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Activation of Sp1-mediated transcription by Rta of Epstein–Barr virus via an interaction with MCAF1

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ABSTRACT

Rta is a transcription factor encoded by BRLF1 of the Epstein–Barr virus (EBV). This factor is expressed during the immediate-early stage of the lytic cycle to activate the genes required for EBV lytic development. Although transcription activation by Rta is frequently associated with the binding of Rta to the Rta-response element (RRE) in promoters, Rta sometimes activates promoters without an RRE. Here we show that Rta interacts with an Sp1-interacting protein, MBD1-containing chromatin-associated factor 1 (MCAF1). This interaction is critical to the formation of an Sp1–MCAF1–Rta complex at Sp1 sites. Therefore, following lytic induction and the expression of Rta, Rta increases Sp1-mediated transcription. The genes that are thus activated include *p16*, *p21*, *SNRPN* and BRLF1. However, the binding of Rta to RRE prevents the interaction between Rta and MCAF1; therefore, transcription activation by RRE depends only on Rta, and not on MCAF1 or Sp1. Furthermore, this study finds that MCAF1 promotes the expression of Rta and Zta from EBV, indicating that MCAF1 participates EBV lytic activation. Our study documents the critical role of Rta in regulating the transcription of the genes that are mediated by Sp1.

INTRODUCTION

Rta, encoded by BRLF1, is a transcription factor expressed by Epstein–Barr virus (EBV) during the immediate-early stage of the lytic cycle. This factor often cooperates with another EBV immediate-early protein, Zta, to activate synergistically the transcription of EBV lytic genes (1–3). These two immediate-early proteins are crucial to EBV lytic activation because mutants that do not produce either one of these two proteins do not produce infectious EBV particles (4). Earlier studies have established that Rta frequently activates transcription by binding to a 17 bp *cis* sequence, the Rta-response element (RRE) (5). The EBV genes that are activated in this way include BMRF1, BHRF1, BHLF1 and BMLF1 (2,3,6–9). However, Rta also activates the transcription of BRLF1, BZLF1, BALF5 and BNLF1 of EBV, which lack an RRE in their promoter (10–13). Although exactly how Rta activates these promoters is unclear, an earlier work demonstrated that the Sp1 sites in the BRLF1 promoter (Rp) are critical to the activation by Rta (10). However, Rta does not appear to interact directly with Sp1 to activate the transcription (10).

MBD1-containing chromatin-associated factor 1 (MCAF1), a human homolog of murine ATFa-associated modulator, is a transcription factor of 1270 amino acids with a transcriptional repression activity (14). MCAF1 enhances SETDB1/ESET-mediated histone H3 methylation (15) to promote the formation of heterochromatin domains (16). MCAF1 also interacts with MBD1 and Sp1 (14). The interaction between MCAF1 and MBD1 is crucial to MBD1-dependent transcriptional repression (14); when it binds to MBD1, MCAF1 forms a repressive complex with MBD1 to prevent transcription from a methylated promoter (14). However, when binding to Sp1, MCAF1 becomes a coactivator to enhance Sp1-mediated transcription (14). This work finds that Rta interacts with MCAF1, which facilitates the formation of an Sp1–MCAF1–Rta complex on the Sp1-binding site to autoregulate the transcription of BRLF1 and regulate the host genes in EBV-infected cells.

MATERIALS AND METHODS

Cell lines and EBV lytic induction

P3HR1 cells were cultured in RPMI 1640 medium that contained 10% fetal calf serum (FCS). 293T and 293 cells were

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cultured in DMEM supplemented with 10% FCS. P3HR1 cells were treated with 3 ng/ml of 12-*O*-tetradecanoylphobol-13-acetate (TPA) and 3 mM sodium butyrate to activate the EBV lytic cycle (17). Maxi-EBV is a laboratory-constructed EBV strain that contains an F replicon (18).

Plasmids

Plasmid pCMV-R contains BRLF1 transcribed from the cytomegalovirus (CMV) immediate-early promoter. Plasmids pcDNA-MCAF1, pHA-Rta, pRRE, pRp-luc, pCMV-R(K213A) and pET-Rta were described elsewhere (17,19). Plasmid pGEX-4T1, which expresses GST, was purchased from Amersham Pharmacia Biotech (Hong Kong). The DNA fragment that encodes p621 (amino acids 650–1270 of MCAF1) was isolated from pcDNA-MCAF1 (14) by HpaI–XhoI digestion and inserted into the SmaI and XhoI sites to yield plasmid pGST-621. The Sp1 gene was amplified by PCR, using GST-Sp1 (5′-CCGGAATTCATGGATG-AAATGACAGCTGTG) and Sp1-His (5′-ACGCGTCGAC-AAGCCATTGCCACTGATA) as primers, and plasmid pCMV-Sp1 (20), which was obtained from Guntram Suske, as a template. The fragment was inserted into the EcoRV and SalI sites of pET32a or pGEX-4T1 to form plasmid pET-Sp1 or pGST-Sp1, respectively. Plasmid pR-Sp1 was constructed by inserting a double-stranded oligonucleotide that contained a sequence that covers the region between −53 and +4 of Rta, into the SmaI site of pGL2-Basic (Promega Corp., Madison, WI). In this sequence (5′-GTCCCGCCCATGCC-AGATATCCATAAAAGACCAGTAATCCATGTCAGCCG-GCCTTT), a ZRE sequence and a nucleotide residue that precedes the sequence was mutated from 5′-ATGGCTCA to 5′-GATATCCA (Fig. 1). The Sp1 site (5′-CCGCCC), the mutated ZRE sequence and the TATA sequence (5-AT-AAAA) are underlined. Plasmid pR-mSp1 includes the same sequence as pR-Sp1, except in that the Sp1 site, 5′-CCGCCC, was changed to 5′-ATTAAT. R-TATA (5′-CATAAAAGAC-CAGTAATCCATGTCAGCCGGCCTTT), which contains a sequence from the region from −32 to +4 in Rp, was inserted into the SmaI site of pGL2-Basic to construct plasmid pTA. Plasmid pSp1-luc contains four of the six Sp1-binding sites from the promoter of *p21* upstream of the firefly luciferase gene (*luc*) in pTA. Plasmid pFlag-Rta that expresses full-length Flag-tagged Rta was constructed by inserting a PCR-amplified BRLF1 fragment into pCMV-Tag2A (Stratagene) at the EcoRI and XhoI sites. The same approach was employed to produce deletions in Flag-tagged Rta (Flag-Stu, Flag-HincII, Flag-HinfI and Flag-EcoRII), which lacked the regions between amino acids 1 and 190, 1 and 254, 1 and 290, and 1 and 360, respectively. Plasmid pEGFP-MCAF1-N, which contains the N-terminal 561 amino acids of MCAF1, was constructed by inserting a PCR-amplified fragment into the HindIII and SmaI sites of pEGFP-C1 (Clontech). Plasmids pEGFP-MCAF1-D1 and pEGFP-MCAF1-D2 were also constructed using the same strategy. An Rta deletion mutant, HA-RN290 that contains the N-terminal 290 amino acids of Rta was constructed by inserting a PCR fragment into the EcoRI and XhoI sites of pcDNA3-HA. Plasmid pEGFP-R255/290, which encodes the region between amino acid 255 and 290 in Rta fused with green fluorescence protein (GFP), was constructed by inserting a PCR-amplified fragment into the EcoRI and SalI sites of pEGFP-C1. Plasmid pp21-luc was obtained from W.-C. Hung. Plasmids pp16-luc and pSNRPN-luc have been described elsewhere (14).

