Published online 12 April 2010 *Nucleic Acids Research, 2010, Vol. 38, No. 14* ***4687–4700***
doi:10.1093/nar/gkq243

MCAF1 and synergistic activation of the transcription of Epstein–Barr virus lytic genes by Rta and Zta

Li-Kwan Chang[1], Jian-Ying Chuang[1], Mitsuyoshi Nakao[2] and Shih-Tung Liu[3,*]

[1]Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei, Taiwan, [2]Department of Regeneration Medicine, Kumamoto University, Kumamoto, Japan and [3]Department of Microbiology and Immunology, Chang-Gung University, Taoyuan, Taiwan

Received October 30, 2009; Revised March 23, 2010; Accepted March 24, 2010

ABSTRACT

Epstein–Barr virus (EBV) expresses two transcription factors, Rta and Zta, during the immediate-early stage of the lytic cycle. The two proteins often collaborate to activate the transcription of EBV lytic genes synergistically. This study demonstrates that Rta and Zta form a complex via an intermediary protein, MCAF1, on Zta response element (ZRE) *in vitro*. The interaction among these three proteins in P3HR1 cells is also verified via coimmunoprecipitation, CHIP analysis and confocal microscopy. The interaction between Rta and Zta *in vitro* depends on the region between amino acid 562 and 816 in MCAF1. In addition, overexpressing MCAF1 enhances and introducing MCAF1 siRNA into the cells markedly reduces the level of the synergistic activation in 293T cells. Moreover, the fact that the synergistic activation depends on ZRE but not on Rta response element (RRE) originates from the fact that Rta and Zta are capable of activating the BMRF1 promoter synergistically after an RRE but not ZREs in the promoter are mutated. The binding of Rta–MCAF1–Zta complex to ZRE but not RRE also explains why Rta and Zta do not use RRE to activate transcription synergistically. Importantly, this study elucidates the mechanism underlying synergistic activation, which is important to the lytic development of EBV.

INTRODUCTION

Epstein–Barr virus (EBV) expresses two transcription factors, Rta and Zta, during the immediate-early stage of the lytic cycle, to activate the transcription of EBV lytic genes (1–5). As is well known, Zta, encoded by BZLF1, activates transcription by binding to Zta response elements (ZRE) in promoters. Since the transcription of many EBV lytic genes depends on Zta, EBV cannot complete its lytic cycle after BZLF1 is mutated (6,7). Meanwhile, Rta commonly binds to a defined sequence, Rta response elements (RRE), in EBV lytic promoters to activate transcription (2,5,8–11). However, Rta may activate transcription through a mechanism that is independent of RRE binding. For example, Rta interacts with Sp1 via an intermediary protein, MCAF1, to form a complex on Sp1-binding sites to autoregulate the transcription of its own gene, BRLF1 (12,13). Furthermore, the transcription of several EBV late genes depends on Rta but not on Zta (6,14–16), explaining why a mutation in BRLF1 is devastating to viral lytic development (6,7).

Earlier studies have established that Rta and Zta often collaborate with each other to activate transcription synergistically of many EBV lytic genes, including BHLF1, BMRF1 and BRLF1 (3,5,9,17–19). Although various studies of the mechanism that underlies synergistic activation have been conducted, exactly how Rta and Zta synergistically activate transcription remains unknown. Since earlier studies have failed to demonstrate an interaction between Rta and Zta (5), Rta and Zta are assumed to bind to their respective binding sites in a promoter to promote synergistic activation. In fact, Quinlivan *et al.* (5) posited that synergistic activation depends on the presence of at least one RRE and one ZRE in a promoter. However, the fact that Zta and Rta synergistically activate the BRLF1 promoter (18), which does not contain an RRE, is evidence against the hypothesis that synergistic activation depends on an RRE. In fact, Ragoczy *et al.* (20) suggested that the synergistic activation of the BRLF1 promoter by Rta and Zta depends on the binding of an unknown protein to the promoter. Giot *et al.* (4) also asserted that

*To whom correspondence should be addressed. Tel/Fax: +886 3 211 8292; Email: cgliu@mail.cgu.edu.tw

rather than binding to an RRE, Rta may form a complex with Zta via an intermediary protein to activate transcription synergistically.

As is well known, MCAF1 promotes SETDB1/ESET-mediated histone H3 methylation, thus facilitating the formation of heterochromatin domains (21,22). MCAF1 also interacts with MBD1 and Sp1 (23). When interacting with MBD1, MCAF1 promotes MBD1-dependent transcriptional repression (23). When interacting with Sp1, MCAF1 becomes a coactivator that enhances Sp1-mediated transcription (12,23,24). An earlier study has demonstrated that Rta interacts with Sp1 through MCAF1 to promote the autoregulation of BRLF1 transcription (12). This study demonstrates that MCAF1 interacts simultaneously with Rta and Zta. This interaction promotes the synergistic activation of the transcription of the EBV lytic genes.

MATERIALS AND METHODS

Cell lines and EBV lytic induction

P3HR1, EBV-negative Akata and BJAB cells were cultured in RPMI 1640 medium that contained 10% fetal calf serum. 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal calf serum. P3HR1 cells were treated with 3 ng/ml of TPA and 3 mM sodium butyrate to activate the EBV lytic cycle (25).

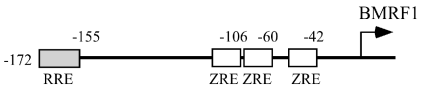
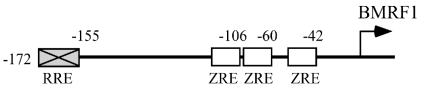
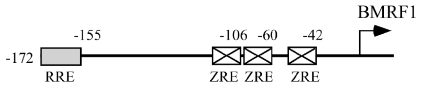
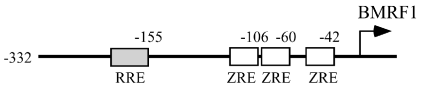
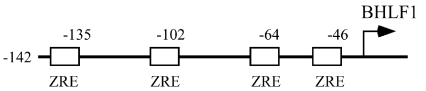
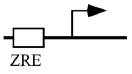
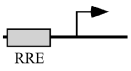
Plasmids

Plasmids pCMV-3, pCMV-R, pCMV-Z, pSp1-luc, pcDNA-MCAF1, pRRE, pEGFP-Rta, pEGFP-MCAF1-N, pET-Rta and pGEX-Rta have been described elsewhere (12,25). Plasmids pEGFP-MCAF1-DM1, pEGFP-MCAF1-M and pEGFP-MCAF1-DM2 were constructed by inserting polymerase chain reaction (PCR)-amplified DNA fragments that encoded the MCAF1 regions from amino acid 562 to 817, 818 to 1153 and 1154 to 1270, respectively, into the ScaI–XhoI sites in pEGFP-C1 (Clontech). Plasmids pEGFP-ZN, pEGFP-ZM and pEGFP-ZC, which expressed truncated Zta, were constructed by inserting PCR-amplified fragments that encoded the amino acid regions from 1 to 86, 87 to 166 and 167 to 245, respectively, into the EcoRI–SalI sites in pEGFP-C1. Plasmids pET-Zta and pGEX-Zta were constructed by inserting full-length BZLF1 (n.t. 103155–102211 in the EBV genome) (GenBank accession number V01555), which was amplified by PCR, into the EcoRI–SalI sites in pET-28a (Novagen) and pGEX-4T1 (Amersham Pharmacia Biotech), respectively. A DNA fragment that encoded the region from amino acid 562 to 884 in MCAF1 was inserted into the HindIII–XhoI sites in pGEX-4T1 and pET-32a to yield pGEX-MCAF-C1 and pET-MCAF1-C1, respectively. Plasmid pGEX-MCAF1-C2 was constructed by inserting a PCR-amplified fragment that encoded the region from amino acid 885 to 1270 in MCAF1 into the HindIII–XhoI sites in pGEX-4T1. The synergistic activation by Rta and Zta was studied using reporter plasmids pEAD, pBMRF1, pBMRF1-mRRE, pBMRF1-mZRE, pBHLF1, pRRE and pZRE (Table 1). Plasmid pEAD was constructed by inserting a PCR-amplified DNA fragment that contained the −332 to +20 region in BMRF1 (n.t. 79537 to 79889 on the EBV genome) into the HindIII–XhoI sites in pGL2-Basic (Promega). A reporter plasmid, pBMRF1, was constructed by inserting a PCR-amplified DNA fragment that contained the −172 to +20 region in BMRF1 (n.t. 79698 to 79889 on the EBV genome) into the HindIII–XhoI sites in pGL2-Basic. Plasmid pBMRF1-mRRE contains the same sequences as pBMRF1, but with the RRE sequence mutated from 5′-ACACCACCCCCCA AGGAC to 5′-GATATCCCCCCCAAGGAC. Plasmid pBMRF1-mZRE has the same sequence as is present in pBMRF1, but the three ZREs in the promoter, from upstream to downstream, were mutated from 5′-TTGCT CA, 5′-TGAGCAA, and 5′-TGAGTCA to 5′-GGATCCA, 5′-TGGATCC and 5′-CAGCTGA, respectively. Another reporter plasmid, pBHLF1, which includes four copies of ZREs (n.t. 52769 to 52931 on the EBV genome) was constructed by inserting a PCR-amplified fragment into the HindIII and XhoI sites in pGL2-Basic. Plasmid pZRE was constructed by inserting a double-stranded oligonucleotide 5′-AAAGGCCGGCTGACATGGATTACTGGTCT TTTATGAGCCATT, which had the sequence from the −39 to +4 region in the BRLF1 promoter, into the SmaI site in pGL2-Basic. Plasmid pCMV-Sp1 was obtained from Guntram Suske (26). Plasmid pTag2-Zta was constructed by inserting a PCR-amplified DNA fragment that encodes BZLF1 (n.t. 103155 to 102211 on the EBV genome) to the EcoRI and XhoI sites of pCMV-Tag2A (Stratagene). Moreover, plasmid pCMV-R(K213A) encoded a mutant Rta that contained a K213A mutation (12).

