Synergistic autoactivation of the Epstein-Barr virus immediate-early BRLF1 promoter by Rta and Zta

Pingfan Liu and Samuel H. Speck*

Division of Microbiology and Immunology, Yerkes National Primate Research Center, Emory University, 954 Gatewood Road, NE, Atlanta, GA 30329, USA

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Abstract

Expression of two Epstein-Barr virus (EBV) immediate-early gene products, Zta (encoded by the BZLF1 gene) and Rta (encoded by the BRLF1 gene), are required for the switch from latent infection to virus replication. We have analyzed the regions of the BRLF1 gene promoter (Rp) that are required for Rta and Zta transactivation of Rp. Notably, significant synergy between the actions of Rta and Zta on Rp was observed in both a B cell line (DG75) and an epithelial cell line (293), suggesting that during induction of the viral lytic cycle low levels of these viral transactivators are likely sufficient to initiate the entire lytic cascade. However, while two Zta binding sites (ZREs) have been identified in Rp, the proximal ZRE was the dominant site for mediating Zta transactivation. Rta activation of Rp was diminished by mutation of the proximal Sp1 binding site, as previously reported (J. Virol. 75 (2001), 5240), but mutation of this site only had a modest impact on transactivation of Rp by Rta in the presence of Zta. Further deletion analyses of Rp failed to identify a critical site for Rta transactivation of Rp in the presence of Zta, with the exception of deleting the TATAA box of Rp, suggesting that a non-DNA binding mechanism may be involved in the observed activation of Rp by Rta. We also observed promiscuous activation of several reporter constructs by Rta, suggesting that Rta activation of gene expression may involve a general non-DNA binding mechanism. Decreasing the amount of transfected Rta expression vector reduced background Rta activation, while retaining specific activation of Rp.

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Introduction

Epstein-Barr virus (EBV) is a lymphotropic human herpesvirus that latently infects B lymphocytes, resulting in a concomitant growth transformation of the infected cell. Infection is closely associated with several human cancers, including nasopharyngeal carcinoma and African Burkitt’s lymphoma, and also plays a role in several lymphoproliferative diseases in immunocompromised individuals. In vitro the transforming potential of EBV is evidenced by its ability to immortalize B lymphocytes. Immortalization is achieved through the expression of a relatively small subset of EBV-encoded genes that serve to establish and maintain cellular transformation.

Propagation of EBV from host to host is dependent on the activation of an estimated 80–100 viral genes, culminating in the production of infectious virions. While these genes remain quiescent during latency, a switch in the genetic program leading to the expression of viral replication associated genes can be accomplished in vitro by treatment of latently infected B lymphocytes with various reagents, including phorbol esters, butyrate, Ca2+ ionophores, and anti-immunoglobulin (Bauer et al., 1982; Faggioni et al., 1986; Kallin et al., 1979; Luka et al., 1979; Tovey et al., 1978; Zur et al., 1978). Activation of the lytic cascade by crosslinking surface immunoglobulin or superinfection results initially in the expression of two viral genes, BZLF1 and BRLF1, which exhibit similar induction kinetics (maximal mRNA levels are reached between 2 and 4 h postinfection) (Biggin et al., 1987; Flemington et al., 1991; Takada and Ono, 1989). Both the BZLF1 gene product (Zta; also referred to as ZEBRA and EB1) and the BRLF1 gene product (Rta) have been shown to be transcriptional activators (reviewed in Speck et al., 1997). Expression of Zta and...
Rta leads to the activation of early genes and ultimately virus replication.

Zta is a sequence-specific transcriptional activator, distantly related to c-fos (Farrell et al., 1989), which binds DNA via degenerate AP-1 and CREB-like binding sites (Farrell et al., 1989; Flemington and Speck, 1990a, 1990b; Urier et al., 1989). Zta homodimerizes through a leucine zipper motif at the carboxy terminus of the protein (Flemington and Speck, 1990c; Kouzarides et al., 1991) and is able to autoactivate its own expression through binding to two adjacent sites within Zp (ZIIIA and ZIIIB sites) (Flemington and Speck, 1990a). Rta has also been shown to specifically bind a GC-rich motif (Gruffat et al., 1990, 1992; Gruffat and Sergeant, 1994); however, it also appears that Rta activation of some promoters occurs through a mechanism that does not involve direct DNA binding (Gutsch et al., 1994; Ragoczy and Miller, 2001). DNA binding-dependent and independent mechanisms of gene activation have also been noted for the Rta homologs encoded by other gamma herpesviruses (Song et al., 2002, 2001; Deng et al., 2002a, 2000b, Zhang et al., 1998; Liang et al., 2002; Lukac et al., 2001).