Yeast two-hybrid screen

The binding partners of Rta were identified by yeast two-hybrid screen with a bait plasmid, pR476, and a human testes cDNA library, following a method described elsewhere (17).

GST-pull-down assay

GST or GST-p621 at a concentration of 40 ng/ml in 500 µl of NETN buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40), containing 10 µg/ml each of leupeptin, aprotinin and 4-(2-aminoethyl)-benzenesulfonyl fluoride, was added to 30 µl of 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 a lysate (300 µl) prepared from *Escherichia coli* BL21(DE3)(pET-Rta) or *E.coli* BL21(DE3)(pET-Sp1). The reaction mixture was incubated on ice for 1 h. The beads were then washed in NETN buffer. An equal volume of 2× 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 finally separated by SDS–PAGE (21).

Immunoprecipitation

P3HR1 cells (1×10^7) treated with TPA and sodium butyrate were washed in phosphate-buffered saline (PBS). Lysate was prepared using radioimmune precipitation assay (RIPA) buffer, following a method described elsewhere (17). To the supernatant, anti-Rta (1:500 dilution) (Argene, Varilhes, France), anti-MCAF1 antibody (1:500 dilution) (14) or anti-Sp1 antibody (1:2000 dilution) (Santa Cruz Biotechnology Inc., Santa Cruze, CA) was added and incubated at 4°C for 1 h. Protein-A/G agarose beads (30 µl) (Oncogene, Boston, MA) were added to the lysate and the mixture was incubated with shaking for 1 h at 4°C. The beads were finally collected by centrifugation and washed three times in RIPA buffer. Proteins that bound to the beads were eluted by adding 20 µl of 2× electrophoresis sample buffer and analyzed by immunoblotting (IB) with anti-Rta, anti-MCAF1 or anti-Sp1 antibody.

DNA-affinity precipitation assay (DAPA)

A 5′-biotin end-labeled double-stranded probe (Rp64/28) that contains a sequence from −64 to −28 in Rp (5′-ATAT-TGCGATTGTCCCGCCCATGCCAATGGCTCATAA), was synthesized by Mission Biotech, Inc. (Taipei, Taiwan). A mutant probe (mRp28/64), with the same sequence except in that the Sp1 site was changed from 5′-CCGCCC to 5′-ATTAAT, was used as a negative control. In both Rp64/28 and Rpm64/28, the ZRE in Rp was mutated from 5′-TGAGCCA to 5′-TGCAGTC (Fig. 1). A biotin-labeled probe that contained an RRE sequence (5′-CATGTCCCTCTATCATGGCG-CAGAC) from the BMLF1 promoter was utilized to demonstrate the binding of Rta to RRE. A cell lysate prepared from P3HR1 cells treated with TPA/sodium butyrate or 293T cells transfected with pCMV-R was mixed with 0.2 µg of a biotin-labeled 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 DTT and 10 μg/ml each of leupeptin, aprotinin and 4-(2-aminoethyl)-benzenesulfonyl fluoride. After it was incubated on ice for 45 min, DNA–protein complexes were then incubated with 30 μl of Streptavidin MagneSphere Paramagnetic particles (Promega Corp.), which were pre-equilibrated in the binding buffer with gentle shaking for 1 h at 4°C. DNA–protein complexes were then washed five times in the binding buffer. Next, 2× electrophoresis sample buffer was added to the precipitated DNA–protein complex and the solution was boiled for 5 min to dissociate the complexes. Finally, the proteins were resolved by PAGE and detected by IB.

Chromatin immunoprecipitation (CHIP) assay

CHIP assay was performed according to a method described previously (19). P3HR1 cells (1×10^7) were transfected with pcDNA-MCAF1, pCMV-R and pR-Sp1 or pR-mSp1. Formaldehyde-fixed DNA–proteins complex was immunoprecipitated with anti-Rta, anti-Sp1 or anti-MCAF1 antibody. The presence of Rp fragments in the precipitates was detected by PCR, using R-TATA1 (5′-AAAGGCCGGCTGACATGGATTACTGGTCTTTTATG) and F1 (5′-GGTACGTGGAGGTTTTAC) as primers. PCR was performed for 28 cycles under the conditions of 30 s at 94, 51 and 72°C.

Immunofluorescence analysis

P3HR1 cells were transfected with pCMV-R. After culturing for 24 h, cells were harvested by centrifugation; plated on poly-L-Lysine (Sigma)-coated coverslips, and fixed with 4% paraformaldehyde in PBS for 30 min. Immunostaining was conducted with anti-Rta monoclonal antibody, rabbit anti-MCAF1 polyclonal antibody and rabbit anti-Sp1 polyclonal antibody. The cells were then treated with fluorescein isothiocyanate-conjugated donkey anti-mouse IgG polyclonal antibody (KPL Inc., Gaithersburg, MD) or rhodamine-conjugated donkey anti-rabbit IgG polyclonal antibody (DAKO, Glostrup, Denmark), following a method described elsewhere (17). Nuclei were visualized by staining with 5 μg/ml of 4′-6-diamidino-2-phenylindole (DAPI). Finally, cells were examined using an Olympus confocal laser-scanning microscope (Model Fluoview 500).