DNA-affinity precipitation assay

A 5′-biotin end-labeled double-stranded DNA probe, Rp-SZ, which contained the sequence from −64 to −28 in the BRLF1 promoter (5′-ATATTGCGATTGTCCCGCCCA TGCCAATGGCTCATAA), was synthesized by Mission Biotech, Inc. (Taipei, Taiwan). This sequence includes an Sp1-binding site (5′-CCGCCC) and a ZRE (5′-TGGCTC A). Probes Rp-mS and Rp-mZ included the same sequence as Rp-SZ except that the Sp1 site and ZRE were mutated to 5′-ATTAAT and 5′-GACTGCA, respectively. In probe Rp-mSZ, both the Sp1 site and the ZRE were mutated. Probe MRp-RZ contained the sequence between the region −174 and −36 in the BMRF1 promoter and, then, was amplified by PCR using biotinylated primers. In this probe, the RRE was mutated from 5′-ACACCACCCCCCAAGGAC to 5′-G ATATCCCCCCCAAGGAC to generate a probe, MRp-mR; the three ZREs, 5′-TTGCTCA, 5′-TGAGCAA and 5′-TGAGTCA to 5′-GGATCCA, 5′-TGGATCC and 5′-C AGCTGA, respectively, to generate MRp-mZ. In the probe MRp-mRZ, RRE and ZREs were all mutated. Probe HLp-Z (5′-GTCTCTGTGTAATACTTTAAGGT TTGCTCAGGAG) contained two ZREs, 5′-TGTGTAA and 5′-TTGCTCA, from the BHLF1 promoter. In probe HLp-mZRE, these two ZRE sites were both mutated to 5′-TGGTATA. Probe MLp-R had an RRE sequence that

Table 1. Reporter plasmids used in this study

Plasmid	Range in promoter[a]	*cis* elements in promoter[b]
pBMRF1	−172 to +20	
pBMRF1-mRRE	−172 to +20	
pBMRF1-mZRE	−172 to +20	
pEAD	−332 to +20	
pBHLF1	−143 to +16	
pBMLF1[c]	−523 to +38	
pZRE	−31 to −37 in BRLF1	
pRRE	−374 to −391 in BMLF1	

[a]The DNA fragment was inserted into a luciferase reporter vector, pGL2-Basic.
[b]Crossed rectangle represents mutated sequence; arrow, transcription start site.
[c]Although the BMLF1 promoter contains a sequence resembling that of ZRE, the promoter is not activated by Zta (30).

was taken from the BMLF1 promoter; in MLp-mR, the RRE sequence was mutated, as described elsewhere (12). A cell lysate prepared from P3HR1 cells that had been treated with sodium butyrate and TPA was mixed with 0.2 μg of a biotinylated probe in a binding buffer that contained 60 mM KCl, 12 mM HEPES, pH 7.9, 4 mM Tris–HCl, 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol and 10 μg/ml each of leupeptin, aprotinin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride. After it had been incubated on ice for 45 min, the reaction mixture was mixed with 30 μl Streptavidin MagneSphere Paramagnetic particles (Promega), which had been pre-equilibrated in the binding buffer for 1 h at 4°C. The beads were then washed five times in the binding buffer. The proteins on the beads were then extracted using 2X electrophoresis sample buffer and boiled for 5 min. Finally, they were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and detected by immunoblotting (25).

Immunoprecipitation

P3HR1 cells (1×10^7) were treated with TPA and sodium butyrate for 24 h. A lysate was prepared from the cell using radioimmune precipitation assay (RIPA) buffer (25), followed by three times of sonication under the conditions of 10-s pulses with 10-s cooling at 20% power using a sonicator (Vibra Cell, Sonics) equipped with a microprobe. The lysate was centrifuged at 13 800 *g* for 5 min and the supernatant was mixed with anti-Rta (1:3000 dilution) (Argene), anti-Zta (1:1000) (Argene) or anti-MCAF1 (1:1000) (Bethyl) antibody at 4°C for 1 h. Protein-A/G agarose beads (30 μl) (Oncogene) were added to the lysate and the mixture was incubated with shaking for 1 h at 4°C. The beads were collected by centrifugation and washed three times in RIPA buffer. Proteins binding to the beads were eluted by adding 20 μl of 2X electrophoresis sample buffer and analyzed by immunoblotting with anti-Rta, anti-Zta, anti-MCAF1 or anti-GFP (Santa Cruze) antibody.

Glutathione S-transferase-pull-down assay

GST, GST-Zta, GST-Rta, GST-MCAF1-C1 or GST-MCAF1-C2 at a concentration of 40 ng/ml in 500 μl NETN buffer [20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40 and 10 μg/ml each of leupeptin, aprotinin and 4-(2-aminoethyl)-benzenesulfonyl fluoride] was added to 30 μl glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The mixture was incubated with shaking for 1 h at 4°C. The beads were washed three times in NETN buffer and added to the *Escherichia coli* BL21(DE3)(pET-MCAF1-C1), *E. coli* BL21(DE3)(pET-Zta) or *E. coli* BL21(DE3)(pET-Rta) lysate. The reaction mixture was incubated on ice for 1 h. The beads were then washed in NETN buffer.

An equal volume of 2X electrophoresis sample buffer was added to the mixture and proteins were extracted from the beads by heating at 95°C for 5 min. Proteins were then separated by SDS-polyacrylamide gel electrophoresis (27).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (CHIP) assay was performed using a method described elsewhere (28). P3HR1 cells (1×10^7) were treated with TPA and sodium butyrate to induce the expression of Rta and Zta. DNA–protein complex that was cross-linked by formaldehyde was immunoprecipitated with anti-Rta, anti-Zta or anti-MCAF1 antibodies after the DNA had been fragmented by sonication. The presence of specific DNA fragments in the precipitates was detected by qPCR. Primers used for amplifying the BHLF1 promoter were 5′-TCGCCTTCTT TTATCCTCTTTTTG and 5′-CCCAACGGGCTAAAA TGACA; BHRF1, 5′-CGCGTGCCTTACTGACTT GTC and 5′-CCAGGAAGTGGCGAGCAT; BMRF1, 5′-GCCCGCTCACCTACATGAC and 5′-GCAGCAGC AGAAGCCAAC; BMLF1, 5′-CCAGATGTCCCTCTA TCA and 5′-AACCTCTTACATCACTCAC. Quantitative PCR was performed on the products of CHIP in triplicate by the SYBR Green method using the Power SYBR Green PCR Master Mix and StepOne Real-Time PCR system (Applied Biosystems). Standard curves were generated using serial dilutions of input DNA (100, 10, 1 and 0.1%). The Ct of each reaction was quantified against the standard curve.

Immunofluorescence analysis

P3HR1 cells were transfected with pEGFP-Rta. After they had been cultured for 24 h, the cells were harvested by centrifugation, plated on poly-L-Lysine (Sigma)-coated coverslips, and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Immunostaining was performed using anti-Zta monoclonal antibody and rabbit anti-MCAF1 polyclonal antibody and then the cells were treated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG polyclonal antibody (KPL), or rhodamine-conjugated donkey anti-rabbit IgG polyclonal antibody (DAKO), using a method described elsewhere (27). Nuclei were visualized by staining with 5 μg/ml 4′-6-diamidino-2-phenylindole (DAPI). After staining, the cells were observed under an Olympus confocal laser-scanning microscope (Model Fluoview 500).

Transient transfection assay

P3HR1 and 293T cells (5×10^6) were transfected with 5 μg of plasmids at 240 V, 975 μF by electroporation using a BTX ECM630 electroporator (BTX Instrument). To suppress MCAF1 expression, 293T cells (1×10^4) were transfected with 0.3 μg pBHLF1, pEAD and 50 pmol siRNA MCAF1 or control siRNA (Invitrogen primer number 130189C06) with Lipofectamin 2000 (Invitrogen). Luciferase assay was performed using a luminometer (Orion II; Berthod) as described elsewhere (29). Each transfection experiment was performed at least three times, and each sample in the experiment was prepared in duplicate.

RESULTS

Binding of Zta, MCAF1 and Rta to a ZRE in lytic promoters

This work used four different biotinylated probes, Rp-SZ, Rp-mS, Rp-mZ and Rp-mSZ (Figure 1A), to study the binding of MCAF1, Rta, Sp1 and Zta to an Sp1 site and a ZRE in the BRLF1 promoter. A lysate was prepared from P3HR1 cells that were treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate to induce the EBV lytic cycle and the expression of Rta and Zta. Immunoblotting revealed that Sp1, MCAF1, Rta and Zta were present in the cell lysate (Figure 1B, lanes 1 and 4). Our earlier study found that Rta, MCAF1 and Sp1 form a complex on Sp1 sites (12). Therefore, as expected, this study found the binding of Rta and MCAF1 to probes Rp-SZ and Rp-mZ (Figure 1B, lanes 2 and 6) because both probes contained an Sp1 site (Figure 1A). Meanwhile, Zta bound to Rp-SZ but not Rp-mZ (Figure 1B, lanes 2 and 6) because in Rp-mZ, the ZRE was mutated (Figure 1A). This study also analyzed the binding of these proteins to Rp-mS, which contained a mutated Sp1 site (Figure 1A). Although Sp1 did not bind to the probe, immunoblot analysis revealed the binding of MCAF1, Rta and Zta (Figure 1B, lane 3). Additionally, these three proteins did not bind to the probe after the Sp1 site and ZRE were both mutated (Rp-mSZ) (Figure 1A and B, lane 7), suggesting that Rta and MCAF1 interact with not only the Sp1 on the Sp1 site but also the Zta on ZRE. This study also investigated the binding of Rta, MCAF1 and Zta in the BMRF1 promoter. The region between −174 and −36 in the BMRF1 promoter, MRp-RZ, contained one RRE and three ZREs (Figure 1C). Immunoblot analysis revealed the binding of Rta, MCAF1 and Zta to a biotinylated MRp-RZ (Figure 1D, lane 2). These results further indicated that mutating the RRE in the probe, MRp-mR (Figure 1C), did not affect the binding of these three proteins (Figure 1D, lane 3). However, MCAF1 and Zta did not bind to the mutant probe, MRp-mZ (Figure 1D, lane 4), in which the three ZREs were mutated (Figure 1C); although the binding of Rta to the mutant probe was detected (Figure 1D, lane 4). Following mutation of the RRE and ZREs in the probe (MRp-mRZ) (Figure 1C), Rta, MCAF1 and Zta no longer bound to the probe (Figure 1D, lane 5). These results suggested that Rta, MCAF1 and Zta formed a complex on ZRE. This study used another ZRE probe, HLp-Z, which contained the region from −74 to −41 in the BHLF1 promoter, and showed the binding of these three proteins to the probe (Figure 1E, lane 2). However, these proteins did not bind to the probe after the ZRE was mutated (HLp-mZ) (Figure 1E, lane 3), verifying the binding of Rta, MCAF1 and Zta to ZRE. When another probe, MLp-R, which contained an RRE from the BMLF1 promoter, was utilized, only the binding of Rta was detected (Figure 1E, lane 5). Furthermore, Rta did not bind to the probe after the RRE was mutated