Several cis elements involved in regulating Rp activity have been identified (Fig. 1). These include sites for binding the cellular transcription factors NF1, Sp1, YY1, and Zif, as well as two binding sites for Zta (ZRE-1 and ZRE-2) (Zalani et al., 1992, 1995, 1997; Glaser et al., 1998; Ragoczy and Miller, 2001) (Fig. 1). A recent analysis of Rta transactivation of Rp, in the context of an EBV latently infected B cell line, has implicated the Sp1 sites in mediating Rta autoactivation of Rp (Ragoczy and Miller, 2001). He we assess Rta and Zta transactivation of Rp in the absence of EBV infection and hence other viral factors. We also show here that unlike Zp, Rp is only weakly responsive to the lytic cycle inducers TPA and ionomycin. The latter result is consistent with the hypothesis that transcription of the BZLF1 gene, and subsequent expression of Zta, precedes transcription of the BRLF1 gene (Flemington et al., 1991; Speck et al., 1997).

**Results**

**Rp is weakly responsive to the combination of phorbol ester and calcium ionophore**

Because the EBV lytic cycle can be induced in many latently infected B cell lines by the addition of TPA, in the presence or the absence of calcium ionophore, we assessed whether Rp was responsive to phorbol ester and calcium...
ionophore (Fig. 2). Rp promoter fragments of various lengths, fused to the firefly luciferase gene (see schematic in Fig. 1A), were transiently transfected into the EBV-negative Burkitt’s lymphoma (BL) cell line DG75. As a positive control, the BZLF1 promoter (Zp) fragment from \( \text{H11002} \) to \( \text{H11001} \) bp (ZpLuc) was also introduced into the DG75 cell line. All Rp-driven luciferase reporter constructs, as well as ZpLuc, exhibited very low basal level activity. However, while the ZpLuc reporter construct was strongly induced by the addition of TPA and ionomycin (>200-fold induction), the Rp-driven luciferase reporter constructs were only weakly responsive to TPA and ionomycin (Fig. 2). This is consistent with previous data indicating that phorbol ester induction of the lytic cycle predominantly targets activation of BZLF1 gene transcription, followed by Zta induction of Rp (Flemington et al., 1991).

Synergistic activation of Rp by Rta and Zta

To assess autoactivation of Rp by the EBV immediate-early transactivators Rta and Zta, Rp-driven luciferase reporter constructs were transiently transfected into the DG75 BL cell line either alone or in conjunction with an Rta expression vector, a Zta expression vector, or both (Fig. 3). In the DG75 EBV-negative BL cell line all the Rp-driven luciferase reporter constructs assayed were responsive to Rta and Zta and also strongly transactivated by the additional of both transactivators (Fig. 3A). Deletion of Rp sequences from \( \text{H11002} \) to \( \text{H11002} \) bp did not have a significant impact on Rta or Zta transactivation of Rp, nor the observed synergistic transactivation by Rta and Zta together (Fig. 3A).

Because Rta has been shown to disrupt latency in some EBV-infected epithelial cell lines (Zalani et al., 1996; Ragoczy et al., 1998), we assessed Zta and Rta transactivation of Rp in the 293 epithelial cell line (Fig. 3B). Notably, Zta alone only weakly transactivated Rp in this cell line, while Rta alone strongly activated all the Rp-driven reporter constructs examined (Fig. 3B). However, even though Zta activation was weak, strong synergistic transactivation was observed when both Rta and Zta were present (Fig. 3B). In addition, deletion of Rp sequences from \( \text{H11002} \) to \( \text{H11002} \) bp resulted in an \( \sim \)threefold reduction in Rta and Rta/Zta transactivation of Rp. Further deletion of sequences to \( \text{H11002} \) bp partially restored the levels of Rta and Rta/Zta transactivation of Rp. A similar pattern was observed in the DG75 cell line, although the effect was much smaller (< twofold) (see Fig. 3A). Importantly, in both the DG75 and 293 cell lines all the necessary cis elements for Rta and Zta transactivation of Rp were present in the promoter fragment.
containing the region from −110 to +56 bp relative to the site of transcription initiation.

