Transcriptional activation of Epstein–Barr virus BRLF1 by USF1 and Rta

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During its lytic cycle, Epstein–Barr virus (EBV) expresses Rta, a factor encoded by BRLF1 that activates the transcription of viral lytic genes. We found that upstream stimulating factor (USF) binds to E1, one of the five E boxes located at −79 in the BRLF1 promoter (Rp), to activate BRLF1 transcription. Furthermore, Rta was shown to interact with USF1 in coimmunoprecipitation and glutathione S-transferase (GST)-pulldown assays, and confocal laser-scanning microscopy further confirmed that these two proteins colocalize in the nucleus. Rta was also found to bind with the E1 sequence in a biotin-labelled E1 probe, but only in the presence of USF1, suggesting that these two proteins likely form a complex on E1. We subsequently constructed p188mSZ, a reporter plasmid that contained the sequence from −188 to +5 in Rp, within which the Sp1 site and Zta response element were mutated. In EBV-negative Akata cells cotransfected with p188mSZ and plasmids expressing USF1 and Rta, synergistic activation of Rp transcription was observed. However, after mutating the E1 sequence in p188mSZ, USF1 and Rta were no longer able to transactivate Rp, indicating that Rta autoregulates BRLF1 transcription via its interaction with USF1 on E1. This study showed that pUSF1 transfection after EBV lytic induction in P3HR1 cells increases Rta expression, indicating that USF1 activates Rta expression after the virus enters the lytic cycle. Together, these results reveal a novel mechanism by which USF interacts with Rta to promote viral lytic development, and provide additional insight into the viral–host interactions of EBV.

INTRODUCTION

During induction of the lytic cycle, Epstein–Barr virus (EBV) expresses a transcription factor, Rta (Flemington et al., 1991), which regulates viral gene expression, cell signalling and autophagy to promote EBV lytic development (Adamson et al., 2000; Hsu et al., 2005; Hung et al., 2014; Lee et al., 2008). Rta is known to bind to a conserved 17 bp sequence, termed the Rta response element (RRE), to activate the transcription of EBV lytic genes (Ragoczy & Miller, 1999); however, Rta can also transactivate its own gene, BRLF1, and an immediate-early gene, BZLF1 (which encodes Zta), via RRE-independent mechanisms (Chang et al., 2005, 2010). For example, Zta is expressed when EBV enters the lytic cycle, and is known to bind to Zta response elements (ZREs) in the BRLF1 promoter (Rp) and the BZLF1 promoter (Zp) to respectively activate BRLF1 and BZLF1 transcription (Ragoczy & Miller, 2001). Rta can form a complex with Zta on ZREs via a mediator protein, MCAF1, to synergistically activate BRLF1 transcription (Chang et al., 2010; Lin et al., 2014); moreover, Rta has been found to form an Rta-MCAF1-Sp1 complex on the Sp1 sites identified in Rp, to autoregulate BRLF1 transcription (Chang et al., 2005). BRLF1 transcription is differentially regulated in epithelial cells and B lymphocytes. An earlier study showed that BRLF1 is constitutively transcribed in epithelial cells, but is
repressed during viral latency in B lymphocytes (Zalani et al., 1992). The inability of host Sp1 to transactivate Rp during viral latency in B lymphocytes suggests the presence of a repressive mechanism that suppresses activation (Ragoczy & Miller, 2001; Zalani et al., 1992).

Upstream stimulating factors (USF1 and USF2) are transcription factors of the bHLH/LZ family (Atchley & Fitch, 1997), and form homo- or heterodimers on E boxes with the consensus sequence 5′-CANNTG (Sirito et al., 1994; Viollet et al., 1996) to activate transcription. Earlier studies have also shown that USF can interact with and functionally enhance IE62, the major transcriptional activator in varicella-zoster virus (VZV), to activate the promoters of the DNA polymerase and major DNA-binding protein genes through an E box (Meier & Straus, 1995; Rahaus et al., 2003; Yang et al., 2004, 2006). In EBV, Rta activates BALF5 transcription via an E box in the gene promoter (Liu et al., 1996). Here, we show that Rta and USF1 can form a complex to autoregulate BRLF1 transcription. These results provide evidence that USF is involved in EBV lytic cycle activation.