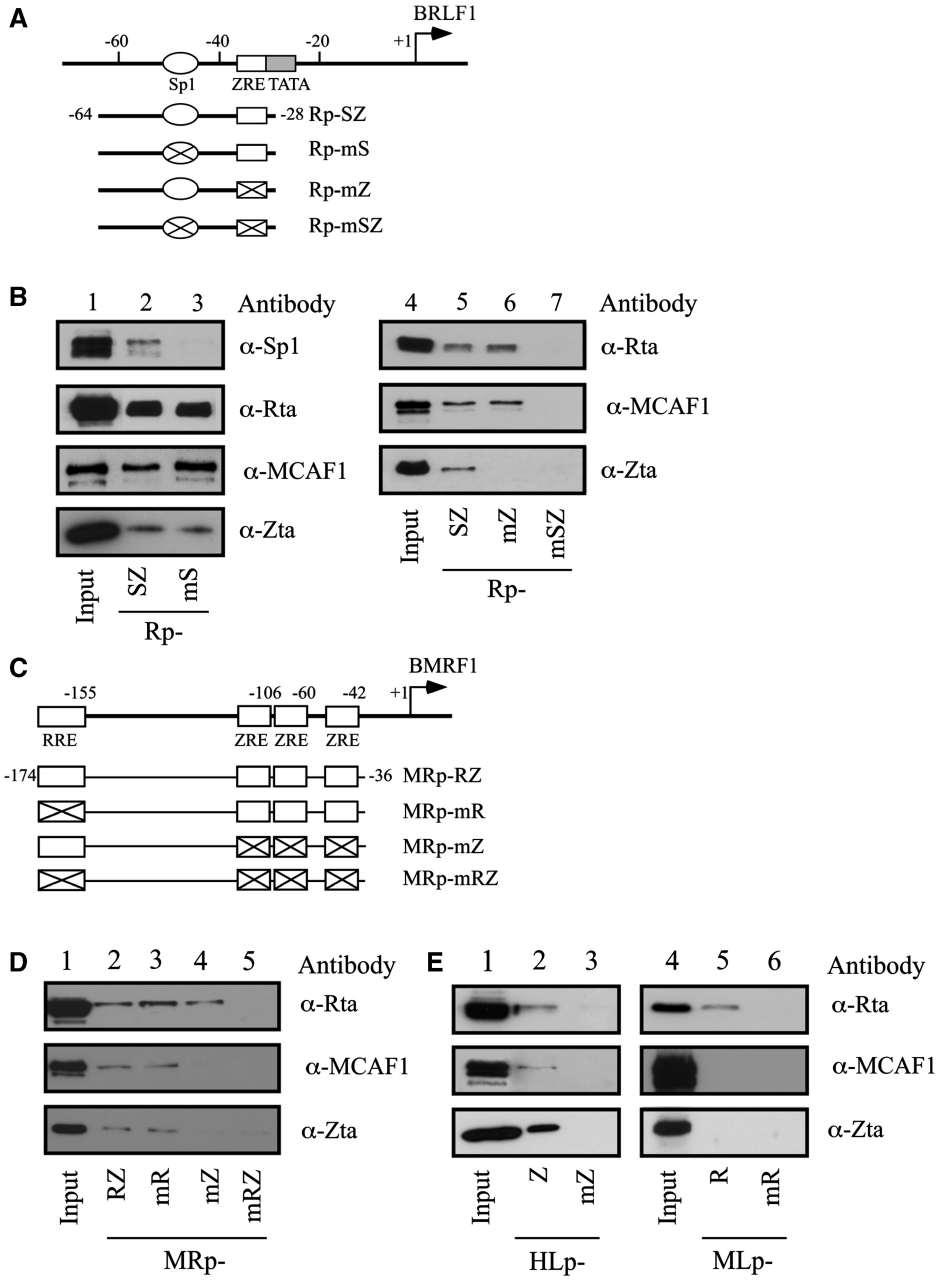


Figure 1. Binding of Zta, MCAF1 and Rta to ZRE. (**A**) Probe Rp-SZ contains the sequence from −64 to −28 in the BRLF1 promoter. It also includes an Sp1 site (oval) and ZRE (rectangle). Probes Rp-mS, Rp-mZ and Rp-mSZ have the same sequence as Rp-SZ, except that the Sp1 site and ZRE in Rp-SZ were mutated as indicated by a crossed rectangle and a crossed oval. The number represents the nucleotide position relative to the transcriptional start site, '+1' (arrow). 'TATA' represents TATA box. (**C**) A probe, MRP-RZ, contained the region between −174 and −36 in the BMRF1 promoter. Probes MRP-mR, MRP-mZ and MRP-mRZ contained a mutated RRE, ZREs and both RRE and ZREs as indicated by crossed rectangles. (**E**) Probe HLp-Z contained a ZRE from the BHLF1 promoter. In HLp-Z, the ZRE was mutated. (**B, D, E**) The probes were added to a lysate from P3HR1 cells that had been treated with TPA and sodium butyrate. Proteins that were bound to the probes were captured using streptavidin magnetic beads and analyzed by immunoblotting with anti-MCAF1, anti-Rta, anti-Sp1 and anti-Zta antibodies.

(Figure 1E, lane 6), indicating that MCAF1 and Zta do not interact with the Rta that binds to an RRE. Furthermore, the BMLF1 promoter is known to contain a sequence resembling that of ZRE (Table 1). However, Zta does not appear to activate the promoter (30). Our result showed that Zta did not bind to the MLp-mR probe, suggesting that the lack of activation is likely attributed to the inability of Zta to bind to this ZRE-like sequence (Figure 1E, lane 6).

Co-immunoprecipitation of Zta, MCAF1 and Rta

Our earlier study demonstrated that Rta and MCAF1 interact and the Rta–MCAF1 complex can be co-immunoprecipitated from a lysate (12). This study conducted a similar study to investigate the interaction between Zta and MCAF1. Immunoblot analysis revealed that MCAF1 in the P3HR1 lysate (Figure 2A, lane 1) was immunoprecipitated by anti-MCAF1 antibody

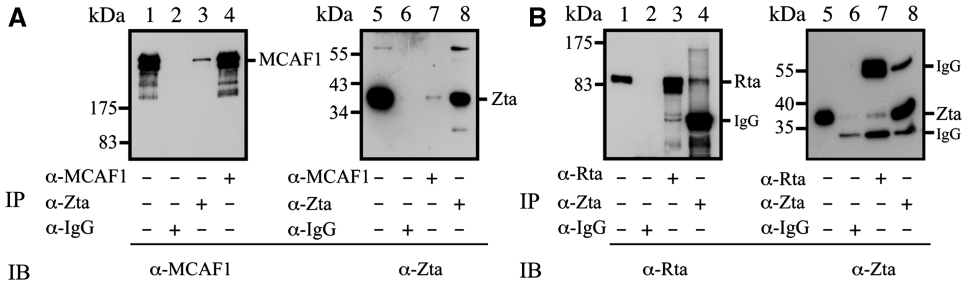


Figure 2. Interaction among Zta, MCAF1 and Rta *in vivo*. (**A**) P3HR1 cells were treated with TPA and sodium butyrate. Proteins in the lysate (lanes 1 and 5) were immunoprecipitated (IP) with anti-IgG (lanes 2 and 6), anti-MCAF1 (lanes 4 and 7) and anti-Zta (lanes 3 and 8) antibodies. Immunoprecipitated proteins were analyzed by immunoblotting (IB) with anti-MCAF1 (lanes 1–4) and anti-Zta (lanes 5–8) antibodies. (**B**) Proteins in the lysate (lanes 1 and 5) were immunoprecipitated with anti-IgG (lanes 2 and 6), anti-Rta (lanes 3 and 7) and anti-Zta (lanes 4 and 8) antibodies. Immunoprecipitated proteins were detected by immunoblotting with anti-Rta (lanes 1–4) and anti-Zta (lanes 5–8) antibodies. Lane 1 in (A) and (B) was loaded with 1% of the cell lysate. Lane 5 in (A) and (B) was loaded with 3% of the cell lysate.

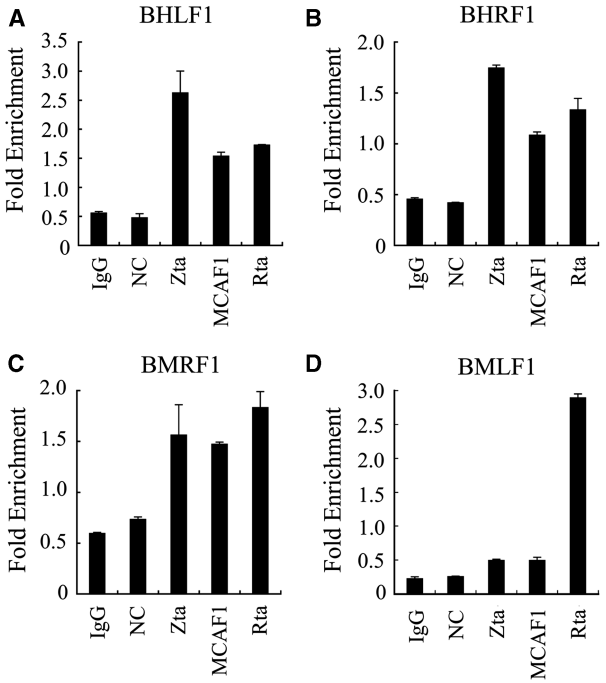


Figure 3. Quantitation of the binding of Zta, MCAF1, and Rta to the ZREs in the promoters of BHLF1 (**A**), BHRF1 (**B**), BMRF1 (**C**) and BMLF1 (**D**). P3HR1 cells were treated with TPA and sodium butyrate. The binding of Zta, MCAF1 and Rta to the ZREs was investigated by CHIP analysis using anti-Zta, anti-MCAF1 and anti-Rta antibodies and by qPCR. The reaction was performed with and without the addition of anti-IgG antibody as negative controls. The error bar represents standard error.

