. Z(166-245) distinguishes between methylated and unmethylated DNAs with a factor of ~ 5 (K_d of 1,800 nM versus 9,700 nM). In addition to substantially enhanced DNA binding, the discrimination of different DNA methylation states increases to ~ 30 -fold (K_d of 62 nM versus 1,500 nM) when the RRTRK motif is included in Z(155-245) (Fig. 2 and Table 1). This analysis shows that the detection of methylated DNA largely depends on this newly identified basic motif.

To validate this effect with proteins expressed in eukaryotic cells, we used EMSA to compare full-length ZEBRA and the Z(155-245) and Z(166-245) truncations binding to doubly methylated and unmethylated RpZRE3 DNA. Z(155-245) again bound preferentially to the doubly methylated RpZRE3, similar to full-length ZEBRA (Fig. 2G). We then used a cold oligonucleotide DNA competition assay to show that even in the presence of a $400\times$ excess of unmethylated RpZRE3, Z(155-245) truncation preferentially bound to the methylated DNA (Fig. S3C). Consistent with our ITC results, while Z(166-245) is competent in DNA binding, it is severely impaired in recognizing the methylation state of DNA. Z(166-245) was impaired in binding to all three methylation states of RpZRE3 (Fig. 2G and Fig. S3A). Together, these measurements support the hypothesis that the additional RRTRK basic region outside the conserved DNA-binding domain is important for substrate recognition and for methylation-dependent DNA binding.

ZEBRA exhibits asymmetrical recognition of DNA methylation sites. The additional basic region of ZEBRA is also important for distinguishing between different positions of methylation within the RpZRE3 DNA. To examine whether ZEBRA prefers methylation at site 1 or site 2 of RpZRE3 DNA (Fig. 2A and Fig. S3A), we compared the amount of probe that was shifted by FLAG-tagged full-length ZEBRA, Z(155-245), Z(166-245), and CMV control with RpZRE3 DNA that was singly methylated at site 1 or site 2. Full-length Z(1-245) and its Z(155-245) truncation have binding efficiency similar to that of site 2-methylated RpZRE3. When only site 1 is methylated, there is a 10-fold decrease in binding by both full-length ZEBRA and Z(155-245) to site 1-methylated DNA (Fig. 3A). We confirmed ZEBRA's preference for site 2-methylated RpZRE3 DNA by measuring its affinity by ITC with Z(155-245). ITC measurements indicate that Z(155-245) binds RpZRE3 site 1-methylated DNA with a K_d of ~ 400 nM (Fig. 3B and Table 1), which is ~ 10 -fold lower than its affinity for site 2-methylated RpZRE3 (Fig. 3C and Table 1). Both experiments, using two different methods (EMSA and ITC) and two different sources of ZEBRA protein, demonstrated that methylation at site 2 is sufficient for ZEBRA's DNA binding, while site 1 methylation provides a minor contribution. On the other hand, Z(166-245) truncation was impaired in binding both site 1- and site 2-methylated RpZRE3 DNA (Fig. 3A). Together, these data support the general role of the RRTRK motif in detection of DNA methylation. Moreover, the RRTRK plays a role in selective recognition of one of the two CpG elements asymmetrically positioned within one ZRE.

Sequence identity and length of DNA is important for high-affinity ZEBRA binding. Since high-affinity binding of ZEBRA is important for viral DNA replication, we further assessed the importance of DNA length, sequence identity, and the flanking nucleotides neighboring the canonical ZEBRA DNA heptad. We compared full-length ZEBRA binding affinity to various lengths of RpZRE3 (Fig. 4A) and ZpIIIIB DNA (Fig. 4B) by EMSA. ZEBRA was prebound to 20-mer or 21-mer radiolabeled DNA in the presence of a 50-fold excess of unlabeled competitor DNA truncations. For both DNA substrates, ZEBRA required DNA longer than 12 or 13 nucleotides to bind with high affinity and to prevent the unlabeled DNA from competing for the ZEBRA binding site (Fig. 4C and D and Fig. S4A). However, further increasing the length of the DNA to greater than 15 nucleotides did not enhance ZEBRA's binding affinity. This indicates that having three to four nucleotides flanking the canonical heptad DNA-binding site is sufficient for ZEBRA high-affinity binding.

FIG 3 ZEBRA exhibits asymmetrical recognition of methylation sites on DNA. (A) Electrophoretic mobility shift assays with 10 μ g of cell extract of HHKB5-B5 cells containing wild-type or N-terminal truncations of ZEBRA. Probes were RpZRE3 (20-mer) site 1 or site 2 methylated. The letter B marks a background band. (B and C) ITC measurement for the binding of Z(155-245) to RpZRE3 14-mer DNA methylated at only site 1 (B) or only site 2 (C). All measurements were completed in triplicate.