RESULTS

Activation of Rp by USF1

Rp contains five E boxes (Ragoczy & Miller, 2001), and to ascertain if USF can regulate Rp through these E-box elements, we used the pGL2-Basic vector to construct a series of Rp reporter plasmids, including pR967, which contains full-length Rp (Fig. S1, available in the online Supplementary Material). Cells were subsequently cotransfected with pUSF1 and the respective Rp reporter plasmid or pGL2-Basic control, and luciferase activity was measured at 24 h post-transfection. Results were normalized against luciferase levels detected in cells cotransfected with pSG5 and the corresponding reporter plasmid or control vector. We found that in EBV-negative Akata cells, pR967 was activated by pUSF1 at a level that was 9.7-fold higher than that of the pGL2-Basic control (Fig. 1a). In P3HR1 and 293 cells, Rp activation by pUSF1 was respectively 7.6-fold and twofold higher than controls (Fig. 1b, c). We also observed that relative light unit (RLU) values exhibited by pR967 in 293 cells were about 60-fold higher than those in P3HR1 cells, consistent with the finding that BRLF1 is constitutively expressed in epithelial cells (Zalani et al., 1992).

Identifying the Rp elements involved in USF1 transactivation

We found that in EBV-negative Akata cells, cotransfection of pUSF1 and pR967 resulted in 40.3-fold higher levels of reporter activity over background levels seen with cotransfection of pSG5 and pR967 (Fig. 2a). Deletion of the region between −967 and −189 in Rp (pR615, pR385 or pR188) did not change activity levels significantly (Figs S1 and 2a). However, luciferase activity decreased to only 14.1-fold over background levels with pR53, which contains the Rp region from −53 to +5 (Fig. 2a). In 293 cells, although...

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Fig. 1. Activation of pR967 by USF1. (a) EBV-negative Akata, (b) P3HR1 and (c) 293 cells were cotransfected with either pSG5 or pUSF1, and the pR967 reporter plasmid or pGL2-Basic control. Luciferase activity was measured at 24 h after transfection. Each transfection experiment was performed three times, and each sample in the experiment was prepared in duplicate. Error bars represent SD. The amounts of USF1 and α-tubulin in transfected cells were analysed by immunoblotting.
Fig. 2. Rp regions involved in transcriptional activation by USF1. (a, c) EBV-negative Akata and (b, d) 293 cells were cotransfected with either pSG5 or pUSF1 and the respective Rp reporter plasmid. Luciferase activity was measured at 24 h after transfection. USF1-activated luciferase activity levels were normalized against background levels observed in cells cotransfected with pSG5 and the corresponding reporter plasmid. The experiments were repeated three times, and each sample in the experiment was prepared in duplicate. Error bars represent SD. The expression of USF1 and α-tubulin in transfected cells was analysed by immunoblotting. In each set of experiments shown in the immunoblots, the left lane was derived from cells cotransfected with a reporter plasmid and pSG5, while the right lane was derived from cells cotransfected with a reporter plasmid and pUSF1.
cotransfection of pUSF1 and pR967, pR615 or pR188 increased reporter activity by 1.7-fold to 2.3-fold over background levels (Fig. 2b), deletion of the region between −188 and −54 in Rp (pR53) led to a reduction in reporter luciferase activity, to about 90% of background levels (Fig. 2b). These results indicate that the Rp sequence between −188 and −54, which contains two E boxes, E1 and E2 (Fig. S1), is required for transactivation by USF1. We found that in EBV-negative Akata cells, mutation of the E1 sequence in pR188 (pR188mE1) reduced USF1-induced reporter activity from 43.1-fold to 13.8-fold over background levels (Fig. 2c). Mutation of both the E1 and E2 sequence (pR188mE2) did not affect USF1 transactivation capabilities (Fig. 2c). Mutation of both the E1 and E2 sequences resulted in a reduction of USF1-induced reporter activity to 13.8-fold over background levels (Fig. 2c). A reduction in reporter activity following mutation of E1 was also observed in 293 cells, in which E1 mutation reduced USF1-mediated reporter activity from 2.4-fold to 0.9-fold over background levels (Fig. 2d). However, mutation of E2 did not affect reporter activity, while mutation of both E1 and E2 decreased USF1-mediated reporter activity to 90% of background levels (Fig. 2d). These results point to a role for E1 in the USF1-mediated activation of Rp.

