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Expression and regulation of the *BKRF2*, *BKRF3* and *BKRF4* genes of Epstein-Barr virus

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ABSTRACT

The *BKRF2*, *BKRF3* and *BKRF4* genes of Epstein-Barr virus (EBV) are located close together in the viral genome, which encode glycoprotein L, uracil-DNA glycosylase and a tegument protein, respectively. Here, we demonstrate that the *BKRF2* gene behaves as a true-late lytic gene, whereas the *BKRF3* and *BKRF4* genes belong to the early lytic gene family. Our results further reveal that both *BKRF3* and *BKRF4* promoters are new synergistic targets of Zta and Rta, two EBV latent-to-lytic switch transactivators. Multiple Rta- and Zta-responsive elements within the *BKRF3* and *BKRF4* promoters were identified and characterized experimentally. Importantly, we show that DNA methylation is absolutely required for activation of the *BKRF4* promoter by Zta alone or in combination with Rta. Moreover, we find that sodium butyrate, an inducing agent of EBV reactivation, is capable of activating the *BKRF4* promoter through a mechanism independent of Zta and Rta. Overall, our studies highlight the complexity of transcriptional regulation of lytic genes within the *BKRF2-BKRF3-BKRF4* gene locus.

1. Introduction

Epstein-Barr virus (EBV) is a human gammaherpesvirus, which can cause infectious mononucleosis and is closely linked to several malignancies of lymphoid and epithelial cell origin (Thompson and Kurzrock, 2004). Like other herpesviruses, EBV displays two distinctive life cycles: latency and lytic replication (Miller, 1989). Upon viral reactivation, the expression of EBV lytic genes is coordinately regulated in an orderly fashion, ultimately leading to the production of infectious virions (zur Hausen et al., 1978). The earliest lytic genes expressed during viral reactivation are *BZLF1* and *BRLF1* genes, which encode lytic cycle activators Zta and Rta, respectively. Although the *BZLF1* and *BRLF1* genes were originally referred to as “immediate-early” genes, it was later shown that the expression of the *BZLF1* and *BRLF1* mRNAs at the onset of EBV reactivation is sensitive to treatment of the protein synthesis inhibitor cycloheximide (Ye et al., 2007). Due to the requirement of *de novo* protein synthesis for the induction of the *BZLF1* and *BRLF1* mRNA expression in EBV-infected cells, both *BZLF1* and *BRLF1* genes are not typical “immediate-early” genes.

Zta (also named as ZEBRA or EB1) is a bZIP transcriptional activator, which critically involves the induction of EBV lytic cycle and is capable of driving the whole lytic cascade to completion in latently infected cells (Chang et al., 1990). At least two subclasses of Zta response elements (ZREs, or Zta binding sites) have been identified in the target gene promoters of Zta. One subclass of the ZREs encompasses AP1-related recognition elements (Chang et al., 1990; Farrell et al., 1989), and the other subclass contains CpG motif (CpG ZREs) where Zta preferentially binds to their methylated form. Rta also triggers viral lytic infection in a subset of latently infected epithelial cell lines (Ragoczy et al., 1998; Zalani et al., 1996). There are at least two different molecular mechanisms by which Rta activates its target genes during viral reactivation: direct binding of Rta to a consensus Rta response element (RRE; $GNCCN_9GGNG$) in the promoters (Gruffat et al., 1990; Gruffat and Sergeant, 1994; Kenney et al., 1989) and indirect binding of Rta to target promoters *via* a protein-protein interaction with other transcriptional factors (Adamson et al., 2000; Darr et al., 2001; Liu et al., 1996; Ragoczy and Miller, 2001). Additionally, Rta and Zta can cooperate to synergistically activate a number of viral lytic genes

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that commonly contain both RRE and ZRE in their gene promoters (Chang et al., 2010a; Quinlivan et al., 1993).

Genome-wide analyses of Zta or Rta binding to the EBV genome have been conducted previously and great progress has been made in the identification of new viral targets of Zta or/and Rta (Heilmann et al., 2012; Lu et al., 2006; Ramasubramanyan et al., 2012). In these experiments, viral genome fragments bound by Zta or Rta in EBV-infected cells were pulled down using specific anti-Zta or anti-Rta antibodies, and the immunoprecipitated DNA fragments were then analyzed by deep sequencing. Due to the potential complexity of Zta- or Rta-associated transcriptional complexes on viral genome, it is possible that a given antibody used in chromatin immunoprecipitation (ChIP) assays might not be able to comprehensively immunoprecipitate all forms of Zta or Rta-associated chromatin complexes. Therefore, the use of alternative methods or experiments *in vivo* and *in vitro*, such as promoter-luciferase reporter assay and electrophoretic mobility shift assay (EMSA), is still important not only for validation of the identified Zta- or Rta-binding targets, but also has an opportunity to find more binding targets of Zta and Rta. Moreover, although numerous Zta- or Rta-binding sites have been described in EBV genome, it is unclear whether all these enhancer-like elements indeed exert effects on transcriptional activation of nearby genes.

The *BKRF2*, *BKRF3* and *BKRF4* genes of EBV are situated adjacent to each other in the viral genome, which are predicted to encode glycoprotein L (gL), uracil-DNA glyosylase (UDG) and a virion tegument protein, respectively. Thus far, the detailed gene structure, expression and regulation of these viral genes are not fully understood. In this study, we clearly demonstrated that the *BKRF2* gene is a true-late lytic gene, whereas the *BKRF3* and *BKRF4* genes belong to the members of the early lytic gene family. Detailed regulations on transcriptional activation of both *BKRF3* and *BKRF4* promoters by Zta or/and Rta were also presented. Importantly, we unexpectedly found that sodium butyrate, a well-known inducing agent of EBV lytic cycle, could activate the *BKRF4* promoter independently of Zta and Rta. These findings may have important implications for the regulation of the viral lytic cycle program.