We further examined whether these four nucleotides are interacting with the four charged amino acids in the RRTRK basic region of ZEBRA. We compared the difference in binding affinity by ITC for the doubly methylated RpZRE3 DNA between the wild-type Z(155-245) protein and the AATAA mutant that lacks the four basic amino acids that putatively interact with the flanking DNA. Sequences underlined in AATAA are the 4 amino acids mutated from the wild-type sequence. Wild-type Z(155-245) that contains the RRTRK region has 15- to 20-fold higher affinity for 14-nucleotide doubly methylated RpZRE3 DNA than does the AATAA mutant (Fig. 2D and 5B). The difference in affinity between the two proteins moderately decreases when the DNA length is shortened to 11 nucleotides with two flanking nucleotides on each side of the heptad (Fig. 4E and Fig. S5A to C). However, the difference in affinities for doubly methylated RpZRE3 between the wild-type and mutant protein drastically decreases when only a single nucleotide flanks both sides of the heptad sequence (Fig. 4E and Fig. S5D and S5E). We conclude from this experiment that the RRTRK amino acids are important for interaction with several DNA nucleotides flanking the heptad sequence.

In addition to the length of the flanking sequence, the identity of the flanking nucleotides is also important for ZEBRA DNA binding. Unlabeled, wild-type DNA substrate successfully competed with identical, labeled DNA for binding to ZEBRA. However, when mutations were introduced in the flanking nucleotides closest to the

FIG 4 Flanking DNA length and sequence identity is important for DNA binding by ZEBRA. Sequence of decreasing lengths of RpZRE3 (A) or ZpZIIIB (B) DNA substrates used as cold competitors. Sites of methylation are highlighted with yellow stars. (C) Summary of cold-competitor electromobility shift assays (Fig. S4A) with 10 μ g extracts of HKB5/B5 cells expressing full-length ZEBRA protein with either radiolabeled 20-mer RpZRE3 site 2 methylated (C) or 21-mer radiolabeled ZpZIIIB (D). Cold competitors (50 $\times$) of oligonucleotides of different lengths of RpZRE3 site 2 methylated or ZpZIIIB were added together with the probe. (E) Fold differences in binding affinity between purified MBP Z(155-245) mutant and wild-type protein for methylated RpZRE3 DNA with various lengths of flanking sequence (Fig. S5). (F) Summary of electromobility shift assays (Fig. S4B) with 10- μ g extracts of HKB5/B5 cells expressing full-length ZEBRA protein with radiolabeled RpZRE3 site 2-methylated 20-mer with different amounts of cold competitors of RpZRE3 site 2-methylated 20-mer with mutations in the flanking sequence.

ZRE heptad, the ability of the mutated DNA to compete for binding exhibited modest to large reductions in most of the mutations (Fig. 4F and Fig. S4B and S4C). Furthermore, since ZEBRA preferentially binds to DNA with site 2 methylation, we asked if ZEBRA prefers the flanking region adjacent to the site 1 or site 2 methylation site (Fig. S6A). The results showed that ZEBRA had similar binding affinity for RpZRE3 DNA with flanking regions on either side of the heptad (Fig. S6B and S6C). Thus, ZEBRA interacts with both sides of the RpZRE3 DNA site equally, indicating that the RRTRK region is important for attaching ZEBRA onto the DNA through its interaction with the DNA regions flanking the recognition site (Fig. 6A). While 14-nucleotide pairs, including the heptad ZRE, is an optimal DNA binding substrate, only the two nucleotides flanking the ZRE are sequence specific (Fig. 4F and Fig. S4B and S4C). This result suggests that ZEBRA interacts with specific bases immediately adjacent to the ZRE but with the phosphate backbone of DNA in the extended flanking sequences.