Transient transfection assay

Plasmids (10 μg) were transfected into P3HR1 or 293T cells by electroporation with a BioRad Gene-Pulser electroporator (Richmond, CA) (22). Luciferase assay was performed, using a luminometer (model LB593; Berthod, Bad Wildbad, Germany), according to a method described elsewhere (22). Each transfection experiment was performed three times and each sample in each experiment was prepared in duplicate.

RESULTS

Interaction among Sp1, p621 and Rta in yeast and *in vitro*

A yeast two-hybrid screen was performed with pR476, which encodes the N-terminal 476 amino acids of Rta, to screen a human testes cDNA library constructed in pACT2. Of the 24 clones that produce a protein interacting with Rta, one cDNA clone encoded p621 (23), which interacts with Sp1 and has a sequence identical to that in the C-terminal 621 amino acid region of a 1270 amino acid protein, MCAF1 (14). The interaction between Rta and p621 was further verified by transforming the plasmid that expresses p621 into the yeast strain Y190(pR476). A GST-pull-down assay was then performed with a bacterially expressed GST–p621 fusion protein. GST or GST–p621 bound to glutathione–Sepharose beads were added to the lysate that had been prepared from P3HR1 cells treated with TPA and sodium butyrate. After extensive washing, proteins bound to the beads were eluted and analyzed by IB with anti-Rta antibody. The results revealed that Rta in the lysate (Figure 2A, lane 1) was retained by GST–p621–glutathione–Sepharose (Figure 2A, lane 3) but not by GST–glutathione–Sepharose beads (Figure 2A, lane 2). IB with anti-Sp1 antibody also produced a similar result, indicating that Sp1 in the lysate (Figure 2B, lane 1) was retained by GST–p621–glutathione–Sepharose (Figure 2B, lane 3) but not by GST–glutathione–Sepharose beads (Figure 2B, lane 2). In parallel experiments, MCAF1 in the P3HR1 lysate (Figure 2C, lane 1) was retained by GST–Rta- and GST–Sp1- (Figure 2C, lanes 3 and 4) but not by GST–glutathione–Sepharose beads (Figure 2C, lane 2). Bacterially expressed His-tagged Sp1, GST–p621 and His-tagged Rta were mixed to elucidate whether Sp1 and Rta simultaneously bind to p621. In a reaction mixture that contained all three proteins, Rta was immunoprecipitated by anti-Sp1 antibody (Figure 2D, lane 5). However, Rta was not immunoprecipitated by anti-Sp1 antibody when GST–p621 was not added to the binding mixture (Figure 2D, lane 4). In negative controls, anti-HA antibody, which was used as a nonspecific antibody, did not immunoprecipitate Rta even when GST–621 was present in the reaction mixture (Figure 2D, lanes 2 and 3). Rta did not inhibit the association of Sp1 with MCAF1, even the addition of 60 μg of Rta in the binding reaction did not influence the binding of Sp1 to MCAF1 (Figure 2E).

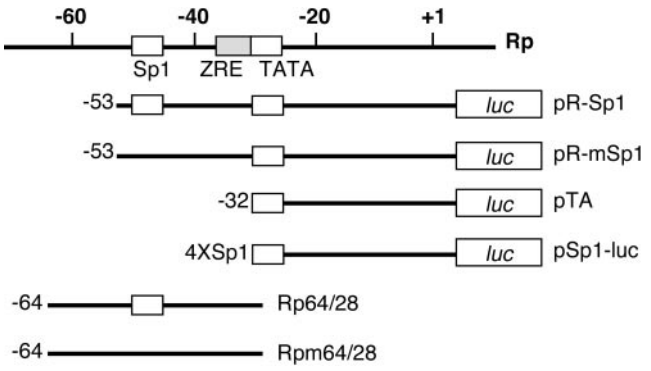

Figure 1. Reporter plasmids and the DNA fragments used in this study. Numbers represent the nucleotide positions in relation to the +1 site of BRLF1. A Zif268 site, located at −45, is not shown. A ZRE located close to the TATA box of Rp was mutated. Rp, the BRLF1 promoter; Sp1, Sp1-binding sequence; ZRE, Zta-response element; TATA, TATA box; 4XSp1: four Sp1 sites in the *p21* promoter.

Coimmunoprecipitation of Sp1, MCAF1 and Rta

293T cells were cotransfected with pCMV-R and pcDNA-MCAF1. A lysate was subsequently prepared and proteins