2. Materials and methods

2.1. Cell cultures and transfections

293 T is a human embryonic kidney cell line transformed with the E1 region of adenovirus and the simian virus 40 T antigen. The 293 T cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS). P3HR1 and Akata(+) are two EBV-positive Burkitt's lymphoma cell lines. Raji, a Burkitt's lymphoma cell line, harbors an EBV strain that is defective for DNA replication and late gene expression (Pulvertaft, 1964). All these EBV-positive cell lines were cultured in RPM1 1640 supplemented with 10% FBS. Transient DNA transfection in 293 T cells was performed using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen), whereas DNA transfection in EBV-positive lymphoma cell lines was conducted by electroporation.

2.2. Northern blot analysis

P3HR1 cells that were treated with 3 mM sodium butyrate in the presence or absence of 200 μg/ml phosphonoacetic acid (PAA) were harvested at 12, 24, 36 and 48 h posttreatment. Total cellular RNAs from the treated cells (1.0×10^7) were extracted using an RNeasy kit (QIAGEN), fractionated on 1% formaldehyde-agarose gels, and transferred to nylon membranes (Hybond-N; Amershann Pharmacia Biotec). Specific DNA probes that cover targeted regions from nt 98,473 to 98,721 (BKRF2), from nt 98,925 to 99,402 (BKRF3), and from nt 99,748 to 100,211 (BKRF4) of the EBV genome (NC_009334) were prepared by PCR and labeled with 0.1 M digoxigenin (DIG) DNA mix (Roche). Hybridization was carried out according to the protocol described previously (Chang et al., 2005).

2.3. Rapid amplification of cDNA ends (RACE)

At 24 h after SB treatment, P3HR1 cells were harvested and total RNAs from the collected cells were prepared. Mapping of the 5′ transcription start sites and the 3′ polyadenylation cleavage site of the *BKRF2*, *BKRF3* and *BKRF4* mRNA transcripts was carried out using the GeneRacer kit (Invitrogen) as described previously (Chang et al., 2013). The nested PCR amplification was performed using the gene-specific primers and the linker primers provided in the kit. The individual gene-specific primers used in amplification of the 5′ ends of cDNAs were 5′-GTGACGTGACAACATGGGTATGCC-3′ (for BKRF2), 5′-CCAGGCCATGCACATGTCCTCCTC-3′ (for BKRF3), and 5′-GACTCATCAGTGTCACTCACGTCC-3′ (for BKRF4). The primer used in the amplification of the 3′ ends of cDNAs was 5′−CCAACATGCTTCAGGTCTGACACC-3′.

2.4. Plasmid construction

To construct the pBKRF2p-Luc, pBKRF3p-Luc and pBKRF4p-Luc reporter plasmids, the promoter region of each viral gene was amplified by PCR using total DNA from P3HR1 cells as a template and cloned into pGL3-Basic (Promega). Deletions in the *BKRF3* and *BKRF4* promoters in pGL3-Basic were subsequently generated. To map the Zta-responsive elements in *BKRF3* promoter, the double-stranded oligonucleotides or PCR amplified DNA fragments were cloned into pE4luc digested with NheI and XhoI. Internal deletions of the *BKRF3* promoter (dRRE and dZRE) were generated through an inverse PCR procedure. Point mutations in the *BKRF3* and *BKRF4* promoters were created using the QuickChange site-directed mutagenesis kit (Stratagene). To define the butyrate-responsive element in the *BKRF4* promoter, the Sp1(II) or Sp1(II)-mt double-stranded oligonucleotides were cloned into pE4-luc digested with NheI and XhoI. The plasmids including pE4-luc, pCMV, pCMV-Z, pCMV-Z(S186 A), pRTS, pRTS-Rta, pCMV-F350, pCMV-F-Sp1 and pCMV-F-Sp1-DBD were described previously (Chang et al., 2010b; Ragoczy and Miller, 1999; Wang et al., 2010).

2.5. Luciferase assays

The reporter assays were carried out according to the manufacturer's protocol for the luciferase reporter assay system (Promega). Briefly, P3HR1 or 293 T cells were transfected with a fixed amount of plasmid DNA that included the reporter plasmid and the expression plasmids encoding Rta or Zta. The reporter assays were routinely performed at 24 h after induction or transfection for P3HR1 cells and at 48 h after induction or transfection for 293 T cells. *In vitro* DNA methylation of the reporter constructs was performed with CpG methyltransferase (MSssI; New England Biolabs) according to the manufacturer's instructions. In order to confirm the completion of DNA methylation, the treated plasmid DNA was digested with BstUI (New England Biobabs), a restriction enzyme that cleaves DNA only at unmethylated sites. Fold activation was calculated as the luciferase activity in the presence of stimuli divided by that in the absence of stimuli. All results shown in the study were obtained from at least three independent experiments.

2.6. Electophoretic mobility shift assay (EMSA)

Total protein extracts for EMSAs were prepared from 293 T cells that were transfected with the expression plasmids. Annealed double-stranded oligonucleotides were end-labeled with biotin-11-UTP using terminal deoxynucleotidyl transferase (PIERCE). In the EMSA binding reaction, 10–15 μg of total protein extract were mixed with 1 ng DNA probe in a solution containing 10 mM HEPES (pH 7.5), 50 mM NaCl, 2 mM $MgCl_2$, 2.5 μM $ZnSO_4$, 0.5 mM EDTA, 1 mM dithiothreitol, 15%