Mutation of the RRTRK basic motif affects DNA binding and methylation recognition. To directly observe the effect of the RRTRK basic region on DNA binding and

FIG 5 Mutations in the RRTRK basic motif of ZEBRA diminish DNA affinity, methylation specificity, and lytic cycle activation. (A to C) ITC measurement for the binding of Z(155-245) AATAA mutant to ZpZIIIB 14-mer DNA (A), RpZRE3 14-mer doubly methylated DNA (B), or RpZRE3 unmethylated 14-mer DNA (C). All measurements were completed in triplicate. Table 1 provides comparisons of the AATAA mutant with the wild type. (D) Electrophoretic mobility shift assays with $10 \mu\text{g}$ of 293T cell extracts expressing FLAG-tagged Z(155-245) constructs with wild-type Z(157-161) amino acid sequence or the indicated alanine substitutions. (E) Immunoblots of BZKO cell extracts transfected with the wild-type Z(1-245) or Z(1-245) construct with the indicated alanine substitution mutations. Blots were probed with antibodies to EBV marker early or late lytic cycle proteins. (F) Quantitative PCR assay for EBV oriLyt abundance in BZKO cells transfected with wild-type CMV FLAG Z(1-245) or mutants in the RRTRK motif. (G) Relative expression of two EBV early and two late mRNAs 48 h after transfection of BZKO cells with wild-type or RRTRK alanine substitution mutants. Expression was analyzed by qRT-PCR. $n = 3$.

FIG 6 (A) ZEBRA RRTRK basic region is important for high-affinity binding to DNA and methylation site recognition to activate DNA replication and EBV late gene protein expression, leading to lytic cycle activation and viral shedding. (B) Schematic models showing the RRTRK basic motif (red dotted lines) N terminal to the canonical bZIP domain (green and cyan ribbons) interacting with the DNA flanking the heptad RpZRE3 (boxed in red) to position and increase ZEBRA's affinity for the DNA (tan). The methylation sites are marked with asterisks. The models are based on the published partial structure (PDB entry 2CDL) (25).

methylation selectivity, we created mutations in the RRTRK motif. We first verified that the quality of the mutant protein with all charged residues mutated to alanine ($^{157}\text{AATAA}^{161}$) is similar to that of the wild-type protein (Fig. S7F). ITC measurements of ZEBRA (155-245)AATAA demonstrated impaired DNA binding to ZpZIIIB DNA, with an approximately 7-fold decrease in binding affinity compared to that of the wild-type protein (Fig. 5A compared to Fig. 1D and Table 1). In addition, these basic residues are necessary for methylation-specific binding, since the AATAA mutant binds doubly methylated DNA RpZRE3 with an approximately 10-fold weaker affinity than wild-type ZEBRA (Fig. 5B compared to Fig. 2D and Table 1). The same DNA substrate RpZRE3 without methylation has only 2-fold lower affinity for the AATAA mutant than wild-type ZEBRA (Fig. 5C compared to Fig. 2C and Table 1). These data support the conclusion that the RRTRK basic residues are more specific for recognition of methylated than unmethylated DNA.

We further assessed the contribution of individual residues in the ZEBRA RRTRK basic motif to methylation sensing. Z(155-245) mutants were expressed at similar levels in 293T cell extracts (Fig. S7F). Using EMSA, we assessed their DNA-binding affinity to doubly methylated RpZRE3 (Fig. 5D). Mutants RRTRA, AATRK, and AATAK bound DNA

with a modest reduction in DNA affinity, 43 to 49% of probe DNA shifted compared to 60% for wild-type ZEBRA (Fig. 5D). This reduction in DNA binding suggests that while these residues individually contribute to DNA binding, each alone is not essential for interaction with methylated DNA. When both residues after the threonine (RRTAA) were mutated, the binding affinity was reduced by 2.6-fold compared to that of the wild-type protein. The larger reduction of RRTAA in binding compared to the AATRK mutant, with the two residues preceding the threonine mutated, suggests that RK are more important determinants in methylation binding. Moreover, when all of the basic residues were mutated to alanine, the binding to methylated DNA was markedly (92%) impaired. We conclude that the four basic residues cooperatively contribute to methylation selectivity.

Mutation of the RRTRK basic motif affects lytic cycle activation. Plasmids encoding wild-type ZEBRA or four different mutants (described above) in the ZEBRA RRTRK motif were introduced into BZKO cells and assessed for their capacity to induce lytic reactivation (Fig. 5E). The activity of all four RRTRK site mutants was comparable to wild-type ZEBRA in expression of two EBV early lytic cycle proteins, Rta, a transcription factor, and EA-D, the EBV DNA polymerase processivity factor (Fig. 5E). The AATAA mutant was unique among the other mutants in being markedly impaired at inducing expression of the EBV BFRF3 late small viral capsid protein (Fig. 5E). The RRTRK site mutants were all comparable to wild-type ZEBRA in activation of expression of EBV BRLF1 (Rta) and BMRF1 (EA-D) RNA, but among all the mutants, the Z(AATAA) mutant was most markedly impaired in activation of expression of RNA of two EBV late genes, BFRF3 and BLRF2 (Fig. 5G and Fig. S8A). The defect of AATAA mutant in promoting late gene expression correlated with a marked defect in inducing lytic viral DNA replication (Fig. 5F and G and Fig. S8A). It is known that expression of the BFRF3 and BLRF2 late gene mRNA is dependent on lytic replication (23). These experiments showed that the marked defect in DNA-binding affinity (~5.5-fold difference) of purified Z(155-245) proteins with the AATAA mutant compared with that of wild-type Z(155-245) (Fig. 1D and 5A and Table 1) was correlated with a marked defect in lytic viral DNA replication and late gene expression when the AATAA mutation was installed in full-length ZEBRA protein.