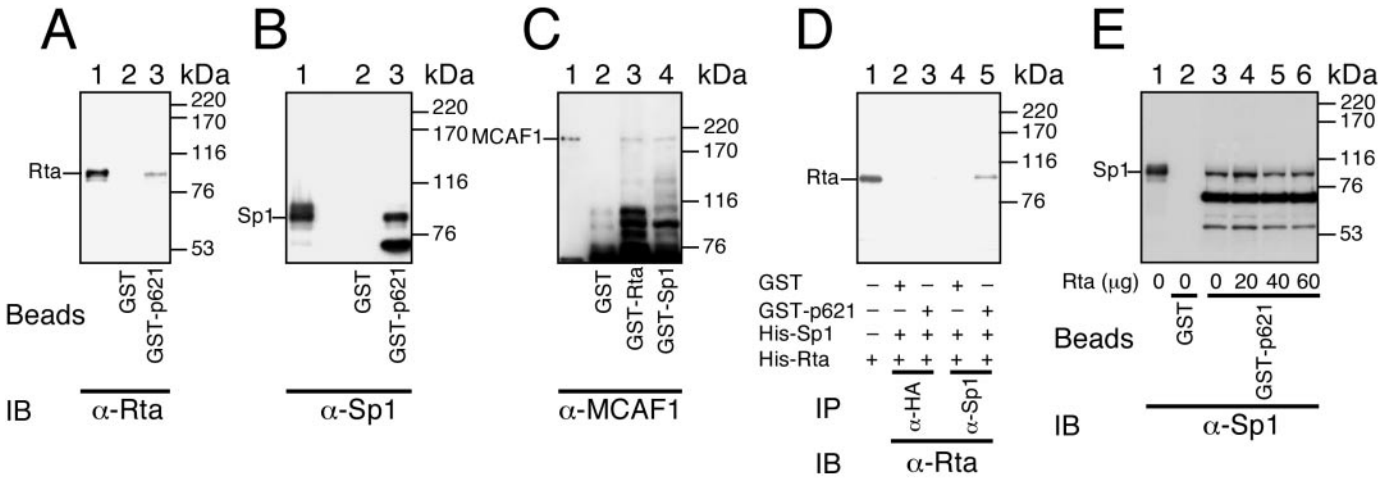

Figure 2. Interaction among Sp1, p621 and Rta *in vitro*. GST–p621 was added to the lysate prepared from P3HR1 cells treated with TPA and sodium butyrate. Proteins bound to GST–p621 were pulled down with glutathione–Sepharose beads (Beads) and analyzed by IB using anti-Rta (**A**) and anti-Sp1 antibody (**B**). Parallel experiments involving GST–Rta (lane 3) and GST–Sp1 (lane 4) were also conducted and IB was performed with anti-MCAF1 antibody (**C**). Meanwhile, anti-HA antibody (lanes 2 and 3) and anti-Sp1 antibody (lanes 4 and 5) were added to mixtures containing Sp1, GST–p621 and Rta to determine whether the interaction between Sp1 and Rta requires p621 (**D**). Various amounts of Rta were added to binding mixtures that contained Sp1 and GST–p621. Proteins were pulled down using GST–p621–glutathione–Sepharose beads and analyzed by IB with anti-Sp1 antibody (**E**). In each part, lane 1 was loaded with cell lysate; in lane 2, proteins were eluted from GST–glutathione–Sepharose beads.

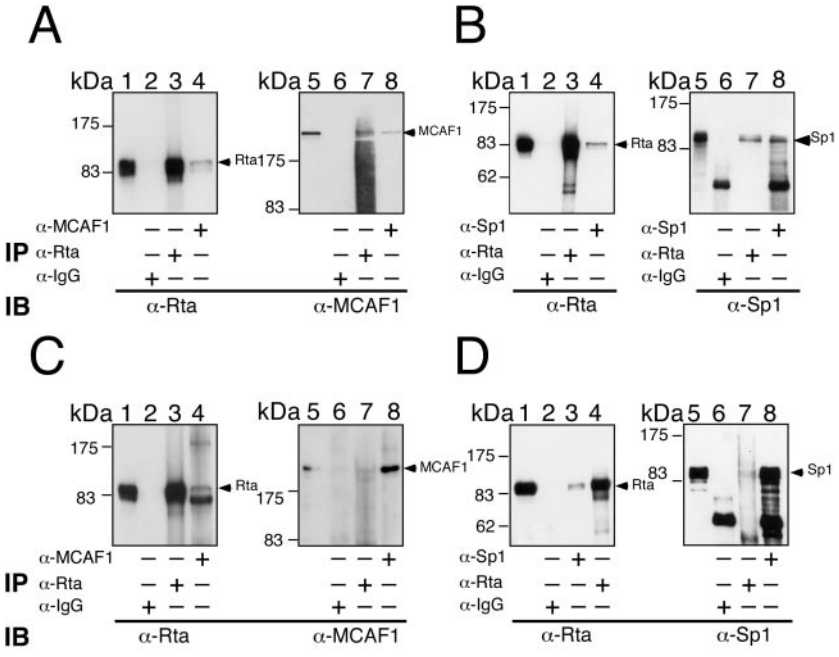

Figure 3. Coimmunoprecipitation of Sp1, MCAF1 and Rta. Anti-Rta and anti-MCAF1 antibody were added to the lysates prepared from 293T cells transfected with pCMV-R and pcDNA-MCAF1 (**A** and **B**) or from P3HR1 cells that were treated with TPA and sodium butyrate (**C** and **D**). Proteins in the lysate immunoprecipitated by the anti-Rta antibody, anti-MCAF1 antibody or anti-Sp1 antibody were analyzed by IB with anti-Rta antibody (A–D, lanes 1–4), anti-MCAF1 antibody (A and C, lanes 5–8) and anti-Sp1 antibody (B and D, lanes 5–8). Reactions involving anti-IgG antibody were used as negative controls.

in the lysate were immunoprecipitated with anti-Rta and anti-MCAF1 antibody. IB revealed that Rta was immunoprecipitated by anti-Rta antibody (Figure 3A, lane 3) and coimmunoprecipitated with MCAF1 by anti-MCAF1 antibody (Figure 3A, lane 4); MCAF1 was also immunoprecipitated by anti-MCAF1 antibody (Figure 3A, lane 8) and coimmunoprecipitated by anti-Rta antibody (Figure 3A, lane 7). Immunoblot analysis also showed that Rta was immunoprecipitated by anti-Rta antibody (Figure 3B, lane 3) and coimmunoprecipitated by anti-Sp1 antibody (Figure 3B, lane 4). In parallel, Sp1 was immunoprecipitated by anti-Sp1 antibody (Figure 3B, lane 8) and coimmunoprecipitated by anti-Rta antibody (Figure 3B, lane 7). A similar study was performed with the lysate from P3HR1 cells that were treated with TPA and sodium butyrate (Figure 3, panels C and D). Immunoblot analysis revealed that Rta was coimmunoprecipitated by