Binding of USF1 to E1

An electrophoretic mobility shift assay (EMSA) revealed that His-USF1 purified from Escherichia coli BL21(DE3) (pET-USF1) shifted a biotin-labelled probe containing the E1 sequence (E1) (Figs S2 and 3a, lane 2). However, USF1 did not shift a probe containing the E2 sequence (E2) (Figs S2 and 3a, lane 6); probe C, which does not contain an E1 or E2 sequence, was also not shifted by USF1 (Figs S2 and 3a, lane 4). We further found that proteins in the P3HR1 nuclear extract shifted a biotin-labelled E1 probe (Fig. 3b, lane 2), and the addition of 0.1–5 ng of an unlabelled E1 probe competed with this binding to reduce band shift (Fig. 3b, lanes 3–6). However, the addition of 5 or 10 ng of unlabelled mE1 probe, which contains a mutated E1 sequence (Fig. S2), did not compete with binding (Fig. 3b, lanes 7, 8). The addition of anti-USF1 or anti-USF2 antibodies to the reactions led to disappearance of the shifted band (Fig. 3c, lanes 3, 4), but addition of IgG did not affect band shift (Fig. 3c, lane 5).

We subsequently transfected P3HR1 cells with pUSF1, and found that proteins in the lysate shifted the E1 probe (Fig. 3d, lane 2), while addition of anti-USF1 antibody resulted in a supershift of the probe (Fig. 3d, lane 3). However, the supershift of the probe by anti-USF2 and IgG was inefficient (Fig. 3d, lanes 4, 5). This suggests that although both USF1 and USF2 in P3HR1 cells can bind to the E1 sequence in Rp, overexpression of USF1 increases binding of an USF1 homodimer to the E1 probe.

**Binding of USF1 to Rp in vivo**

P3HR1 cells were treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) and sodium butyrate for 24 h to activate the EBV lytic cycle. Cells were then treated with formaldehyde and sonicated, and the DNA–protein complexes were immunoprecipitated using anti-EBNA1, anti-RNA polymerase II, anti-USF1, anti-USF2 and anti-Rta antibodies.

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**Fig. 3.** Binding of USF to the E1 sequence in Rp. (a) His-USF1, which was purified from an E. coli lysate, was mixed with probes E1, E2 and C (Fig. S2). (b) Biotin-labelled E1 probe was mixed with the nuclear extract (NE) from P3HR1 cells. Non-biotin-labelled E1 (U-E1) (lanes 3–6) and mE1 (U-mE1) (lanes 7, 8) probes were added to compete with the binding of USF1 to the E1 probe in EMSA. (c) Probe E1 was mixed with P3HR1 nuclear extract (lane 2). Anti-USF1 (lane 3), anti-USF2 (lane 4) and IgG (lane 5) antibodies were added to the mixture to supershift the binding. (d) Probe E1 was mixed with a nuclear extract from P3HR1 cells that had been transfected with pUSF1. Anti-USF1 antibody, anti-USF2 antibody and IgG were added to the mixture to supershift the binding.
Quantitative (q)PCR analysis revealed that the amount of Rp captured by anti-RNA polymerase II and anti-Rta antibodies was, respectively, 14.7- and 3.4-fold higher than that seen with anti-EBNA1 antibody (Fig. 4a). In addition, the amount of Rp immunoprecipitated by anti-USF1 and anti-USF2 antibodies was 8.6- and 10.6-fold higher than that captured by anti-EBNA1 antibody (Fig. 4a). Anti-EBNA1 antibody captured DNA containing the dyad symmetry component (DS) of oriP (Tempera et al., 2011) (Fig. 4b). However, the amounts of DS sequence captured by anti-RNA polymerase II, anti-USF1, anti-USF2 and anti-Rta antibodies were low, at levels ranging from 6 to 28% of that captured by anti-EBNA1 antibody (Fig. 4b). We also found that the expression of USF1 by transfection in induced P3HR1 cells resulted in an increase from 5.8-fold to 33.8-fold in the binding of USF1 to Rp, and Rta binding to Rp increased from 3.8-fold to 13.8-fold as well (Fig. 4c). Additionally, a chromatin immunoprecipitation (ChIP) study in latent P3HR1 cells revealed that the levels of Rp immunoprecipitated by anti-USF1 and anti-USF2 antibodies was 1.4- and 1.8-fold higher, while Rp levels immunoprecipitated by Rta were 80% lower than those immunoprecipitated by the anti-EBNA1 antibody (Fig. 4d).

**Interaction of Rta with USF1**

An earlier study found that USF binds to an E box in the BALF5 promoter, and Rta was also shown to activate the BALF5 promoter through this E box (Liu et al., 1996). This study has shown that USF binds to E1 in Rp (Figs. 3 and 4), and we sought to determine whether a similar interaction may regulate BRLF1 transcription. Therefore, we used a lysate from P3HR1 cells that had been cotransfected with pCMV-Rta and pUSF1. We found that USF1 was immunoprecipitated by anti-USF1 antibody (Fig. 5a, lane 3) and coimmunoprecipitated with Rta by anti-Rta antibody (Fig. 5a, lane 4). Rta was also immunoprecipitated by anti-Rta antibody (Fig. 5a, lane 7) and coimmunoprecipitated with USF1 by anti-USF1 antibody (Fig. 5a, lane 8).