The AATAA mutant maintains the capacity to activate expression of viral early lytic cycle genes. *In vitro* assays using high-affinity ZEBRA DNA binding sites located in the BRLF1 and BZLF1 promoters demonstrated a marked defect in the capacity of the AATAA mutant, relative to wild-type ZEBRA, to bind naked DNA (Fig. 5A to D and Table 1). However, the AATAA mutant manifests an unexpected unique biological phenotype; it activated expression of RNA and protein from early lytic genes BRLF1 and BMRF1 to levels comparable to those observed with wild-type ZEBRA (Fig. 5E and G and Fig. S8A). The main functional defect of the AATAA mutant that correlated with the DNA binding defect was failure to support the process of viral genome amplification and late gene expression (Fig. 5F and G and Fig. S8A). These results prompted us to compare the association of wild-type ZEBRA and the AATAA mutant with Rp, the BRLF1 promoter, and with oriLyt, the origin of lytic replication, using chromatin immunoprecipitation. The experiment was performed in BMRF1 knockout cells. Since BMRF1 protein is an essential component of the viral replication machinery, performing chromatin immunoprecipitation (ChIP) in BMRF1 knockout cells eliminates differences in input viral DNA due to the capacity of the wild type, but not the AATAA mutant, to promote genome amplification. We found that both wild-type ZEBRA and the AATAA mutant exhibited similar associations with Rp and oriLyt (Fig. S8B). These results suggest that additional factors, other than binding to naked DNA, contribute to the ability of ZEBRA to activate transcription of early viral lytic cycle genes and to promote lytic viral DNA replication. These factors include cooperativity of binding among various ZREs, DNA modifications, and protein-protein interactions on the same regulatory sites where binding is impaired on naked DNA.

DISCUSSION

In this report, we describe several novel features of the interaction of EBV ZEBRA with its DNA targets. (i) We identified a region with 4 basic amino acids, RRTRK, upstream of the bZIP domain, that mediates high-affinity DNA binding and recognition of methylated DNA. Using three different assays, namely, ITC, FP, and EMSA, and two different methods of protein preparation, we consistently found that RRTRK significantly augments DNA binding of ZEBRA to well-documented naked ZEBRA-binding sites (Table 1). (ii) Mutation of the RRTRK motif to AATAA markedly reduces DNA binding affinity. This mutant is impaired in activation of lytic EBV DNA replication and late gene expression. (iii) ZEBRA selectively binds to one of two asymmetrically placed methylated CpG residues in a ZEBRA response element in the promoter of the EBV BRLF1 gene, one of the most proximal targets of ZEBRA. (iv) ZEBRA recognizes DNA via two classical heptad ZREs, and sequences flanking these ZREs are also required for optimal binding. It is likely that the RRTRK motif plays a role in the direct interaction with the flanking sequences (Fig. 6B). However, it is also conceivable that the RRTRK motif promotes a conformation change of the ZEBRA protein that enhances binding to DNA or interaction with other proteins that are important in transcription and replication.

Relevance of these observations to previous crystal structures of ZEBRA. Previous work demonstrated that ZEBRA's serine 186 (S186) is the major contributor to recognition of methylated DNA (12). Upon mutation of this residue to alanine, ZEBRA recognition of methylated DNA is ablated, essentially eliminating its function in activation of the EBV lytic cascade (24). Several crystal structures have attempted to explain ZEBRA's selectivity for methylated DNA. The structure of the ZEBRA S186A mutant bound to an AP-1 DNA sequence (25) confirmed the bZIP architecture of the viral protein. However, due to the mutation of the critical S186 residue and use of an unmethylated AP-1 DNA substrate, this structure did not elucidate the methylation-dependent DNA selectivity mechanism of ZEBRA.