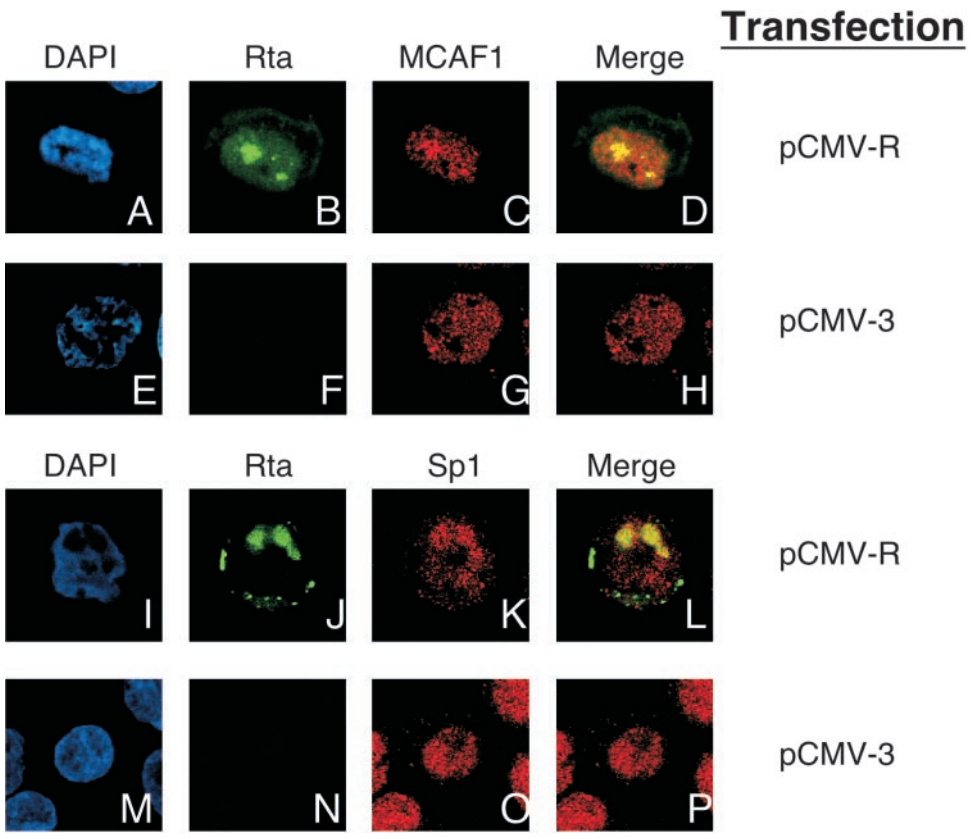

Figure 4. Indirect immunofluorescence analysis. P3HR1 cells were transfected with pCMV-R (**A–D**, **I–L**) or an empty vector (**E–H**, **M–P**). Cells were incubated with monoclonal anti-Rta antibody (B, F, J and N), rabbit anti-MCAF1 polyclonal antibody (C and G) and rabbit anti-Sp1 polyclonal antibody (K and O). DAPI staining revealed the positions of the nucleus. Finally, cells were examined using a confocal laser-scanning microscope. D, H, L and P are merged images.

anti-MCAF1 antibody (Figure 3C lane 4) and vice versa (Figure 3C, lane 7). Additionally, Rta was coimmunoprecipitated by anti-Sp1 antibody (Figure 3D, lane 3) and vice versa (Figure 3D, lane 7).

Subcellular localization of Rta, MCAF1 and Sp1

Immunofluorescence analysis of P3HR1 cells was performed with confocal microscopy to locate Sp1, MCAF1 and Rta in the cell. After transfection with pCMV-R, Rta was detected in the nucleus and in the cytoplasm (Figure 4B and J). However, MCAF1 and Sp1 were detected only in the nucleus (Figure 4C, G, K and O). Merged pictures revealed that MCAF1 (Figure 4D) and Sp1 (Figure 4L) were colocalized with Rta in the nucleus. Colocalization was not observed, however, in the cells that had been transfected with an empty vector (Figure 4H and P).

Binding of the Sp1–MCAF1–Rta complex to the Sp1 site

A double-stranded 37 bp biotin-labeled Sp1 probe, Rp64/28 (Figure 1), which contains the sequence in the region between −64 and −28 of Rp, was added to a lysate prepared from P3HR1 cells treated with TPA and sodium butyrate. Immunoblot analysis revealed that Sp1, MCAF1 and Rta were bound to the probe (Figure 5), but these proteins did not bind to a mutant probe, Rpm64/28 (Figure 5), whose sequence is identical to that of Rp64/28, except for a mutated Sp1-binding sequence (Figure 1).

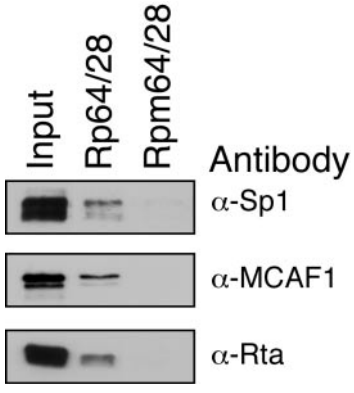

Figure 5. Binding of the Sp1, MCAF1 and Rta to an Sp1-binding site on Rp. A biotin-labeled double-stranded Sp1 probe (Rp64/28) that contains the sequence from −64 and −28 of Rp was added to a lysate prepared from P3HR1 cells treated with TPA and sodium butyrate. A probe with an identical sequence, except for a mutated Sp1-binding sequence (Rpm64/28), was used as a negative control. Proteins bound to the probes were captured with streptavidin magnetic beads, extracted and detected by IB with anti-Sp1, anti-MCAF1 and anti-Rta antibodies.

Binding of the Sp1–MCAF1–Rta complex to the Sp1 sites *in vivo*

CHIP analysis was performed to demonstrate the binding of Sp1, MCAF1 and Rta, to the Sp1 site on pR-Sp1 (Figure 6). Meanwhile, a parallel experiment was conducted with