![Figure 4](http://vir.sgmjournals.org)  
**Fig. 4.** Chromatin immunoprecipitation (ChIP) analysis of USF and Rta binding to Rp. P3HR1 cells were (a, b, c) treated with TPA and sodium butyrate or (d) left untreated. (c) Cells were transfected with pSG5 or pUSF1 prior to lytic induction. Protein–DNA complexes were immunoprecipitated using the antibodies indicated, and analysed by qPCR, using primers specific to Rp (a, c, d) and the DS component in oriP (b). The experiments were repeated three times. Error bars represent sd.
Fig. 5. Interaction between Rta and USF1. (a) Proteins in lysates prepared from P3HR1 cells transfected with pCMV-Rta and pUSF1 were immunoprecipitated (IP) with the antibodies indicated and detected by immunoblotting (IB). Input lanes were loaded with 2% of the cell lysate. NS, proteins non-specifically detected by anti-USF1 antibody. IgG-H, heavy chain of Rta, USF1. (b) GST pull-down assay: GST-Rta and GST-USF1 were incubated with GST beads or GST-Rta beads. Input lanes were loaded with 2% of the cell lysate. NS, proteins non-specifically detected by anti-USF1 antibody. IgG-H, heavy chain of Rta, USF1. (c) Immunofluorescence assay: P3HR1 cells were infected with EBV-negative Akata and treated with pCMV-Rta. DAPI, USF1, Rta, and Merge were detected. (d) EBV-negative Akata cells were transfected with pCMV-Rta and treated with pCMV-Rta. DAPI, USF1, Rta, and Merge were detected. (e) EBV-negative Akata cells were transfected with pCMV-Rta and treated with pCMV-Rta. DAPI, USF1, and Merge were detected.
IgG. (b) GST- and GST-Rta-Sepharose beads were mixed with *E. coli* BL21(DE3)(pHis-USF1) lysate (lanes 2 and 3). GST- and GST-USF1-Sepharose beads were mixed with *E. coli* BL21(DE3)(pHis-Rta) lysate (lanes 5 and 6). Proteins pulled down by the beads were analysed by immunoblotting. Input lanes were loaded with 0.1% of the lysate. P3HR1 cells were treated with TPA and sodium butyrate (lytic) (c), and EBV-negative Akata cells were transfected with pCMV-Rta (d) or treated with TPA and sodium butyrate (T/S) (e). Rta was stained using monoclonal anti-Rta antibody and goat anti-mouse antibody conjugated with Alexa Fluor 488. USF1 was stained using polyclonal anti-USF1 antibody and goat anti-rabbit antibody conjugated with Alexa Fluor 594. Rta and USF1 cellular localization was observed under a confocal laser-scanning microscope. Bars, 10 μm.

A parallel experiment showed that rabbit IgG did not immunoprecipitate these two proteins (Fig. 5a, lanes 2, 6). The results indicate that Rta and USF1 interact in *vivo*. A glutathione S-transferase (GST)-pulldown assay using bacterially expressed proteins showed that GST-Rta-glutathione-Sepharose beads pulled down His-USF1 (Fig. 5b, lane 3), while GST-USF1-glutathione-Sepharose beads pulled down His-Rta (Fig. 5b, lane 6). In parallel, GST-glutathione-Sepharose beads did not pull down His-Rta or His-USF1 (Fig. 5b, lanes 2, 5), indicating that Rta and USF1 interact in *vitro*. Confocal laser-scanning microscopy revealed that Rta and USF1 colocalized in the nuclei of P3HR1 cells (Fig. 5c) following EBV lytic induction. Moreover, USF1 was dispersed in the nuclei of latent P3HR1 (Fig. 5c) and EBV-negative Akata cells (Fig. 5e). The expression of Rta from pCMV-Rta in transfected EBV-negative Akata cells appeared to cause USF1 to form dots and colocalize with Rta (Fig. 5d). However, treatment of EBV-negative Akata cells with TPA and sodium butyrate did not change the distribution pattern of USF1 in the nuclei (Fig. 5e), indicating Rta expression causes USF1 distribution changes.