The recent structure of Z(175-236) bound to singly methylated RpZRE2 DNA in which S186 is intact (PDB entry [5T01](#)) (26), shows that residue S186 in ZEBRA interacts with the methyl group in DNA. This study reports that mutating S186 to alanine reduces the affinity of ZEBRA to methylated DNA by 12-fold (26). A comparison of this structure with the previous crystal structures, i.e., ZEBRA S186A mutant in complex with unmethylated DNA (PDB entry 2CDL) (25) and Jun homodimer in complexes with unmethylated or methylated DNA (PDB entries [2H7H](#) and [5T01](#)), revealed that all of these structures are similar (26). The lack of differences observed among these structures in the presence and absence of methylation suggests that they do not capture the full mode of methylation-specific binding of ZEBRA to DNA. This is potentially due to the lack of a previously unappreciated DNA-binding basic motif of ZEBRA in these structures, which we show here is also essential for recognition of DNA methylation. Moreover, the previous crystal structures do not capture C171, a residue previously implicated in recognition of ZREs (16).

Relevance of findings to recognition of methylated DNA. The canonical, minimal DNA-binding domain of Z(166-245) is not sufficient to recapitulate the methylation-specific DNA binding observed with full-length viral protein. Here, we used a combination of methods (ITC, FP, and EMSA) to demonstrate that an expanded ZEBRA construct containing the segment of positively charged residues, RRTRK, upstream of the canonical DNA-binding domain is required to achieve this selectivity. The additional charged residues in the RRTRK basic motif are essential for both DNA methylation selectivity and high-affinity binding to nonmethylated ZEBRA response elements. Thus, this basic motif is important for both substrate recognition and methylation-dependent DNA binding. The inclusion of the basic motif in the minimal binding domain allows ZEBRA to bind the canonical methylated DNA in a specific manner at an affinity similar to that of full-length ZEBRA.

We propose a model where the additional residues in the RRTRK basic motif interact electrostatically with the flanking regions of the DNA to position and increase ZEBRA's affinity for the DNA. This ensures that ZEBRA remains bound to DNA to allow it to scan the DNA for methylated CpG preferentially recognizing methylation at site 2 (Fig. 6B). In the case of Fos-Jun, a segment of basic residues N terminal to the basic DNA-binding domain of Fos is crucial for bending of the DNA and ultimately facilitating protein binding (27). It is possible that ZEBRA uses a similar mechanism to remain bound to DNA. ZEBRA may form electrostatic interactions to facilitate binding that is stable enough to achieve conformational changes necessary for recognition of the methylation state of DNA.

Relevance of findings to EBV lytic reactivation by ZEBRA. The basic motif that we characterized is essential for EBV lytic reactivation by ZEBRA, which plays a pivotal role in transcription of viral lytic cycle genes and in DNA replication. The impact of DNA methylation on EBV transcription is paramount. Classically, methylation at cellular promoters is known to downregulate or silence transcription of the corresponding gene. However, in the case of EBV, promoter DNA methylation is required for ZEBRA to activate the transcription of a specific subset of genes. This activating effect of methylation is believed to be a mechanism that prevents premature lysis of the host cells, allowing the virus to establish persistent latent infection (28). Furthermore, high-affinity binding of ZEBRA to nonmethylated ZEBRA response elements in the EBV origin of lytic replication is needed for inducing DNA amplification (29). As little as a 2-fold decrease in affinity for ZREs in oriLyt is associated with a defect in DNA replication (30). Mutation of the RRTRK motif to AATAA was associated with profound defects in DNA binding, DNA replication, and late gene expression (Fig. 5 and 6 and Table 1; see also Fig. S8A in the supplemental material).

Considering the importance of the DNA-binding activity of ZEBRA in EB viral lytic cycle activation, one would expect that the Z(AATAA) mutant form of ZEBRA lacking the RRTRK motif, which is markedly defective in binding to DNA, would fail to activate expression of the BRLF1 gene, the earliest downstream transcriptional target of ZEBRA. Surprisingly, however, we did not detect defects in transcription or protein expression of the two early genes studied in response to the mutant Z(AATAA). Activation of the BRLF1 and BMRF1 promoters by the Z(AATAA) mutant can be explained by indirect interactions with DNA, as demonstrated by chromatin immunoprecipitation (Fig. S8B). Further analysis will be needed to examine the role of the RRTRK motif in expression of the entire spectrum of viral and cellular genes that may play functional roles in the EBV lytic cycle.

In conclusion, our study highlights the importance of the noncanonical ZEBRA RRTRK basic motif upstream of the classical bZIP domain in conversion of EBV to its lytic state. Our experiments emphasize the importance of studying ZEBRA DNA recognition both *in vitro*, using pure protein and naked DNA, and *in vivo* in the context of DNA modifications and protein-protein interactions.