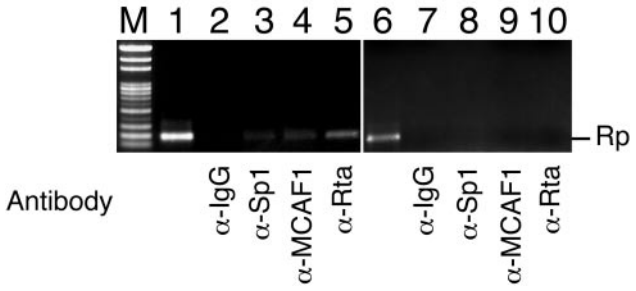

Figure 6. CHIP analysis of the binding of Sp1, MCAF1 and Rta to an Sp1 site on Rp. P3HR1 cells were cotransfected with pCMV-R, pcDNA-MCAF1 and pR-Sp1 (lanes 1–5). Meanwhile, in separate experiments, pR-mSp1 (lanes 6–10) rather than pR-Sp1, was cotransfected. Binding of Sp1, MCAF1 and Rta to the Sp1 site on pR-Sp1 was studied by CHIP analysis using anti-IgG antibody (lanes 2 and 7), anti-Sp1 antibody (lanes 3 and 8), anti-MCAF1 antibody (lanes 4 and 9) and anti-Rta antibody (lanes 5 and 10). Meanwhile, in positive controls, DNA in the cell lysate was used as a template for PCR to amplify the Rp sequence (lanes 1 and 6); Rp, an Rp fragment amplified from pR-Sp1; M, 100 bp ladder.

pR-mSp1 whose sequence is identical to that of pR-Sp1, except in that the Sp1 sequence in Rp was mutated (Figure 1). P3HR1 cells were cotransfected with pcDNA-MCAF1, pCMV-R and pR-Sp1. After the cells were treated with formaldehyde to cross-link DNA and proteins, DNA was isolated, sonicated and immunoprecipitated with anti-Sp1, anti-MCAF1 and anti-Rta antibody. PCR amplification of immunoprecipitated DNA with primers that are complementary to the vector sequences that flank the Rp segment in pR-Sp1 demonstrated that these three antibodies immunoprecipitated the Rp segment (Figure 6, lanes 3–5). The DNA fragment was not amplified by PCR if anti-IgG antibody was utilized to immunoprecipitate DNA (Figure 6, lane 2). The Rp sequence was undetected when pR-mSp1 was cotransfected into the cells (Figure 6, lanes 7–10). In this work, the ZRE sequence in Rp in both pR-Sp1 and pR-mSp1 was purposely mutated (Figure 1). Mutating this ZRE sequence was necessary to detect the binding of Sp1, MCAF1 and Rta to the Sp1 site by CHIP analysis, because according to our unpublished results, MCAF1 and Rta interact with Zta and form a complex on ZRE.

Mapping the interaction domains in MCAF1 and Rta

Plasmids that expressed Rta and derivatives of MCAF1 fused to GFP (Figure 7A) were cotransfected into 293T cells. Immunoblot analysis revealed that a GFP fusion protein, which contained the region between amino acids 562 and 981 of MCAF1 (GFP-MCAF1-D1), was coimmunoprecipitated with Rta by anti-Rta antibody (Figure 7B, lane 7). However, GFP alone (Figure 7B, lane 5) and a GFP fusion protein that contained the region from amino acid 1 to 561 (Figure 7B, lane 6) or from 982 to 1270 (Figure 7B, lane 8) in MCAF1 were not coimmunoprecipitated by anti-Rta antibody. Thus, the region between amino acid 562 and 981 in MCAF1 interacts with Rta. Additionally, GST–p621, which contains the region between amino acid 650 and 1270 in MCAF1, binds to Rta (Figure 2), indicating that the region between amino acid 650 and 981 in MCAF1 interacts with Rta. We performed a similar study using Flag-tagged Rta, HA-tagged Rta and GFP–Rta fusion proteins (Figure 7C) to identify the regions in Rta that interact with MCAF1. Coimmunoprecipitation with anti-MCAF1 antibody revealed that full-length Rta (Figure 7D, lanes 2, 8 and 11), the N-terminal 290 amino acid region (HA-RN290) (Figure 7D, lane 9), Rta without the N-terminal 190 amino acids (Figure 7D, lane 3) and Rta without the N-terminal 254 amino acids (Figure 7D, lane 4), interact with MCAF1. Further deleting Rta into the C-terminal region prevented the proteins from being coimmunoprecipitated by anti-MCAF1 antibody (Figure 7D, lanes 5 and 6), indicating that the region between amino acids 255 and 290 in Rta interacts with MCAF1. Furthermore, immunoblot analysis revealed that a faint band, which migrated to a position slightly higher than that of the 62 kDa marker, was nonspecifically detected by anti-Rta antibody (Figure 7D, lanes 5 and 6). To confirm that the 255–290 amino acid region indeed interacted with MCAF1, a plasmid, which expresses a protein that contains a GFP sequence in the N-terminal region fused with the amino acid 255–290 region of Rta (Figure 7C, lane 12), was transfected into cells. IB with anti-GFP antibody revealed that anti-MCAF1 antibody coimmunoprecipitated the fusion protein with MCAF1 (Figure 7D, lane 12) but not GFP (Figure 7D, lane 10).

Involvement of MCAF1 in Rta autoregulation

A reporter plasmid, pRp containing a full-length Rp, from −963 to +5 in BRLF1, was transfected into P3HR1 cells. The reporter plasmid exhibited a background level of luciferase activity (Figure 8A). Meanwhile, cotransfecting 1 μg of pcDNA-MCAF1 had little effect on the transcription of *luc* from the reporter plasmid (Figure 8A). However, as expected, transfecting pCMV-R activated the transcription of *luc* from the reporter plasmid (Figure 8A). Furthermore, transfecting pcDNA-MCAF1 enhanced the transcription of the luciferase gene activated by Rta in a dose-dependent manner (Figure 8A). The activity increased to ∼130, 180 and 300% of the original value when 1, 3 and 5 μg of pcDNA-MCAF1 were cotransfected, respectively (Figure 8A). Similar results were obtained when another reporter plasmid, pR-Sp1, which contained the region between −53 and +8 in Rp (Figure 1), was transfected (Figure 8B). Luciferase activity exhibited by the reporter plasmid increased to ∼200% when pCMV-R and 5 μg of pcDNA-MCAF1 were cotransfected into the cells (Figure 8B). However, although transfecting pCMV-R activated an RRE-reporter plasmid, pRRE (Figure 8C), cotransfecting pcDNA-MCAF1 did not further increase the transcription activity in a dose-dependent manner (Figure 8C), indicating that MCAF1 cannot increase the activity of Rta on RRE. Rta also activated an RRE promoter (pRRE) to a level of ∼1.5 times that of an Sp1 promoter (pR-Sp1) (Figure 8D), indicating that Rta activates an Sp1 promoter almost as efficiently as it does an RRE promoter.