Mapping the interaction domains in Rta and USF1

A GST-pulldown experiment found that GST-Rta-glutathione-Sepharose beads pulled down a GFP-fusion protein, G-UC, which contains a C-terminal region from aa 201 to 310 in USF1 (Fig. 6a, b, lane 9); however, the beads did not pull down two GFP-fusion proteins, G-UN and G-UM, which respectively contain the USF1 regions from aa 1 to 100 and aa 101 to 200 (Fig. 6a, b, lanes 3, 6). Meanwhile, GST-USF1-glutathione-Sepharose beads pulled down RM, which contains the region from aa 191 to 415 in Rta fused to HA (Fig. 6d, e, lane 6), but did not pull down RN or RC (Fig. 6d, e, lanes 3, 9). GST-Rta and GST-USF1 on the glutathione-Sepharose beads were also examined by immunoblotting with anti-GST antibody, and the results showed that these two proteins were abundantly present on the beads (Fig. 6c, f), indicating that the lack of pulldown of G-UN, G-UM, HA-RN and HA-RC was not due to lack of GST-Rta or GST-USF1 binding to glutathione-Sepharose beads. These results show that the Rta region between aa 191 and 415 interacts with the USF1 region between aa 201 and 310.

Autoregulation of Rp

We expressed GST-USF1 and His-Rta in *E. coli* BL21(DE3)(pGEX-USF1) and *E. coli* BL21(DE3)(pET-Rta), respectively. The lysates were mixed, and a biotin-labelled E1 probe (Fig. S2) was then added to the mixture and captured using streptavidin magnetic beads. Immunoblot analysis revealed that His-Rta bound to the probe (Fig. 7a, lane 3); however, no binding was observed when the probe was added to an *E. coli* BL21(DE3)(pGST-4T1) and *E. coli* BL21(DE3)(pET-Rta) lysate mixture (Fig. 7a, lane 2), indicating that Rta does not bind to E1 without USF1. We also found that His-Rta in the *E. coli* BL21(DE3)(pGEX-USF1)-*E. coli* BL21(DE3)(pET-Rta) lysate mixtures did not bind the mE1 probe (Figs S2 and 7a, lanes 4, 5), demonstrating that binding of USF1 and Rta requires the E1 sequence.

Additionally, in ChIP analysis, we found that anti-Rta antibody captured Rp at a level that was 3.4-fold higher than that seen with the anti-EBNA1 antibody (Fig. 4a). Moreover, after transfecting P3HR1 cells with pUSF1, the amounts of Rp captured by anti-USF1 and anti-Rta antibodies increased from 5.8-fold to 33.8-fold, and from 3.8-fold to 13.8-fold, respectively (Fig. 4c). The results showed that overexpressing USF1 promotes the binding of Rta to Rp. We further used pR188, which contains an Rp sequence encompassing E1, E2, an Sp1 site and a ZRE (Chang *et al.*, 2005, 2010), and we therefore mutated the Sp1 site and the ZRE in pR188 to create pR188mSZ (Fig. S1). We found that cotransfection of pUSF1 activated pGL2-Basic by 4.7-fold over background levels (Fig. 7b), while pR188 reporter activity was increased 51.7-fold (Fig. 7b). Cotransfection of pUSF1 also activated Rp in pR188mSZ by 37.4-fold (Fig. 7b). Crucially, after the E1, Sp1 and ZRE sequences were all mutated, cotransfection of pUSF1 only increased reporter activity by 7.5-fold over background levels (Fig. 7b), demonstrating that E1 is involved in the activation of Rp by USF1. We further cotransfected the cells with pCMV-Rta, and found that Rta expression respectively activated pGL2-Basic, pR188 and pR188mSZ reporter activity by 15.7-fold, 159.5-fold and 77.9-fold over background levels (Fig. 7b). However, reporter activity decreased to 29.8-fold over background levels (Fig. 7b) after the E1 sequence in pR188mSZ was mutated (pR188mE1SZ), confirming that the activation of Rp in
Fig. 6. Mapping the interaction domains of USF1 and Rta. (a) USF1 was deleted, fused to GFP, and expressed in 293T cells. USR, USF-specific region; b, basic domain; HLH, helix–loop–helix domain; LZ, leucine-zipper domain. (b) Proteins in the 293T lysates were mixed with GST-Rta-glutathione-Sepharose beads. Proteins binding to the beads were eluted and detected by immunoblotting (IB) with anti-GFP (b) and anti-GST (c) antibodies. (d) Three regions in Rta were fused to HA. 293T cells were transfected with plasmids that expressed these proteins. Lysates were prepared and mixed with GST-USF1-glutathione Sepharose beads. Proteins bound to the beads were detected by immunoblotting with anti-HA (e) and anti-GST (f) antibodies.