(Figure 2A, lane 4) and co-immunoprecipitated by anti-Zta antibody (Figure 2A, lane 3). A parallel experiment found that Zta in the lysate (Figure 2A, lane 5) was immunoprecipitated by anti-Zta antibody (Figure 2A, lane 8) and co-immunoprecipitated with MCAF1 by anti-MCAF1 antibody (Figure 2A, lane 7). On the other hand, Zta and MCAF1 were not immunoprecipitated by anti-IgG antibody (Figure 2A, lanes 2 and 6). These results showed Zta and MCAF1 interact *in vivo*. A similar study revealed that Rta in the lysate (Figure 2B, lane 1) was immunoprecipitated by anti-Rta antibody (Figure 2B, lane 3) and co-immunoprecipitated by anti-Zta antibody (Figure 2B, lane 4). Zta in the cell lysate (Figure 2B, lane 5) was immunoprecipitated by anti-Zta antibody (Figure 2B, lane 8) and co-immunoprecipitated with Rta by anti-Rta antibody (Figure 2B, lane 7). In negative controls, neither Rta nor Zta was immunoprecipitated by anti-IgG antibody (Figure 2B, lanes 2 and 6).

Binding of Zta–MCAF1–Rta complex to ZRE *in vivo*

P3HR1 cells were treated with TPA and sodium butyrate to induce the expression of Rta and Zta. After cross-linked protein–DNA complex was immunoprecipitated with anti-Zta, anti-MCAF1 and anti-Rta antibodies, PCR with primers that were complimentary to the sequences that flanked the ZRE region in the BHLF1 promoter revealed that DNA fragments that contained the BHLF1 promoter were immunoprecipitated by these three antibodies. The amount of promoter captured by anti-Zta, anti-MCAF1 and anti-Rta antibody exceeded that from the reaction without adding an antibody, the negative control (Figure 3A). Adding the anti-IgG antibody immunoprecipitated the promoter DNA at a level comparable to that of the negative control (Figure 3A). Similar results were also found for the BHRF1 and BMRF1 promoters (Figure 3B and C). However, qPCR revealed that amount of BMLF1 promoter DNA immunoprecipitated by anti-Rta antibody was significantly higher than that immunoprecipitated by anti-Zta and anti-MCAF1 antibodies (Figure 3D). Zta and MCAF1 did not appear to bind to the ZRE in the promoter.

Subcellular localization of Rta, MCAF1 and Zta

Our earlier study demonstrated the colocalization between Rta and MCAF1 by confocal microscopy (12). The same analysis was performed to study the localization of Zta, MCAF1 and Rta. After pEGFP-Rta was transfected into TPA-sodium butyrate-treated P3HR1 cells, GFP-Rta was observed in both the nucleus and the cytoplasm (Figure 4B); Zta was present only in the nucleus (Figure 4C). The merged image revealed that GFP-Rta and Zta colocalized in the nucleus (Figure 4D). GFP expressed from pEGFP was distributed in both the nucleus and the cytoplasm (Figure 4F) and no colocalization between Zta and GFP

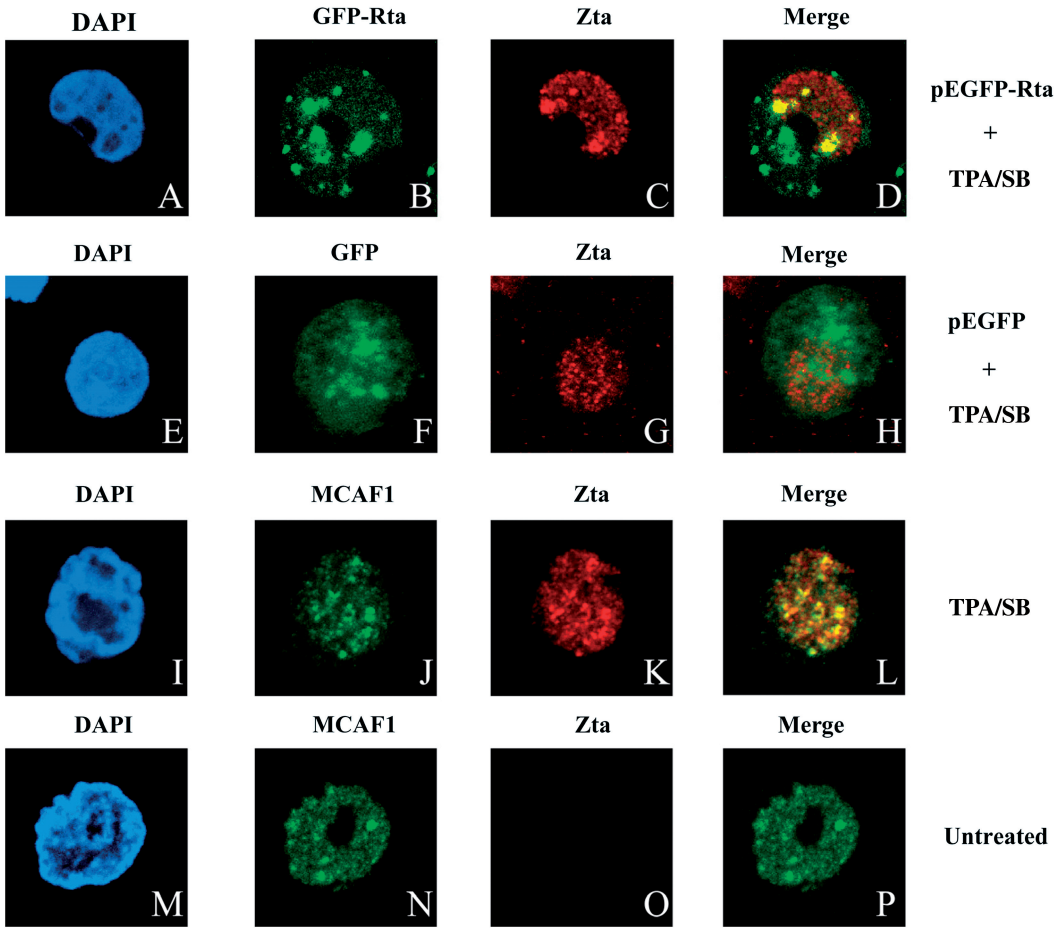


Figure 4. Subcellular localization of MCAF1, Rta and Zta. P3HR1 cells were transfected with pEGFP-Rta (**A–D**) or an empty vector, pEGFP (**E–H**), and treated with TPA and sodium butyrate (SB). Cells were also treated (**I–L**) or untreated (**M–P**) with TPA and sodium butyrate without transfection. Cells were incubated with monoclonal anti-Zta antibody (C, G, K and O) and rabbit anti-MCAF1 polyclonal antibody (J and N). DAPI staining revealed the nucleus (A, E, I, M). Cells were observed under a confocal laser-scanning microscope. D, H, L and P are merged images.

was observed (Figure 4H). Meanwhile, after TPA and sodium butyrate treatment, Zta that was expressed from the EBV genome was found to colocalize with MCAF1 in the nucleus (Figure 4J–L). Colocalization was not observed when cells were untreated with TPA and sodium butyrate (Figure 4N–P).

Mapping interaction domains in Zta and MCAF1

A co-immunoprecipitation experiment was performed to delineate the regions in Zta that interact with MCAF1. 293T cells were cotransfected with plasmids that expressed GFP-ZN, GFP-ZM and GFP-ZC (Figure 5A). Immunoblotting using anti-GFP antibody revealed the presence of GFP-ZN (Figure 5B, lane 1), GFP-ZM (Figure 5B, lane 2) and GFP-ZC (Figure 5B, lane 3) in the lysate, and the co-immunoprecipitation of GFP-ZN (Figure 5B, lane 4), GFP-ZC (Figure 5B, lane 6) but not GFP-ZM (Figure 5B, lane 5) with MCAF1 by anti-MCAF1 antibody (Figure 5B, lanes 4–6), indicating that MCAF1 interacts with the transactivation domain and the region that contains the DNA-binding and dimerization domains in Zta (Figure 5A). Exactly how MCAF1 fragments interact with Zta was also investigated. To do so, plasmids that expressed truncated GFP-MCAF1 fusion proteins (Figure 5C) were transfected into P3HR1 cells that had been treated with TPA and sodium butyrate. After transfection, these proteins were expressed and present in the cell lysate (Figure 5D, lanes 1–4). Immunoblot analysis indicated that GFP-fusion proteins that contain the regions from amino acid 562 to 816 and 1154 to 1270 in MCAF1 (GFP-MCAF1-DM1 and GFP-MCAF1-DM2) were co-immunoprecipitated with Zta by anti-Zta antibody (Figure 5D, lanes 6 and 8). However, GFP fusion proteins that contained the regions from amino acid 1 to 561 (GFP-MCAF1-N) (Figure 5D, lane 5) and from 817 to 1153 (GFP-MCAF1-M) (Figure 5D, lane 7) in MCAF1 were not co-immunoprecipitated, indicating that Zta interacts with domains 1 and 2 in MCAF1 that also interact with Sp1, MBD1, and TFIIE (23,24). Additionally, in GST-pull-down assay, both GST-MCAF1-C1- and GST-MCAF1-C2-glutathione-Sepharose beads (Figure 5C) were found to pull down His-Zta from an *E. coli* lysate (Figure 5E, lanes 4 and 5). However, GST- or GST-Rta-glutathione-Sepharose beads did not pull down His-Zta (Figure 5E, lanes 2 and 3). GST-Zta-glutathione-Sepharose beads also