**Rta promiscuously activates transcription from reporter plasmids**

In the course of optimizing Rta and Zta transactivation of Rp-driven reporter constructs, we noted that Rta could transactivate the parent luciferase reporter construct (pGL2Luc) lacking any Rp sequences (Fig. 4). Although the fold induction of pGL2Luc was substantially below the fold induction of the Rp-driven reporter constructs (see Figs. 5A and B), this observation raised the concern that Rta transactivation of the vector might confound the analysis of Rta activation of Rp. Furthermore, Rta transactivation was not limited to the pGL2Luc reporter construct, but was also observed with several other reporter constructs (data not shown). In addition, we also noted a low, but measurable, level of Zta transactivation of the pGL2Luc construct. Notably, there was little or no observed synergistic transactivation of the pGL2Luc vector by Rta and Zta (Fig. 5A), while Rta and Zta strongly synergized to activate Rp (−324RpLuc) (see Figs. 4 and 5B). To avoid the concerns raised by Zta and Rta transactivation of the vector, we titrated the amount of Zta and Rta expression vectors transfected (Fig. 5). Reducing the amount of Zta expression vector transfected to 100 ng completely eliminated any detectable transactivation of the parent reporter construct (data not shown), while retaining the ability to potently synergize with Rta (see Figs. 5 B and C). The amount of Rta expression vector transfected was titrated from 0.5 ng to 1 μg, alone and in conjunction with 100 ng of Zta expression vector, and transactivation of both the pGL2Luc and −324RpLuc reporter constructs was determined (Fig. 5). There was a parallel reduction in the fold induction by Rta of the pGL2Luc and −324RpLuc reporter constructs. To eliminate detectable Rta transactivation of the pGL2Luc parent reporter construct required reducing the amount of transfected Rta expression vector to <5.0 ng (Fig. 5A). This amount of Rta expression vector only weakly activated the −324RpLuc reporter construct (Fig. 5C). However, in conjunction with 100 ng of Zta expression vector (which alone only transactivates the −324RpLuc construct ~10-fold; see Fig. 6), low levels of Rta expression vector were still able to potently synergize with Zta to activate Rp (Fig. 5C).

**The proximal Zta binding site is the dominant site for Zta transactivation of Rp**

To assess the role of known and putative cis elements in the regulation of Rp by Rta and Zta, a panel of site-directed mutant Rp reporter constructs was generated (see Figs. 1B and 6). With respect to Zta transactivation of Rp, mutation of the proximal Zta binding site (ZRE1) eliminated detectable Zta transactivation of Rp and also the ability of Zta to synergize with Rta in the transactivation of Rp (Fig. 6; see −324RpΔZRE1). In contrast, mutation of the distal Zta binding site (−324RpΔZRE2) had little impact on either Zta transactivation of Rp or Zta/Rta synergistic transactivation of Rp (Fig. 6; see −324RpΔZRE2). Mutation of other cis elements within Rp had little or no impact on Zta transactivation of Rp (Fig. 6). It should be noted that nearly identical results were obtained when higher levels (2 μg) of Rta and Zta expression vectors were transfected into the DG75 EBV-negative BL cell line or the 293 epithelial cell line (data not shown), although, as shown in Fig. 5, the fold inductions were overall substantially higher. This indicates that decreasing the amount of Rta and Zta expression plasmid transfected did not alter the specificity of Zta and Rta activation of Rp and that there was no observed differences in the requirements for Zta activation of Rp in B cells and epithelial cells. Thus, the proximal ZRE appears to be the critical site for Zta activation of Rp in both B cells and epithelial cells.

**The proximal Sp1 site is critical for Rta activation of Rp, but not for synergistic activation of Rp by Rta and Zta**

With respect to Rta transactivation of Rp, mutation of the proximal Sp1 site (−324RpΔSp1-p) greatly diminished Rta transactivation of Rp in the DG75 cell line (Fig. 6; see inset). In contrast, mutation of the distal Sp1 site (−324RpΔSp1-d) had little or no impact on Rta activation of Rp (Fig. 6; see inset). Notably, mutation of the proximal Sp1 site had only a slight impact on synergistic activation of Rp by Rta in conjunction with Zta (Fig. 6). The latter result
raises the possibility that Rta transactivation of Rp requires the cooperation of an Rp-bound factor and that Zta binding to the ZRE1 site in Rp can, at least partially, substitute for the role of Sp1 binding to the proximal Sp1 site. Thus, the proximal Sp1 binding sites appears to play an important role in Rta activation of Rp in the absence of Zta. None of the other cis elements examined were critical for Rta transactivation of Rp (Fig. 6; see inset).