pR188mSZ by Rta depends on E1. Finally, we cotransfected the cells with pUSF1 and pCMV-Rta, and the combined expression of USF1 and Rta respectively increased reporter activity in pGL2-Basic, pR188 and pR188mSZ by 17.6-fold, 361.7-fold and 269.3-fold over background levels (Fig. 7b). However, after E1, the Sp1 site and ZRE were mutated (pR188mE1SZ), USF1 and Rta increased reporter activity by 36.2-fold over background levels (Fig. 7b). The decrease from 269.3-fold to 36.2-fold over background levels indicates that E1 is required for the activation of Rp by Rta and USF1. Interestingly, Rta increased reporter activity in pGL2-TATA 37.4-fold, but enhanced promoter activity 93.8-fold in a similar plasmid containing three copies of the USF-binding sequence from the adenovirus major late promoter (Fig. 7c), indicating that Rta can promote USF1-mediated transcriptional activation of a gene that is unrelated to EBV.

**USF1 and EBV lytic activation**

We transfected P3HR1 cells with 5 μg of pUSF1 or pSG5, and then treated the cells with sodium butyrate to activate the EBV lytic cycle. We found that transfecting the cells with pUSF1 did not induce Rta expression at a level detectable by immunoblotting if the cells were not previously treated with sodium butyrate (Fig. 8a, lane 2), indicating that pUSF1 does not activate Rp from the EBV genome during viral latency. However, a low level of Rta expression was evident after the cells were treated with 1 mM sodium butyrate for 24 h (Fig. 8a, lane 3), and the transfection of pUSF1 subsequently increased Rta expression (Fig. 8a, lane 4). An increase in Rta expression following pUSF1 transfection was also observed after cells were treated with 2 or 3 mM sodium butyrate (Fig. 8a, lanes 5–8). These results suggest that the expression of USF1 promotes BRLF1 transcription after lytic activation. We also introduced USF1 and USF2 small interfering RNA (siRNA) into P3HR1 cells by transfection, and subsequently induced EBV lytic activation at 24 h after transfection. We found that transfection of USF1 siRNA or USF2 siRNA reduced the expression of USF1 or USF2 (Fig. 8b, lanes 2, 3), and the expression of Rta and early antigen diffuse component (EA-D) was also reduced in both instances (Fig. 8b, lanes 2, 3). When cells were transfected with both USF1 and USF2 siRNA, the amount of Rta expression was further reduced.

![Graph](https://example.com/graph.png)
the expression of Rta and EA-D was reduced substantially (Fig. 8b, lane 4). These results suggest that the expression of key EBV lytic proteins is affected by USF.

**DISCUSSION**

Rta is known to activate BALF5 transcription through an E box in the promoter (Liu et al., 1996). Since Rp contains five E boxes (Ragoczy & Miller, 2001), we were interested as to whether Rta interacts with USF to autoregulate BRLF1 transcription. In a transient transfection study, we showed that USF1, expressed from pUSF1, activated Rp in pR967 (Fig. 1), demonstrating that USF1 is capable of activating Rp. However, the expression of USF1 probably does not influence BRLF1 transcription from the EBV genome during EBV latency, as transfecting P3HR1 cells with pUSF1 did not activate the expression of Rta at a level detectable by immunoblotting (Fig. 8a, lane 2). The lack of Rp activation by USF during viral latency (Fig. 8a, lane 2) is not surprising; Rp contains at least 3 Sp1 sites, and Sp1 is abundantly present in B-cells, but Sp1 also fails to activate Rp during viral latency (Zalani et al., 1992).

Interestingly, we found that transfecting induced P3HR1 cells with pUSF1 increased Rta expression (Fig. 8a, lanes 3–8). Furthermore, the transfection of both USF1 and USF2 siRNA reduced the expression of Rta and EA-D after lytic induction (Fig. 8b, lane 4), suggesting that USF activates Rp during the EBV lytic cycle. Our deletion studies revealed that the three upstream E boxes, E3, E4 and E5 (Fig. S1), play no role in USF-mediated BRLF1 transcription (Fig. 2a, b). Furthermore, in pR188, which contains both E1 and E2 (Fig. S1), only mutation of the E1 sequence affected the ability of USF1 to activate Rp (Fig. 2c, d). We also showed via EMSA that USF1 binds to the E1 but not the E2 sequence (Fig. 3). Moreover, the probe was supershifted by both anti-USF1 and anti-USF2 antibodies when a nuclear extract from P3HR1 cells was used (Fig. 3c), but the probe was primarily shifted by anti-USF1 when a nuclear extract from P3HR1 cells over-expressing USF1 was used (Fig. 3d), suggesting that although both USF homodimers and heterodimers can bind to the E1 sequence, binding of USF1 homodimers may increase when USF1 is overexpressed.