MATERIALS AND METHODS

Cloning and purification of ZEBRA protein from *E. coli*. The MBP-tagged ZEBRA fusion constructs consisted of an N-terminal 6× His tag followed by maltose binding protein (MBP), a SARS-CoV M^{Pro} cleavage site, and ZEBRA [Z(1-245), Z(155-245), Z(166-245), or Z(175-245)] in the pMAT9s expression vector. Tagged proteins were expressed in *E. coli* NiCo21(DE3) competent cells (New England Biolabs) in Terrific broth. Cells were induced with 0.1 mM isopropyl β-D-thiogalactopyranoside at the optical density at 600 nm (OD₆₀₀) of 0.8 and grown at 16°C overnight. Cells were pelleted at 5,000 × *g* for 10 min and frozen in liquid nitrogen.

Protein purification. Cells were resuspended in nickel-nitrilotriacetic acid (Ni-NTA) buffer A [50 mM Tris, pH 8.0, 500 mM NaCl, 5% glycerol, 0.1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)] and lysed via microfluidization. Cell lysates were clarified by centrifugation at 13,000 rpm at 4°C for 45 min. Protein was purified by an Ni-NTA affinity gravity column. Protein was eluted with Ni-NTA buffer B (50 mM Tris, pH 8.0, 1 M NaCl, 400 mM imidazole, 0.1 mM TCEP). Eluted protein was collected and stored in the presence of 20% (wt/vol) glycerol for long-term storage at –80°C.

Preparation of fluorescently labeled DNA substrates. Oligonucleotide substrates were ordered from IDT, including those containing methylation at CpG or a fluorescently labeled 5' strand. Single-stranded oligonucleotides were resuspended in buffer (20 mM Tris, pH 8, 100 mM NaCl, 5 mM MgCl₂);

complementary strands were annealed by heating to 100°C and slow cooling overnight to room temperature. Concentration was analyzed using NanoDrop spectroscopy.

Fluorescence polarization assay. MBP-ZEBRA constructs were incubated with fluorescently labeled substrates prior to dilution with the FP buffer (20 mM Na₂PO₄, pH 7.5, 100 mM KCl, 2 mM MgCl₂, 10% glycerol, 0.1 mM TCEP). Samples were incubated on ice to reach binding equilibrium and then briefly spun to remove any precipitated protein. Two hundred μ l of sample was analyzed on a Horiba/PTI QuantaMaster fluorescence machine. Data were collected over ZEBRA concentrations ranging from 0.1 μ M to 15 μ M. Each protein concentration and DNA substrate concentration was run in triplicate. Data were analyzed in Prism6 to determine binding constants.

Isothermal titration calorimetry. The affinity of the MBP ZEBRA constructs for different DNA substrates was determined using isothermal titration calorimetry (NanoITC; TA Instruments). ZEBRA and DNA were separately dialyzed against 20 mM Na₂PO₄, pH 7.5, 50 mM NaCl, 0.1 mM TCEP. The DNA (120 to 500 μ M concentration in the syringe) was added by 30 injections of 1.6 μ l each into a 30 to 50 μ M solution of dialyzed ZEBRA at 25°C in the cell. Data were analyzed using the NanoAnalyze software from TA Instruments.

Experiments in eukaryotic cells. (i) Cell lines. BZKO are 293 cells stably transfected with EBV B95-8 bacmid in which the BZLF1 gene has been inactivated by insertion of a hygromycin resistance cassette (9). Cells were maintained on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin-streptomycin, 1 μ g/ml amphotericin B, and 100 μ g/ml hygromycin (9).

HKB5/B5 is a hybrid cell line derived from fusion of HH514-16 Burkitt lymphoma cells with human embryonic kidney cell line 293 (kindly provided by M.-S. Cho, Bayer Corporation, Berkeley, Calif.) maintained on RPMI 1640 supplemented with 10% FBS, 50 U/ml penicillin-streptomycin, and 1 μ g/ml amphotericin B. 293T cells, human embryonic kidney cells which were transformed with sheared human adenovirus DNA, and simian virus 40 T antigen were maintained on DMEM with 10% FBS (30, 31).

(ii) Eukaryotic expression vectors. To generate FLAG-gZ, two HindIII sites in the bzlf1 open reading frame were destroyed by site-directed mutagenesis (QuikChange, Stratagene). Neither change resulted in any amino acid alterations. The HindIII-resistant sequences were inserted into pHM201-FLAG (a gift from M. Hochstrasser, Yale University) as a HindIII-NotI fragment at compatible sites (32). To construct FLAG Z(155-245), FLAG Z(160-245), FLAG Z(166-245), FLAG Z(172-245), and FLAG Z(175-245) eukaryotic expression vectors, the corresponding DNA fragments were amplified by PCR using full-length ZEBRA as a template. The forward primer had a synthetic EcoRI site at the 5' end, and the reverse primer had a synthetic XbaI site at the 3' end. The DNA fragments encoding truncated ZEBRA proteins were cloned into pCMV/FLAG (Sigma-Aldrich) using the indicated restriction enzyme sites.