Activation of Rta expression and the EBV lytic cycle by MCAF1

To examine how MCAF1 influences the expression of Rta, 20 μg of pcDNA3.1 or pcDNA-MCAF1 was transfected into 5×10^6 of P3HR1 cells. Immunoblot analysis with anti-Rta antibody did not detect the expression of Rta at 48 h after the transfection (Figure 9A, lanes 2 and 3). Cotransfecting the

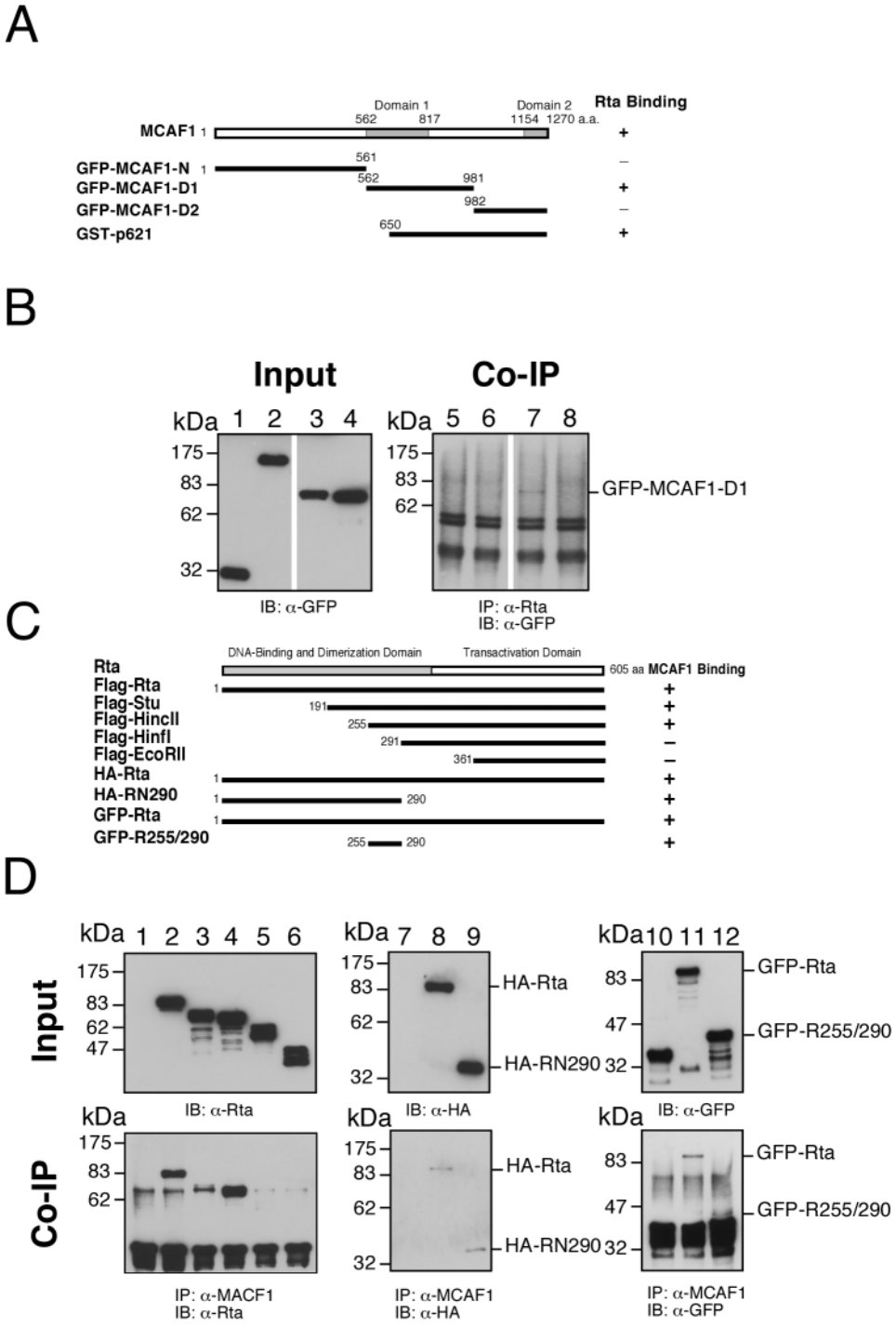

Figure 7. Mapping the interaction domains on MCAF1 and Rta. Plasmid that expresses GFP-MCAF1 and its deletion derivatives (**A**) was cotransfected with pCMV-R into 293T cells. (**B**) Proteins in the lysates prepared from cells that express GFP (lanes 1 and 5), GFP-MCAF-N (lanes 2 and 6), GFP-MCAF1-D1 (lanes 3 and 7) and GFP-MCAF1-D2 (lanes 4 and 8) were analyzed by IB using anti-GFP antibody (Input) and coimmunoprecipitation (Co-IP) with anti-Rta antibody and studied by IB with anti-GFP antibody. (**C**) Plasmids that express Flag-Rta, HA-Rta, GFP-Rta or Rta deletion mutants were cotransfected with pcDNA-MCAF1 into 293T cells. (**D**) Cell lysates that contain Flag-Rta (lane 2), Flag-Stu (lane 3), Flag-HincII (lane 4), Flag-HinfI (lane 5), Flag-EcoRII (lane 6), HA-Rta (lane 8), HA-RN290 (lane 9), GFP-Rta (lane 11) and GFP-R255/290 (lane 12) were immunoprecipitated with anti-MCAF1 antibody and analyzed by IB with anti-Rta antibody (lanes 1–6), anti-HA antibody (lanes 7–9) and anti-GFP antibody (lanes 10–12). Lanes 1, 7 and 10 were loaded with the proteins from the cells transfected with an empty vector.

cells with 0.5 μg of pCMV-R and 1 or 5 μg of pcDNA-MCAF1 also did not result in the synthesis of Rta at a level detectable by IB (Figure 9A, lane 4 and 5). However, Rta was detected if the cells were cotransfected with 0.5 μg of pCMV-R and >10 μg of pcDNA-MCAF1 (Figure 9A, lanes 6 and 7). A similar study was also performed in 293 cells that were infected with maxi-EBV. Because EBV expresses Rta constitutively in epithelial cells (Figure 9B, lane 2), a low level of