This study demonstrates that Rta interacts with USF1 in vivo and in vitro (Fig. 5), via the region between aa 191 and 415 in Rta and the bHLH-LZ domain from aa 201 to 310 in USF1 (Fig. 6). The viral protein IE62 of the VZV is also known to interact with the USF1 bHLH-LZ domain (Yang et al., 2006). Here we showed that the interaction between USF1 and Rta facilitates the autoregulation of BRLF1 transcription by Rta (Fig. 7b). Enhancement of USF1 transactivation capabilities by Rta is probably not limited to Rp, as Rta also activates a promoter containing USF-binding sites from the adenovirus major late promoter (Fig. 7c).

An earlier study showed that Rp was strongly and constitutively activated in epithelial latent C33 cells, and the proximal Sp1-binding site in Rp was found to be required for such activity; however, the study noted that Rp essentially exhibited no activity in latent lymphoid cell lines, despite the presence of Sp1 (Zalani et al., 1992). In this study, we also observed that luciferase activity was 60-fold higher in pR967-transfected 293 epithelial cells, as compared with P3HR1 lymphoma cells. This suggests that in lymphoid cells, repressive mechanisms preventing the transcription of BRLF1 potentially exist, and may be worthy of additional research.
Earlier studies as well as the results presented herein show that Rta is a transcription factor that can either directly bind to DNA (Chen et al., 2005; Cox et al., 1990; Gruffat et al., 1990; Hung & Liu, 1999) or interact with many transcription factors to activate transcription (Chang et al., 2005, 2010; Lin et al., 2014). In this study, ChIP results showed that USF1 and Rta bind to Rp after lytic activation (Fig. 4a), while overexpression of USF1 increases both USF1 and Rta binding to Rp (Fig. 4c). These results allow us to add USF1 to the list of transcription factors that interact with Rta, and elucidate how USF1 and Rta can act in tandem via E boxes to autoregulate BRLF1 transcription during EBV lytic activation. A recent study using the ChIP-seq method did not reveal such indirect binding of Rta to EBV lytic promoters (Heilmann et al., 2012); however, we do not believe that these results are incompatible with our study, and the different approaches used may even be mutually supportive. For example, the ChIP-seq study found low Rta occupancy of the Rp and Zp promoters, and the authors believed that this may reflect low affinity of Rta interactions with non-consensus Rta binding sites, or perhaps indirect associations. Therefore, it is conceivable that when Rta is recruited to promoter binding sites by host proteins such as MCAF1 or USF, the ChIP-seq method may not be able to identify such interactions, due to the indirect association of Rta with the target promoter. Our approach, using EMSA and DNA affinity precipitation assay (DAPA), provides strong evidence of the formation of Rta-MCAF1-Sp1 and Rta-USF1-Zta complexes on Sp1 sites and ZRE in Rp, as well as Rta-USF1 complex formation on the E1 sequence; such bindings were confirmed not only in vitro and in vivo, but also by confocal microscopy (Chang et al., 2005, 2010). Rta has been shown to influence signal transduction, and may indirectly influence EBV lytic transcription (Darr et al., 2001; Heilmann et al., 2010; Hsu et al., 2005), but our earlier studies confirm that Rta can influence the transcription of promoters that lack an RRE via its interaction with MCAF1, and the results presented in this study further show that Rta can interact with USF1 to influence BRLF1 transcription, thus revealing another novel mechanism by which Rta can recruit host cell proteins to promote EBV lytic development.

**METHODS**

**Cell lines and culture conditions.** P3HR1 (ATCC HTB-62) and EBV-negative Akata cells were cultured in RPMI 1640 medium containing 10% FCS. 293 (ATCC CRL-1573) and 293T cells (ATCC CRL-11268) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS. P3HR1 cells were treated with TPA and sodium butyrate to activate the EBV lytic cycle.