(iii) EMSA extracts. HKB5/B5, BZKO, and 293T cells were used for transient transfection to produce extracts for EMSA. Optimal transfection conditions were achieved using DMRIEC reagent (Invitrogen) according to the manufacturer's protocol. Cells were harvested at 48 h. Cell pellets were flash frozen 3 $\times$ in dry ice-ethanol and suspended in 100 μ l whole-cell extract buffer (20 mM HEPES, pH 7.5, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF [phenylmethylsulfonyl fluoride], 0.5 mM dithiothreitol [DTT], and 2 μ g/ml aprotinin). Samples were centrifuged at 90,000 rpm for 15 min at 4°C in a TLA 100 rotor in a Beckman TLX ultracentrifuge. Supernatant fluids were flash frozen and stored at -70°C. Protein concentrations were determined by Bradford assay (31).

(iv) Annealing and labeling oligonucleotide probes. Two single-stranded oligonucleotides with complementary sequences were prepared at 10 μ M. Oligonucleotide mixtures were diluted to 1 pM/ μ l with 1 $\times$ TM buffer (10 mM Tris, 10 mM MgCl₂) and annealed in a Thermocycler (T100; Bio-Rad) at 95°C for 5 min, 65°C for 10 min, 37°C for 10 min, and 25°C for 30 min. Two μ l of annealed oligonucleotide at 1 pM/ μ l, 2 μ l 10 $\times$ polynucleotide kinase buffer (BioLabs), 10 μ l water, 5 μ l [γ -³²P]ATP (6,000 c/mmol 10 mCi/ μ l; Perkin Elmer), and 1 μ l T4 polynucleotide kinase were mixed and incubated at 37°C for 1 h, followed by using a Qiagen QIAquick nucleotide removal kit. The final volume was 150 μ l for a 0.014 pM/ μ l concentration.

(v) EMSA. EMSA binding reaction mixtures contained 10 μ g protein from cell extracts, 10 μ l 1 $\times$ gel shift buffer (10 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM MgCl₂, 2.5 μ M ZnSO₄, 0.5 mM EDTA, 1 mM DTT, 15% glycerol), 1 μ l poly(dI-dC), 10 g Affymetrix, and 1 μ l radioactive duplex oligonucleotide probe at 0.014 pM/ μ l. The final volume was brought to 20 μ l with whole-cell extract buffer. Protein and buffers were mixed first on ice. Probe-dI-dC mix was incubated at room temperature for 15 min and then loaded into a 4% polyacrylamide gel and run at 200 V for 1 h in 0.5 $\times$ TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). Gel was soaked in 10% methanol, 10% acetic acid for 30 min, transferred to Whatman paper, and dried in a Bio-Rad gel dryer model 583 at 80°C for 1 to 2 h. Gels were exposed to Kodak XAR film at -70°C (31).

(vi) Competition EMSA. Cold annealed oligonucleotides were at 1 pM/ μ l. Probes were at 0.014 pM/ μ l. One μ l radioactive probe, 1 μ l dI-dC, and cold oligonucleotide were mixed at desired concentrations (100 $\times$ cold oligonucleotide would be 1.4 pM, and the volume would be 1.4 μ l). The probe-cold oligonucleotide mixture was mixed with 10 μ g protein, gel shift buffer, and whole-cell buffer to a total volume of 20 μ l and incubated at room temperature for 15 min. The sample was then loaded into a 4% polyacrylamide gel and run at 200 V in 0.5 $\times$ TBE for about 1 to 2 h (31).