**Deletion of Rp sequences from \(-30\) to \(-10\) bp ablates Rta transactivation of Rp**

In an attempt to systematically map sequences within Rp that mediate Rta transactivation, several 20-bp internal deletions of Rp were made, and the effects of Rta on reporters containing these deletions were measured (Fig. 6). The deletion of Rp sequences from \(-30\) to \(-10\) bp ablates Rta transactivation of Rp.
Fig. 7. Rta and Zta transactivation of Rp deletion mutants. Transfection of DG75 cells with 2 μg of the indicated reporter constructs alone or cotransfected with the Zta expression plasmid (100 ng) and/or the Rta expression plasmid (1 ng), as indicated. The inset shown on an expanded scale the basal activities of the reporter constructs, as well as the fold activation by Zta and Rta alone. Fold induction represents the fold activation relative to that of the undeleted −100RpLuc reporter construct in the absence of Rta and Zta (defined as 1.0). Data were compiled from four independent experiments and the standard error of the mean is shown.

Deletions were generated in the context of the −110RpLuc reporter construct (see Materials and Methods). These deletions were transiently transfected into the DG75 BL cell line along with the Rta and Zta expression vectors, as described above. No sequences within the region from −110 to −50 bp of Rp appeared critical for Rta transactivation of Rp (Fig. 7; see 110RpΔ−110/−90, 110RpΔ−90/−70, and 110RpΔ−70/−50). Deletion of sequences from −50 to −30 bp, which removes the proximal Sp1 (Sp1-p) and Zta (ZRE1) binding sites, eliminated Zta activation of Rp (Fig. 7). Consistent with the loss of Zta and Rta activation upon deletion of the sequences from −50 to −30 bp, no synergistic induction of the −110RpΔ−50/−30 by Rta in conjunction with Zta was observed (Fig. 7). Furthermore, deletion of the region containing the Rp TATAA box [−110RpΔ−30/−10)] completely abrogated detectable transactivation of Rp by Zta and Rta (alone and together) (Fig. 7).

Discussion

Both Rta and Zta are essential for EBV replication (Feederle et al., 2000), and independently each transactivator has been shown capable of triggering virus reactivation from latency (Countryman et al., 1987; Countryman and Miller, 1985; Zalani et al., 1996; Ragoczy et al., 1998). A number of studies have demonstrated responsiveness of Zp to stimuli that trigger virus reactivation from latency (Flemington and Speck, 1990d; Countryman et al., 1987; Countryman and Miller, 1985; Schwarzmann et al., 1994; Montalvo et al., 1991), indicating that initial induction of the virus lytic cycle likely involves direct activation of BZLF1 gene transcription. However, it is less clear whether these reactivation stimuli also directly activate Rp or whether induction of Rp is dependent on activation of Zp followed by expression of Zta and subsequent activation of Rp. Here we examined the responsiveness of Rp to two known inducers of the EBV lytic cycle, phorbol ester and calcium ionophore, and demonstrated that Rp is only weakly responsive to these stimuli compared to Zp (see Fig. 2). This is consistent with the hypothesis that reactivation signals may preferentially target Zp.

While Rp responded weakly to induction by phorbol ester and calcium ionophore, it was very responsive to induction by Zta and Rta. Zta activation of Rp was shown to predominantly occur through the proximal ZRE site (ZRE-1), while Rta activation of Rp did not clearly map to a single site. Indeed, the only mutation that ablated detectable Rta activation involved deleting the Rp TATA box. Consistent with a previous report (Ragoczy and Miller, 2001), mutation of the proximal Sp1 binding site reduced, but did not eliminate, Rta activation of Rp. However, the inhibition of Rta activation of Rp by mutating the proximal Sp1 site did not affect the overall synergistic induction of Rp by Rta and Zta. The latter suggests that Zta binding to ZRE-1 may functionally compensate for the loss of Sp1 binding to the proximal Sp1 site in Rta activation of Rp. In addition, unlike the results of Ragoczy and Miller (2001), we did not observe any contribution of the distal Sp1 site to Rta activation of Rp in either 293 or DG75 cells. An important distinction between the studies reported here and those of Ragoczy and Miller (2001) is that the latter studies were carried out in the context of a latent EBV infection (HH514-16 Burkitt’s lymphoma cell line). Thus, the impact of specific mutations in Rp on Rta activation in this context could reflect indirect actions of Rta (e.g., through induction of other viral genes that may participate in regulating transcription from Rp). In addition, it is of note that Ragoczy and Miller (2001) failed to observe any impact of mutating the Zta binding sites in Rp on Rta activation. This suggests that Rta does not induce significant expression of Zta in HH514-16 cells, a conclusion that is supported by a previous study (Ragoczy et al., 1998).