**Plasmids and DNA probes.** Plasmids pCMV-Rta, pGEX-Rta, pET-Rta and pR967, also termed pR8, were previously described (Chang et al., 2005). Plasmids pUSF1 and pSG5 were obtained from Dr Ann-Joy Cheng. Plasmid pR615 was generated by inserting a PCR-amplified DNA fragment at the Smal–XhoI sites in pGL2-Basic (Promega) to generate pR385. The same PCR fragment was also cut with HindIII, repaired with a Klenow fragment, and then cut with XhoI and inserted into the Smal–XhoI sites in pGL2-Basic to generate pR188. DNA fragments containing the sequences from −53 to +5 and −32 to +5 in Rp were synthesized chemically, and inserted into the Smal site in pGL2-Basic to generate pR53 and pR32, respectively. The E-box 1 (E1) sequence in pR188 was mutated from 5′-CATGTG to 5′-ACTGTT (mE1) by site-directed mutagenesis (Zheng et al., 2004) to derive pR188mE1. The E-box 2 (E2) sequence from −168 to −163 was similarly mutated from 5′-CAGATTG to 5′-ACTGTT (mE2) to generate pR188mE2. The E1 and E2 sequences in pR188 were both mutated to generate pR188mE1E2. The region from −50 to −31 in pR188, which contains an Sp1 site and a ZRE, was mutated from 5′-CCGCGATCATGCAATGCTCA to 5′-ATGCCCCATGCAAAGTG-CTGA (mSZ) to generate pR188mSZ. Additionally, the E1 sequence in pR188mE1SZ was mutated to generate pR188mE1SZ. The SV40 promoter in pGL2-Promoter (Promega) was deleted using XhoI and HindIII to generate pGL2-TATA. A double-stranded oligonucleotide sequence containing three E box sequences from the adenovirus major late promoter, 5′-CCGCGATCATGCAATGCTCA to 5′-ATGCCCCATGCAAAGTG-CTGA (mSZ) to generate pR188mSZ. Additionally, the E1 sequence in pR188mE1SZ was mutated to generate pR188mE1SZ.

**DNA affinity precipitation assay (DAPA).** Binding of USF1 and Rta to biotin-labelled DNA probes was analysed by DAPA according to a method reported previously (Chang et al., 2005). Proteins bound to streptavidin magnetic beads were eluted and analysed by immunoblot analysis, using anti-GST and anti-Rta antibodies.

**Electrophoretic mobility shift assay (EMSA).** Biotin-labelled probes (0.1 ng) were mixed with 750 ng His-USF1 purified from E. coli BL21(DE3)(pET-USF1) or with the P3HR1 nuclear extract in EMSA binding buffer (Liu et al., 1996). Additionally, 2 μl of 12.5-fold diluted rabbit IgG (Millipore), anti-USF1 (Santa Cruz Biotechnology) and anti-USF2 (Santa Cruz Biotechnology) were respectively added in a supershift experiment. The band shift was detected by autoradiography, using a LightShift Chemiluminescent EMSA kit (Pierce).

**Chromatin immunoprecipitation assay (ChIP).** A ChIP assay was conducted using a kit from Millipore (Bedford) (Chang, Liu, 2000). The DNA–protein complex was immunoprecipitated with anti-EBNA1 antibody (obtained from Dr. Bill Sugden), anti-RNA polymerase II (Millipore), anti-USF1 (Santa Cruz Biotechnology), anti-USF2 (Santa Cruz Biotechnology) or anti-Rta (Argene) antibodies. Immunoprecipitated Rp DNA was quantified by qPCR using primers 5′-CTCACAGAAAGTGACATTCA and 5′-GCTGACATTCA to amplify the region containing the RS region of oriP and anti-USF1 antibody.

**GST pulldown.** The interaction between Rta and USF1 was demonstrated by GST pulldown, according to a method described elsewhere (Chang et al., 2005). Proteins binding the beads were analysed by immunoblotting with anti-Rta or anti-USF1 antibody.

**Com immunoprecipitation.** Coimmunoprecipitation was conducted with anti-USF1 and anti-Rta antibodies, according to a method described elsewhere (Chang et al., 2005).
Transient transfection and luciferase assay. Cell lysates were prepared at 24 h after transfection. Luciferase activity was measured using a tube luminometer GloMax 20/20 (Promega) according to a method described previously (Chang et al., 1998).

Knockdown of USF expression and EBV lytic activation. P3HR1 cells (2 × 10⁶) were respectively transfected with a 30 pmol control, USF1 siRNA, USF2 siRNA, or both USF1 and USF2 siRNA (GE Dharmacon), using an Amaxa Nucleofector II electroporator with the EL4 program. EBV proteins in the lysates were analysed by immunoblot analysis.

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