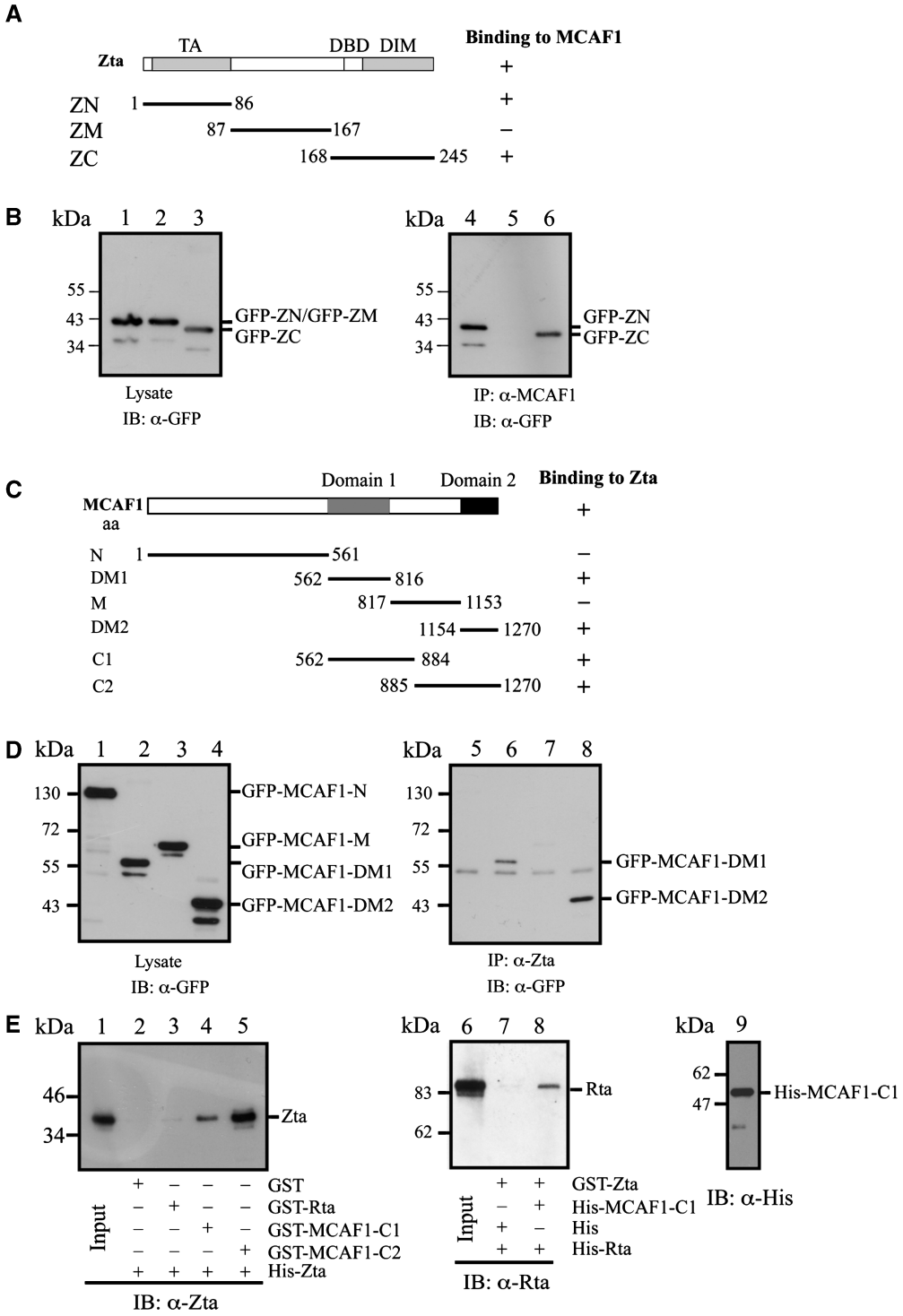


Figure 5. Mapping the interaction domains in MCAF1 and Zta. (**A**) Various regions in Zta were fused with GFP. Numbers represent the positions of amino acid in Zta. TA, transactivation domain; DBD, DNA-binding domain; DIM, dimerization domain. (**B**) Plasmids that expressed GFP-ZN (lanes 1 and 4), GFP-ZM (lanes 2 and 5) and GFP-ZC (lanes 3 and 6) were transfected into 293T cells. Proteins in the lysates were analyzed by immunoblotting (IB) with anti-GFP antibody (lanes 1–3). Proteins in the lysate were immunoprecipitated with anti-MCAF1 antibody and examined by IB with anti-GFP antibody (lanes 4–6). (**C**) Plasmids that expressed a segment of MCAF1 that was fused with GFP were used to identify the region in MCAF1 that interacted with Zta. Numbers represent positions of amino acids in MCAF1. Domains 1 and 2 were delineated by Fujita *et al.* (23). (**D**) The plasmids were transfected into P3HR1 cells that were treated with TPA and sodium butyrate. Proteins in the cell lysate that contained GFP-MCAF1-N (lane 1), GFP-MCAF1-DM1 (lane 2), GFP-MCAF1-M (lane 3) and GFP-MCAF1-DM2 (lane 4) were detected by IB with anti-GFP antibody (lanes 1–4). Proteins in the lysate were immunoprecipitated with anti-Zta antibody and analyzed by immunoblotting with anti-GFP antibody (lanes 5–8). (**E**) The lysate from *E. coli* BL21(DE3)(pET-Zta) was mixed with GST-Rta-, GST-MCAF1-C1-, GST-MCAF1-C2- or GST-glutathione-Sepharose-beads. Proteins that were bound to beads were detected by immunoblotting using anti-Zta antibody (lanes 1–5). *E. coli* lysates that contained His-Rta, His-MCAF1-C1 and His were mixed with GST-Zta-glutathione-Sepharose beads. Proteins that were pulled down by the beads were examined by IB with anti-Rta antibody (lanes 6–8). Lanes 1 and 6 were loaded with 0.5% of the lysate. Lane 9 shows the immunoblot of His-MCAF1-C1 that was purified by Ni-NTA agarose beads.

did not pull down His-Rta from bacterial lysate (Figure 5E, lane 7), suggesting that Rta and Zta do not interact. Our earlier work demonstrated that Rta interacts with domain 1 in MCAF1, which also interacts with Zta (Figure 5D, lane 6), therefore, a bacterial lysate containing His-MCAF1-C1 was added to the lysate mixture that contained GST-Zta and His-Rta. Immunoblotting revealed that adding His-MCAF1-C1 enabled GST-Zta-glutathione-Sepharose beads to pull down His-Rta (Figure 5E, lane 8), indicating that the interaction between Rta and Zta involves domain 1 in MCAF1.

ZRE and synergistic activation by Rta and Zta

A reporter plasmid, pBMRF1, which contains a firefly luciferase gene transcribed from the BMRF1 promoter was adopted to elucidate how Rta and Zta synergistically activate transcription. The BMRF1 promoter in this reporter plasmid contained an RRE and three ZREs (Figure 6A). In a negative control, this study found that in 293T cells, transfecting an empty vector, pCMV-3, did not activate the BMRF1 promoter in pBMRF1; the luciferase activity exhibited by the reporter plasmid was at a background level (Figure 6B). However, the luciferase activity exhibited by the reporter plasmid increased 58-fold when the cells were cotransfected with pCMV-R (Figure 6B), indicating that Rta activates the BMRF1 promoter. A similar experiment demonstrated that transfecting pCMV-Z increased the luciferase activity exhibited by the reporter plasmid 30-fold (Figure 6B), indicating that Zta also activate the BMRF1 promoter. Additionally, the luciferase activity was 784-fold higher than that of the negative control after cotransfecting with pCMV-R and pCMV-Z (Figure 6B), indicating that Rta and Zta synergistically activate the BMRF1 promoter. A similar study was performed with pBMRF1-mRRE (Figure 6A). In this reporter plasmid, the RRE in the BMRF1 promoter in pBMRF1 was mutated (Figure 6A). After cotransfecting with this reporter plasmid and pCMV-R into 293T cells, the luciferase activity exhibited by the reporter plasmid was 28-fold higher than that exhibited by the negative control (Figure 6B). Meanwhile, luciferase activity increased 21-fold after the cells were cotransfected with pCMV-Z (Figure 6B). The 28-fold activation by Rta of the mutant promoter is unsurprising since Rta often nonspecifically activates transcription from an empty vector (18). Despite the RRE mutation, cotransfecting 293T cells with pCMV-R and pCMV-Z synergistically increased the activity of the promoter by a factor of 400 (Figure 6B). Moreover, the three ZREs in the promoter were mutated (pBMRF1-mZRE) (Figure 6A). Although pCMV-Z did not activate the mutant promoter, transfecting pCMV-R increased the promoter activity 45-fold in 293T cells (Figure 6B), a level resembling that achieved using pBMRF1. On the other hand, cotransfecting pCMV-R and pCMV-Z into the cells increased the luciferase activity of the reporter plasmid by only 19-fold; less than that achieved using pCMV-R (Figure 6B). The drop in Rta-activated promoter activity from 45-fold to 19-fold associated with transfecting pCMV-Z (Figure 6B) is consistent with the finding of Giot and Quinlivan *et al.* (4,5), which showed that Zta unbound to ZRE inhibits transcription activation of the BMRF1 promoter by Rta. The DNA-binding domain in Rta is located in the N-terminal region of a protein from amino acid 1 to 320 (31). Our earlier study demonstrated that an Rta mutant containing a mutation with a K213A mutation in this region does not bind to RRE (12). This study further demonstrated that the synergy is independent of the binding of Rta to RRE by conducting similar reporter analysis of the BMRF1 promoter in 293T cells with a plasmid, pCMV-R(K213A), that expressed Rta(K213A). Luciferase assay revealed that pCMV-R(K213A) alone activated the BMRF1 promoter 3-fold (Figure 6C). However, Flag-Zta expressed from pTag2-Zta, activated the promoter 194-fold (Figure 6C). Additionally, cotransfecting pCMV-R(K213A) and pTag2-Zta synergistically activated the promoter 685-fold (Figure 6C). Moreover, two reporter plasmids, pRRE and pZRE, which contain an RRE and a ZRE from the BMLF1 and BRLF1 promoters, respectively, were also used. Cotransfecting 293T cells with pRRE and pCMV-R increased the activity of the promoter by a factor of 11 (Figure 6D); cotransfecting pCMV-Z did not increase the activity of the RRE promoter (Figure 6C). The activity of the RRE promoter increased 12-fold after cotransfecting pCMV-R and pCMV-Z (Figure 6D) into the cells, revealing that Rta and Zta do not activate the RRE promoter synergistically. However, although pCMV-R and pCMV-Z increased the promoter activity of pZRE 4.6-fold and 13-fold, respectively, after both plasmids were cotransfected, the activity of the ZRE promoter increased by 107-fold, indicating that Rta and Zta synergistically increase the activity of the ZRE promoter. Furthermore, immunoblot analysis revealed that after cotransfecting pCMV-R and pCMV-Z, pCMV-R did not affect the expression of Zta from pCMV-Z and *vice versa* (Figure 6E), suggesting that the synergistic activation observed is not attributable to an elevated Rta or Zta expression after cotransfection.