(vii) Immunoblotting. Transient transfection of BZKO cells was used to produce extracts for immunoblotting and assay of DNA and RNA by quantitative PCR. Cells in 25-cm² flasks, 80 to 90% confluent, were transfected with 2 μ g plasmid DNA and DMRIE-C reagent (Invitrogen). Cells were incubated at 37°C and 5% CO₂ for 48 h and then harvested. Samples were centrifuged and diluted in phosphate-buffered saline (PBS). Cells were divided into tubes with 2 $\times$ 10⁶ to 3 $\times$ 10⁶ cells. Cell pellets

were stored at -80°C . To assay for viral proteins, one pellet was thawed and suspended at 10^6 cells/ $20\ \mu\text{l}$ SDS sample buffer. Cells were sonicated for 30 s and heated at 100°C for 5 min. Twenty μl was loaded per lane of a 10% SDS–polyacrylamide gel. After electrophoresis, the protein was transferred to a nitrocellulose membrane by electroblotting in a wet transfer apparatus (Bio-Rad Trans Blot cell) at 0.4 A for 2 h. The blots were blocked with 5% nonfat dry milk for 1 h and were incubated for 1 to 2 h with rabbit or mouse antibodies to EBV lytic cycle proteins diluted in 5% nonfat dry milk. Blots were washed twice in Tris saline (10 mM Tris, pH 7.5, 200 mM NaCl, 5% Tween 20). If needed, a secondary antibody incubation was done for 1 to 2 h with 2 washes to follow. To detect immune-reactive bands, blots were incubated with 1 μCi ^{125}I -protein A (Perkin Elmer) in nonfat dry milk for 1 h and then washed twice. The blots were exposed overnight with intensifying screens to Kodak XAR-5 film at -70°C (29, 31).

(viii) Antibodies for immunoblotting. Rta polyclonal rabbit serum was raised to a 320-amino-acid (aa) N-terminal fragment of Rta using the pET expression system (Novagen). ZEBRA polyclonal rabbit serum was raised to a TrpE BZLF1 fusion protein. FR3 polyclonal rabbit serum was raised to a TrpE BFRF3 fusion protein expressed and purified from *E. coli*. LR2 polyclonal rabbit serum was raised to a TrpE BLRF2 fusion protein expressed and purified from *E. coli*. R3.1, a mouse monoclonal antibody to BMRF1 (EA-D) (33), was used with a secondary rabbit anti-mouse IgG (H+L) bridge antibody (ThermoFisher) (29, 31).

(ix) EBV lytic replication assay. BZKO cells were lysed and sonicated in 50 mM Tris, pH 8, 10% SDS, 10 mM EDTA. Cell lysates were centrifuged and supernatants were diluted 10-fold in 16.7 mM Tris, pH 8, 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, and 1.2 mM EDTA. Proteins were digested using proteinase K (Roche), followed by phenol-chloroform extraction. The DNA was purified with a Qiagen PCR purification kit. Viral genome amplification was measured using iQ SYBR green supermix (Bio-Rad) with primers targeting oriLyt (34). See Table S1 in the supplemental material for primer sequences.

(x) Quantitative reverse transcription PCR (qRT-PCR). RNA was purified from BZKO cells 48 h after transfection using QIAshredder and RNeasy Plus products from Qiagen. The concentration of RNA in each sample was determined by measuring optical density at 260 nm. Viral transcript levels were assessed from 100 ng total RNA using an iTaq universal SYBR green one-step kit (Bio-Rad) in a total volume of 25 μl . The level of 18S RNA was measured to normalize the total amount of RNA. Each sample was analyzed in triplicate (30, 35). See Table S1 for sequences of primers.

(xi) ChIP. To compare the capacity of ZEBRA and Z(AATAA) to associate with viral DNA, we used BMRF1 knockout cells (Fig. S8B). These cells contain an EBV bacmid that is defective in viral genome amplification due to the knockout of BMRF1, a gene encoding an essential replication protein. DNA-protein complexes formed in ZEBRA-transfected BMRF1 knockout cells were cross-linked by adding 1% formaldehyde to the growth medium. The cells were incubated for 10 min at 37°C and then washed once in phosphate-buffered saline containing phosphatase and protease inhibitors (Roche). Cells were resuspended in SDS lysis buffer (50 mM Tris-HCl [pH 8.1], 1% SDS, and 10 mM EDTA) and sonicated four times using a Sonifier 450 apparatus (Branson). Cell lysates were cleared by centrifugation, and the collected supernatants were diluted 10-fold in chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mM Tris-HCl [pH 8.1], 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, and 1.2 mM EDTA). ZEBRA-associated DNA was immunoprecipitated using a rabbit polyclonal antibody (S1605-F) generated against the full-length protein. The immune complexes were collected on protein A/G magnetic beads (Sigma/Millipore). The beads were washed using low-salt, high-salt, lithium chloride, and Tris-EDTA buffers. Crosslinking was reversed and immunoprecipitated DNA was purified and quantitated by real-time PCR using a CFX thermocycler (Bio-Rad). The amount of BRLF1 promoter DNA and oriLyt DNA present in each immunoprecipitated sample was corrected for the amount of corresponding input DNA present in each sample. Primers are described in Table S1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00724-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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