Although Rta has been shown to be a sequence-specific DNA binding protein, Rta responsive promoters (including
Rp) have been identified that lack obvious Rta binding sites (Ragoczy and Miller, 2001; Gutsch et al., 1994). This also appears to be true for the KSHV Rta homolog, which has been shown to activate promoters through either direct DNA binding (Lukac et al., 2001; Deng et al., 2002b; Song et al., 2002, 2001) or through interaction with specific cellular factors (e.g., Sp1 and RBP-Jk/CBF-1) (Liang et al., 2002; Zhang et al., 1998). This suggests that a basic property of Rta homologs may be their ability to interact with a number of cellular transcription factors to facilitate gene activation, as well as targeting genes through direct DNA binding. Why the Rta homologs exhibit this dual functionality remains unclear.

Materials and methods

Cell culture, transfections, and reporter gene assays

The EBV-negative Burkitt’s lymphoma B cell line DG75 was grown at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin, and 100 mg of streptomycin per milliliter. DG75 cells were transfected using DEAE–dextran/DMSO shock, as previously described (Liu et al., 1997). Briefly, 10^7 cells per transfection were washed once with phosphate-buffered saline (PBS), and the cell pellet was resuspended in 0.5 ml of RPMI 1640 medium without serum. Cells were added to 0.5 ml of RPMI 1640 medium with DEAE–dextran (1 mg/ml), 2 µg of reporter plasmid DNA, and the indicated amount of Rta and/or Zta expression plasmid (refer to individual figures). After incubation at room temperature for 30 min, cells were subjected to DMSO shock (addition of 0.5 ml of 20% DMSO for 2 min). Following washing with PBS, cells were resuspended in 10 ml of RPMI 1640 with 10% serum and cultured at 37°C in a 5% CO2 incubator. For induction by TPA, ionomycin, or both, the final concentrations of reagents were TPA at 20 ng/ml and 1 μM ionomycin.

The 293 cell line (available from ATCC) was originally established by transforming human embryonic kidney epithelium with adenovirus DNA (Graham et al., 1977). 293 cells were cultured in Iscove’s modified Dulbecco’s medium with 10% fetal calf serum, glutamine, and penicillin/streptomycin. The 293 cell line was transfected using Superfect (Qiagen) according to the manufacturer’s protocol.

Transfected cells were harvested 48–72 h posttransfection and washed once in PBS. Firefly luciferase activity was determined by resuspended transfected cells in cell lysis buffer and the assay was performed according to the manufacturer’s protocol (Promega). The luciferase activities of reporter constructs were standardized to the parent uninduced wild-type reporter construct, which was set at a value of 1.0. Thus, the activities were expressed as a fold-induction relative to those of the uninduced wild-type reporter construct.

Plasmids

The −221ZpLuc construct was generated from the −221ZpCAT plasmid (Flemington and Speck, 1990d) and was generated as described by Liu et al., (1997). The Rp-directed luciferase (RpLuc) reporter constructs were generated by PCR amplification, using the EBV BamHI R fragment as a template, and cloned into the pGL2Luc reporter plasmid (Promega). All RpLuc reporter constructs contained sequences to +56 bp relative to the site of transcription initiation (see Fig. 1). Mutations in Rp were introduced by PCR-based mutagenesis and incorporated a diagnostic restriction endonuclease cleavage site (see Fig. 1B). The sequences of the mutated promoters was confirmed by DNA sequencing. For those Rp reporter constructs containing mutations in specific cis elements, diagnostic restriction endonuclease cleavage sites were introduced as follows: an XbaI site was introduced into the −321RpΔSp1-p, −321RpΔSp1-d, −321RpΔZif, and −321Rp ΔYY1 mutant reporter constructs; a BamHI site was introduced in −321RpAZRE1; an EcoRI site was introduced in −321RpΔZRE2; an XbaI site was introduced in −321RpΔR1; and a SacI site was introduced into −321RpΔR2 (summarized in Fig. 1B). For all the −110RpLuc deletion constructs, an XbaI site was created at the junction of the internal deletions. The Zta expression vector (pSV40-BZLF1), containing the BZLF1 open reading frame under the control of the SV40 early promoter and enhancer, was constructed as previously described (Flemington and Speck, 1990a). The Rta expression vector (pMH48), containing the BRLF1 open reading frame under the control of the SV40 early promoter and enhancer, was constructed as previously described (Hardwick et al., 1988).

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