Involvement of MCAF1 in synergistic activation of EBV lytic promoters by Rta and Zta

Two reporter plasmids, pBHLF1 and pEAD, were employed herein to study how MCAF1 affects synergistic activation. In the case of pBHLF1, pCMV-R increased the activity of the promoter 4.7-fold; pCMV-Z increased its activity 94-fold; pCMV-R and pCMV-Z, when cotransfected, increased its activity 524-fold (Figure 7A) in 293T cells. Meanwhile, pCMV-R increased the activity of the BMRF1 promoter in pEAD 83-fold, pCMV-Z increased it 113-fold and pCMV-R and pCMV-Z increased it 558-fold (Figure 7A) in 293T cells. These results indicated that Rta and Zta synergistically activate both the BHLF1 and BMRF1 promoters. Additionally, transfecting the cells with pcDNA-MCAF1 increased Zta-activated BHLF1 transcription from 41.6-fold to 73.7-fold (Figure 7A), indicating that MCAF1, although does not collaborate with Zta to activate transcription synergistically, promotes the transcription that is activated

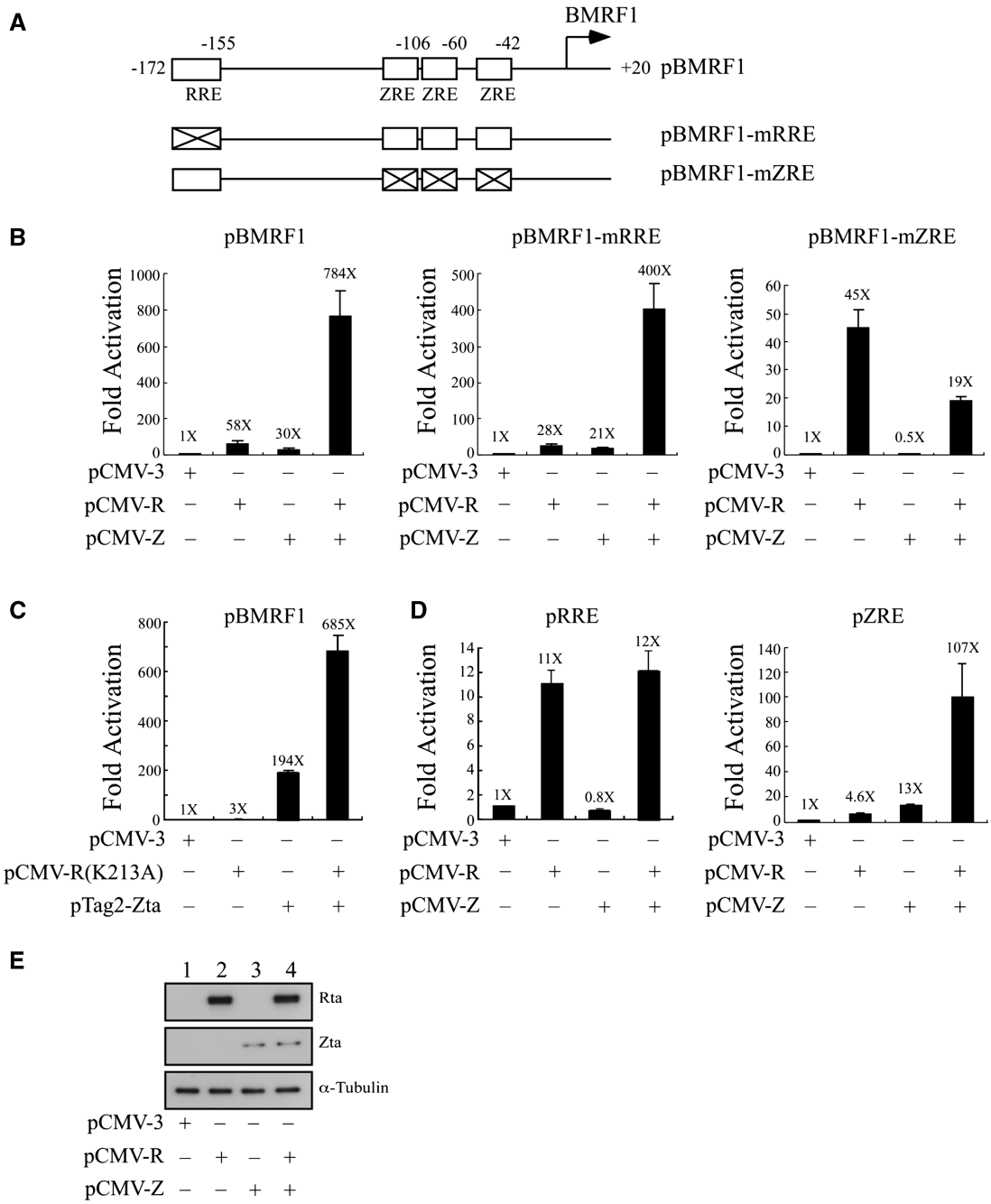


Figure 6. ZRE and synergistic activation by Rta and Zta. (**A**) A reporter plasmid, pBMRF1, contains a luciferase gene that is transcribed from the −172 to +20 region in BMRF1. In this region, the BMRF1 promoter contains an RRE and three ZREs. In pBMRF1-mRRE and pBMRF1-mZRE, the RRE and ZREs, respectively, are mutated. RRE and ZRE are represented as empty boxes. Mutant sequences are shown as crossed rectangles. (**B**) 293T cells were cotransfected with the reporter plasmids, pCMV-R and pCMV-Z. Cells that were transfected with pCMV-3 were used as a control. (**C**) 293T cells were also cotransfected with pBMRF1 and pCMV-R(K213A), pTag2-Zta or pCMV-3. (**D**) Plasmid pRRE contained an RRE from the BMLF1 promoter and pZRE contained a ZRE from the BRLF1 promoter. 293T cells were cotransfected with the reporter plasmids, pCMV-R, pCMV-Z and pCMV-3. The luciferase activity of the cells was determined at 24 h after transfection. Each transfection experiment was performed three times, and each sample in the experiment was prepared in duplicate. (**E**) 293T cells were transfected with pCMV-3, pCMV-R, pCMV-Z or cotransfected with pCMV-R and pCMV-Z. Rta, Zta and α-tubulin expressed by the cells were examined by immunoblotting.

by Zta. Meanwhile, cotransfecting the cells with pcDNA-MCAF1 increased the transcriptional activity of the BHLF1 and the BMRF1 promoters that is synergistically activated by Rta and Zta in a dose-dependent manner. When cells were transfected with 0.5 μg of pcDNA-MCAF1, the promoter activity increased 2.8-fold and 3.8-fold, respectively (Figure 7B). A similar phenomenon was also found in a B lymphocyte cell line, BJAB. Overexpressing Rta and Zta in the cells activated the BMRF1 promoter in pEAD 35- and 15-fold,

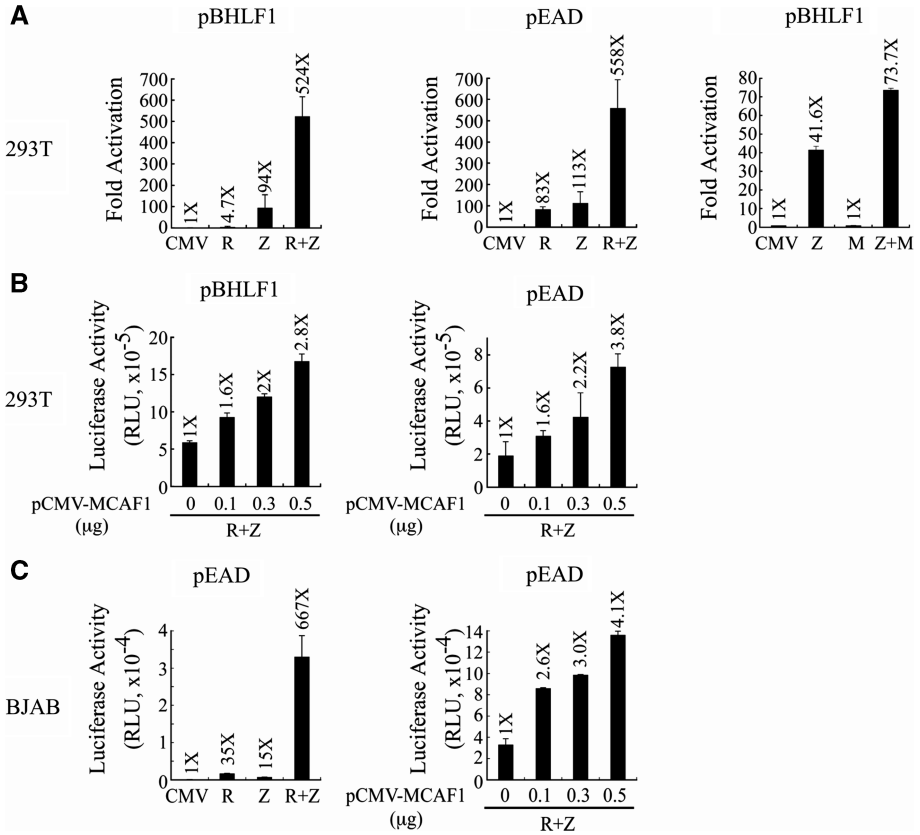


Figure 7. MCAF1 and synCergistic activation of transcription by Rta and Zta. (**A**) 293T cells were cotransfected with a reporter plasmid, pBHLF1 or pEAD, and an empty vector, pCMV-3 (CMV), plasmids that expressed Rta (R), Zta (Z) and MCAF1 (M). (**B**) Cells were also cotransfected with pCMV-R, pCMV-Z and 0–0.5 μg of pcDNA-MCAF1. (**C**) A similar study was conducted in BJAB cells. The luciferase activity of the cells was determined 48 h following transfection. Each transfection experiment was performed three times, and each sample in the experiment was prepared in duplicate.

respectively; in addition, overexpressing both proteins synergistically activated the promoter 667-fold (Figure 7C). Expressing MCAF1 by transfection also enhances the synergistic activation in a dose-dependent manner. In a cotransfection experiment, the synergistic activation was further enhanced 2.6-, 3.0- and 4.1-fold when 0.1, 0.3 and 0.5 μg of pcDNA-MCAF1 was also transfected (Figure 7C). Additionally, transfecting MCAF1 siRNA into 293T cells markedly reduced the intracellular level of MCAF1 (Figure 8A) and decreased the transcriptional activity of the BHLF1 and BMRF1 promoters that is synergistically activated by Rta and Zta 67% and 74%, respectively (Figure 8B). However, MCAF1 siRNA did not affect the activity of the BMLF1 promoter (Figure 8B).

DISCUSSION

As is generally known, viruses commonly express abundant amounts of lytic proteins during the productive cycle. To achieve this, viruses often use viral-encoded transcription factors to promote the transcription of viral genes. Furthermore, these transcription factors may collaborate with each other to activate transcription synergistically to achieve high levels of expression of proteins that are needed for viral lytic development. For example, the two human cytomegalovirus (HCMV) immediate-early proteins, IE1 and IE2, synergistically activate the HCMV lytic genes (32,33). The human immunodeficiency virus (HIV) Tat protein is known to interact with PCAF to activate transcription synergistically (34). In EBV, Rta and Zta are expressed at the onset of the lytic cycle. These two proteins commonly cooperate with each other and activate transcription synergistically (3,5,9,17,18). Since the binding between Rta and Zta has not been proven experimentally, one model proposes that the simultaneous binding of Rta and Zta to RRE and ZRE, respectively, in a promoter is a prerequisite for synergistic activation (5,19). However, the model may not accurately explain how the transcription is synergistically activated, since Rta and Zta activate synergistically the BRLF1 promoter, which does not have an RRE (18). Accordingly, an alternative model suggests that Rta and Zta form a complex through an unidentified protein to activate transcription synergistically (4).

If the synergistic activation by Rta and Zta involves an intermediary protein, then this protein is likely to be MCAF1 since Rta and Zta simultaneously interact with MCAF1. The evidence for this interaction is that Rta, MCAF1 and Zta bind to ZRE probes *in vitro* (Figure 1B, lanes 3 and 7; Figure 1). This interaction

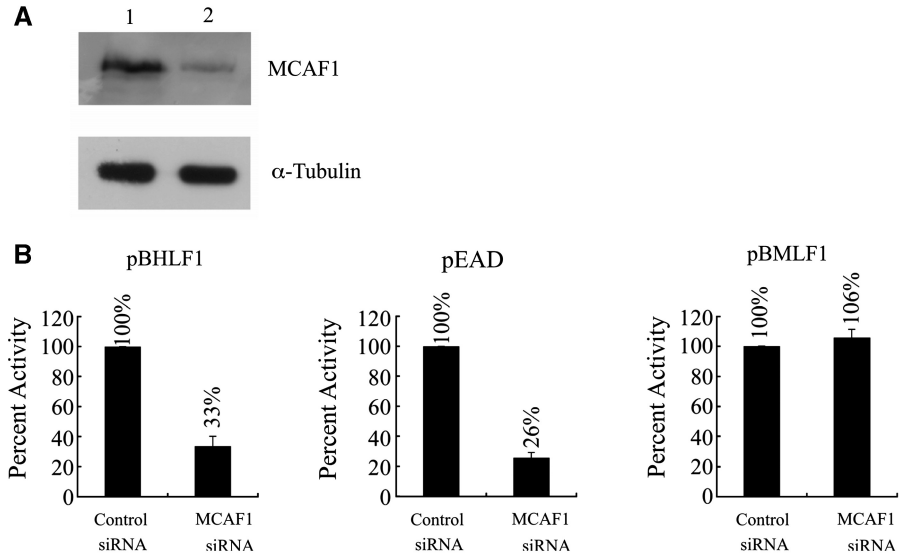


Figure 8. Knockdown of MCAF1 and synergistic activation. (**A**) 293T cells were transfected with MCAF1 siRNA (lane 2) or control siRNA (lane 1). The expression of MCAF1 was determined by immunoblotting using anti-MCAF1 antibody at 48 h following transfection. The amount of α-tubulin in the cell was used as a control. (**B**) 293T cells were cotransfected with pBHLF1, pEAD, or pBMLF1 with pCMV-R, pCMV-Z and MCAF1 siRNA or control siRNA. The luciferase activity of the cells was determined 48 h following transfection. Each transfection experiment was performed three times, and each sample in the experiment was prepared in duplicate.

also proceeds *in vivo* since these three proteins can be co-immunoprecipitated (Figure 2); bind to the ZREs in the BHLF1, BHRF1 and BMRF1 promoters, as demonstrated by CHIP analysis (Figure 3); and colocalize in the nucleus (Figure 4). The fact that the BHLF1 promoter includes four ZREs in the *oriLyt*, which are critically involved in the EBV lytic replication, implies that Rta may participate in EBV lytic replication by interacting with MCAF1 and Zta. This study also establishes that the interaction between Rta and Zta depends on domain 1 region in MCAF1, where MBD1, Sp1, and TFIIE also bind (23,24), since adding a peptide that contains the sequence in this region causes the interaction (Figure 5E).

This study demonstrates that synergistic activation of transcription by Rta and Zta depends on ZRE but not RRE. In the BMRF1 promoter, the region from −172 to +20 contains an RRE and three ZREs (Figure 6A). Results of this study show that mutating the RRE reduces the increase in synergistic activity from 784-fold to 400-fold (Figure 6B). However, the 400-fold increase considerably exceeds those achieved using pCMV-R or pCMV-Z (Figure 6B), indicating that Rta and Zta synergistically activate the mutant promoter in pBMRF1-mRRE. In fact, a similar phenomenon was also observed by an earlier study (5). This result suggests that, rather than participating in synergistic activation, Rta, when it binds to RRE, acts alone but does not cooperate with Zta to activate transcription. Furthermore, mutating the three ZREs in a promoter, such as pBMRF1-mZRE (Figure 6A), eliminates the increase in synergistic activity from 784-fold to 19-fold (Figure 6B), indicating that after ZREs are mutated, pCMV-R and pCMV-Z no longer activate the promoter synergistically. Moreover, the synergistic activation of the ZRE promoter in pZRE but not the RRE promoter in pRRE (Figure 6D) further supports the fact that the synergistic activation depends on ZRE but not RRE. Additionally, Giot *et al.* (4) indicated that the DNA-binding domain in Zta is critical to the synergistic activation, supporting the conclusion that ZRE participates in synergistic activation. Earlier studies of synergistic activation have demonstrated that after ZRE is mutated in the BMRF1 promoter, Zta no longer binds to the promoter and Zta that is unbound to the mutant promoter actually inhibits the transcription that is activated by Rta (4,5). In HCMV, unbound IE1 was also found to inhibit the transcription that is activated by IE2 (32,33). The inhibition by unbound Zta may explain why cotransfecting pCMV-R and pCMV-Z activates the BMRF1 promoter in pBMRF1-mZRE to an extent that is lower than achieved using pCMV-R (Figure 6B). Since the interaction of Rta with MCAF1 prevents the binding of Rta to RRE (Figure 1C, lane 5) (12), after the mutation of ZRE, the unbound Zta may form a complex with MCAF1 and Rta, reducing the level of Rta that binds to RRE to activate transcription.

This study demonstrates that MCAF1 participates in synergistic activation by Rta and Zta. In a transient transfection study, expressing MCAF1 increases the synergistic activation of the BHLF1 and BMRF1 promoters in a dose-dependent manner in both 293T and BJAB cells (Figure 7B and C). As MCAF1 is expressed constitutively and abundant in cells (Figure 4), this may explain why overexpressing MCAF1 does not substantially increase the synergy (Figure 7B and C). Additionally, introducing MCAF1 siRNA into the cell reduces the increment in synergistic activity 67% and 74%, respectively (Figure 8B). This finding suggests that Rta and Zta cannot synergistically activate transcription when MCAF1 is insufficient. The reduction of the activation by MCAF1 siRNA cannot be attributed to the likelihood that the siRNA

nonspecifically inhibits global transcription since the transcription of BMLF1 is unaffected (Figure 8B). This study also attempted to demonstrate the inhibition of transcription of BMRF1 from the EBV genome by using the same siRNA approach (Figure S1). However, the inhibition was less profound than the results from the transfection study. This observation may be owing to that, in contrast with the plasmids transfected into the cell, the copy number of the EBV DNA is relatively low. The amount of MCAF1 remaining in the cell after siRNA knockdown is probably adequate to sustain the transcription of BMRF1. This study further demonstrates that MCAF1 enhances the transcription of BHLF1 that is activated by Zta from 41.6-fold to 73.7-fold (Figure 7A). Moreover, an MCAF1 knockdown by siRNA reduces the enhancement (Figure S2), indicating that MCAF1 promotes Zta-activated transcription, although not synergistically. Furthermore, although Rta interacts with Sp1 through MCAF1 and enhances Sp1-mediated transcription (12), Rta and Sp1 do not activate synergistically the transcription of an Sp1 reporter plasmid (data not shown). Similarly, our unpublished data also show that Rta interacts with AP-1 and ATF2 via MCAF1. However, the interaction does not appear to activate transcription synergistically (data not shown). These findings indicate that the interaction between the MCAF1–Rta complex with a protein binding to a promoter does not always result in synergistic activation. Additionally, Francis *et al*. (19) showed that although an S186A mutation in Zta abolishes the transactivation ability, the mutant protein remains capable of binding to ZRE and collaborates with Rta to activate the transcription of BMRF1 and BRLF1 synergistically. The finding suggests that the binding of Zta to ZRE but not the transactivation capability of Zta is important to the synergistic activation.

Based on our results, we conclude that Rta interacts with Zta on ZRE through MCAF1. In this protein complex, Rta and Zta may act together or recruit additional transcription factors to promote synergistic activation. Synergistic activation of EBV lytic transcription by Rta and Zta is critical to the efficient expression of viral lytic proteins in the lytic cycle. This study demonstrates that formation of a Zta–MCAF1–Rta complex on ZRE is the underlying mechanism that causes synergistic activation, which is critical to EBV lytic development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors would like to thank Chang Jou-Wei and Chen Chien-Chang for their technical support.

FUNDING

The National Health Research Institute of the Republic of China, Taiwan (Contract no. NHRI-EX98-9503BC); the National Science Council of the Republic of China, Taiwan (Contract no. NSC98-3112-B-182-005); and Chang-Gung Memorial Hospital (Contract no. CMRP-D160113). Funding for open access charge: National Science Council of the Republic of China, Taiwan.

REFERENCES

1. Chevallier-Greco,A., Manet,E., Chavrier,P., Mosnier,C., Daillie,J. and Sergeant,A. (1986) Both Epstein-Barr virus (EBV)-encoded trans-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. *EMBO J.*, **5**, 3243–3249.
2. Kenney,S., Holley-Guthrie,E., Mar,E.C. and Smith,M. (1989) The Epstein-Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. *J. Virol.*, **63**, 38783883.
3. Holley-Guthrie,E.A., Quinlivan,E.B., Mar,E.C. and Kenney,S. (1990) The Epstein-Barr virus (EBV) BMRF1 promoter for early antigen (EA-D) is regulated by the EBV transactivators, BRLF1 and BZLF1, in a cell-specific manner. *J. Virol.*, **64**, 3753–3759.
4. Giot,J.F., Mikaelian,I., Buisson,M., Manet,E., Joab,I., Nicolas,J.C. and Sergeant,A. (1991) Transcriptional interference between the EBV transcription factors EB1 and R: both DNA-binding and activation domains of EB1 are required. *Nucleic Acids Res.*, **19**, 1251–1258.
5. Quinlivan,E.B., Holley-Guthrie,E.A., Norris,M., Gutsch,D., Bachenheimer,S.L. and Kenney,S.C. (1993) Direct BRLF1 binding is required for cooperative BZLF1/BRLF1 activation of the Epstein-Barr virus early promoter, BMRF1. *Nucleic Acids Res.*, **21**, 1999–2007.
6. Feederle,R., Kost,M., Baumann,M., Janz,A., Drouet,E., Hammerschmidt,W. and Delecluse,H.J. (2000) The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators. *EMBO J.*, **19**, 3080–3089.
7. Chiu,Y.F., Tung,C.P., Lee,Y.H., Wang,W.H., Li,C., Hung,J.Y., Wang,C.Y., Kawaguchi,Y. and Liu,S.T. (2007) A comprehensive library of mutations of Epstein Barr virus. *J. Gen. Virol.*, **88**, 2463–2472.
8. Chevallier-Greco,A., Gruffat,H., Manet,E., Calender,A. and Sergeant,A. (1989) The Epstein-Barr virus (EBV) DR enhancer contains two functionally different domains: domain A is constitutive and cell specific, domain B is transactivated by the EBV early protein R. *J. Virol.*, **63**, 615–623.
9. Cox,M.A., Leahy,J. and Hardwick,J.M. (1990) An enhancer within the divergent promoter of Epstein-Barr virus responds synergistically to the R and Z transactivators. *J. Virol.*, **64**, 313–321.
10. Chen,L.W., Chang,P.J., Delecluse,H.J. and Miller,G. (2005) Marked variation in response of consensus binding elements for the Rta protein of Epstein-Barr virus. *J. Virol.*, **79**, 9635–9650.
11. Granato,M., Farina,A., Gonnella,R., Santarelli,R., Frati,L., Faggioni,A. and Angeloni,A. (2006) Regulation of the expression of the Epstein-Barr virus early gene BFRF1. *Virology*, **347**, 109–116.
12. Chang,L.K., Chung,J.Y., Hong,Y.R., Ichimura,T., Nakao,M. and Liu,S.T. (2005) Activation of Sp1-mediated transcription by Rta of Epstein-Barr virus via an interaction with MCAF1. *Nucleic Acids Res.*, **33**, 6528–6539.
13. Ragoczy,T. and Miller,G. (2001) Autostimulation of the Epstein-Barr virus BRLF1 promoter is mediated through consensus Sp1 and Sp3 binding sites. *J. Virol.*, **75**, 5240–5251.
14. Ragoczy,T. and Miller,G. (1999) Role of the Epstein-Barr virus RTA protein in activation of distinct classes of viral lytic cycle genes. *J. Virol.*, **73**, 9858–9866.
15. Chua,H.H., Lee,H.H., Chang,S.S., Lu,C.C., Yeh,T.H., Hsu,T.Y., Cheng,T.H., Cheng,J.T., Chen,M.R. and Tsai,C.H. (2007) Role of the TSG101 gene in Epstein-Barr virus late gene transcription. *J. Virol.*, **81**, 2459–2471.
16. El-Guindy,A.S. and Miller,G. (2004) Phosphorylation of Epstein-Barr virus ZEBRA protein at its casein kinase 2 sites mediates its

ability to repress activation of a viral lytic cycle late gene by Rta. *J. Virol.*, **78**, 7634–7644.
17. Gruffat,H., Moreno,N. and Sergeant,A. (1990) The Epstein-Barr virus (EBV) ORI1yt enhancer is not B-cell specific and does not respond synergistically to the EBV transcription factors R and Z. *J. Virol.*, **64**, 2810–2818.
18. Liu,P. and Speck,S.H. (2003) Synergistic autoactivation of the Epstein-Barr virus immediate-early BRLF1 promoter by Rta and Zta. *Virology*, **310**, 199–206.
19. Francis,A., Ragoczy,T., Gradoville,L., Heston,L., El-Guindy,A., Endo,Y. and Miller,G. (1999) Amino acid substitutions reveal distinct functions of serine 186 of the ZEBRA protein in activation of early lytic cycle genes and synergy with the Epstein-Barr virus R transactivator. *J. Virol.*, **73**, 4543–4551.
20. Ragoczy,T., Heston,L. and Miller,G. (1998) The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes. *J. Virol.*, **72**, 7978–7984.
21. Wang,H., An,W., Cao,R., Xia,L., Erdjument-Bromage,H., Chatton,B., Tempst,P., Roeder,R.G. and Zhang,Y. (2003) mAM facilitates conversion by ESET of dimethyl to trimethyl lysine 9 of histone H3 to cause transcriptional repression. *Mol. Cell*, **12**, 475–487.
22. Ichimura,T., Watanabe,S., Sakamoto,Y., Aoto,T., Fujita,N. and Nakao,M. (2005) Transcriptional repression and heterochromatin formation by MBD1 and MCAF/AM family proteins. *J. Biol. Chem.*, **280**, 13928–13935.
23. Fujita,N., Watanabe,S., Ichimura,T., Ohkuma,Y., Chiba,T., Saya,H. and Nakao,M. (2003) MCAF mediates MBD1-dependent transcriptional repression. *Mol. Cell Biol.*, **23**, 2834–2843.
24. Liu,L., Ishihara,K., Ichimura,T., Fujita,N., Hino,S., Tomita,S., Watanabe,S., Saitoh,N., Ito,T. and Nakao,M. (2009) MCAF1/AM is involved in Sp1-mediated maintenance of cancer-associated telomerase activity. *J. Biol. Chem.*, **284**, 5165–5174.
25. Chang,L.K., Lee,Y.H., Cheng,T.S., Hong,Y.R., Lu,P.J., Wang,J.J., Wang,W.H., Kuo,C.W., Li,S.S. and Liu,S.T. (2004) Post-translational modification of Rta of Epstein-Barr virus by SUMO-1. *J. Biol. Chem.*, **279**, 38803–38812.
26. Philipsen,S. and Suske,G. (1999) A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res.*, **27**, 2991–3000.
27. Chang,L.K., Liu,S.T., Kuo,C.W., Wang,W.H., Chuang,J.Y., Bianchi,E. and Hong,Y.R. (2008) Enhancement of transactivation activity of Rta of Epstein-Barr virus by RanBPM. *J. Mol. Biol.*, **379**, 231–242.
28. Chang,L.K. and Liu,S.T. (2000) Activation of the BRLF1 promoter and lytic cycle of Epstein-Barr virus by histone acetylation. *Nucleic Acids Res.*, **28**, 3918–3925.
29. Chang,P.J., Chang,Y.S. and Liu,S.T. (1998) Role of Rta in the translation of bicistronic BZLF1 of Epstein-Barr virus. *J. Virol.*, **72**, 5128–5136.
30. Kenney,S., Holley-Guthrie,E., Mar,E.C. and Smith,M. (1989) The Epstein-Barr virus BMLF1 promoter contains an enhancer element that is responsive to the BZLF1 and BRLF1 transactivators. *J. Virol.*, **63**, 3878–3883.
31. Manet,E., Rigolet,A., Gruffat,H., Giot,J.F. and Sergeant,A. (1991) Domains of the Epstein-Barr virus (EBV) transcription factor R required for dimerization, DNA binding and activation. *Nucleic Acids Res.*, **19**, 2661–2667.
32. Kim,J.M., Hong,Y. and Kim,S. (2000) Artificial recruitment of Sp1 or TBP can replace the role of IE1 in the synergistic transactivation by IE1 and IE2. *Biochem. Biophys. Res. Commun.*, **269**, 302–308.
33. Lukac,D.M., Manuppello,J.R. and Alwine,J.C. (1994) Transcriptional activation by the human cytomegalovirus immediate-early proteins: requirements for simple promoter structures and interactions with multiple components of the transcription complex. *J. Virol.*, **68**, 5184–5193.
34. Dorr,A., Kiermer,V., Pedal,A., Rackwitz,H.R., Henklein,P., Schubert,U., Zhou,M.M., Verdin,E. and Ott,M. (2002) Transcriptional synergy between Tat and PCAF is dependent on the binding of acetylated Tat to the PCAF bromodomain. *EMBO J.*, **21**, 2715–2723.