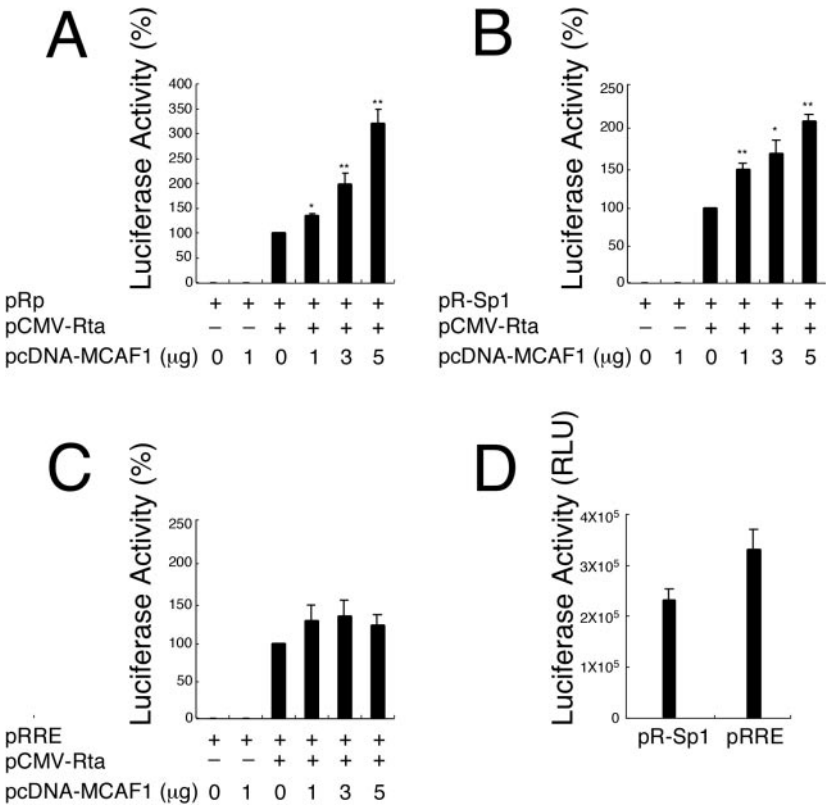

Figure 8. Involvement of MCAF1 in Rp autoregulation. Reporter plasmids, pRp (**A**), pR-Spl (**B**) and pRRE (**C**) were cotransfected with pCMV-R and pcDNA-MCAF1 into P3HR1 cells. (**D**) Displays the luciferase activity expressed by pRRE and pR-Sp1 after cotransfecting pCMV-R. Luciferase activity was determined at 24 h after transfection. Each transfection experiment was performed at least three times, and each sample in the experiments was prepared in duplicate. The values obtained from the experiment were analyzed statistically by the least square means method. *, $P < 0.05$; **, $P < 0.01$.

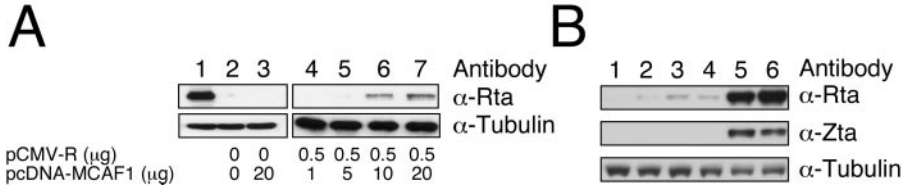

Figure 9. Activation of the expression of EBV immediate-early genes by MCAF1. (**A**) P3HR1 cells (5×10^6) were treated with TPA and sodium butyrate to activate the expression of Rta (lane 1). Cells untreated with TPA and sodium butyrate were transfected with 20 μg of pcDNA3.1 (lanes 2) and pcDNA-MCAF1 (lanes 3). Cotransfection was also performed by transfecting 0.5 μg of pCMV-R and 1–20 μg of pcDNA-MCAF1 (lanes 4–7). Lysates were prepared at 48 h after transfection. Immunoblot analysis was performed with anti-Rta and anti-α-tubulin antibodies. (**B**) 293 cells (5×10^6) that were infected by maxi-EBV (lane 2) were transfected with 20 μg of pcDNA3.1 (lanes 3 and 4) and pcDNA-MCAF1 (lanes 5 and 6). Proteins were prepared from the cells at 24 h (lanes 3 and 5) and 48 h (lanes 4 and 6) after transfection. Lane 1 was loaded with the lysate from 293 cells. Immunoblot analysis was performed using anti-Rta, anti-Zta and anti-α-tubulin antibodies.

Rta expression was detected in the cells that were transfected with an empty vector (Figure 9B, lanes 3 and 4). However, the expression of Rta was markedly higher 1 and 2 days after the cells had been transfected with 20 μg of pcDNA-MCAF1 (Figure 9B, lanes 5 and 6). Meanwhile, the transfection also led to the expression of another EBV immediate-early protein, Zta (Figure 9B, lanes 5 and 6). Meanwhile, pcDNA-MCAF1, pCMV-R and pRRE were also transfected into 293T cells. Transfecting pcDNA-MCAF1 did not increase the capacity of pCMV-R to activate pRRE (data not shown), suggesting that MCAF1 did not activate the expression of Rta from the CMV promoter (data not shown). This finding shows that the increased Rta expression observed in this study is caused by the autoregulation of BRLF1 transcription rather than the activation of the CMV promoter by MCAF1.

Activation of an RRE promoter by Rta is independent of MCAF1 and Sp1

Transfecting pcDNA-MCAF1 had little effect on the activation of an RRE-reporter plasmid by Rta, so we tested whether the Rta–MCAF1–Sp1 complex could bind to RRE. A DAPA experiment with a biotin-labeled RRE probe showed that streptavidin beads could capture Rta (Figure 10A, lane 2). The beads did not capture Rta when a mutant probe (mRRE), in which the RRE sequence was mutated, was utilized

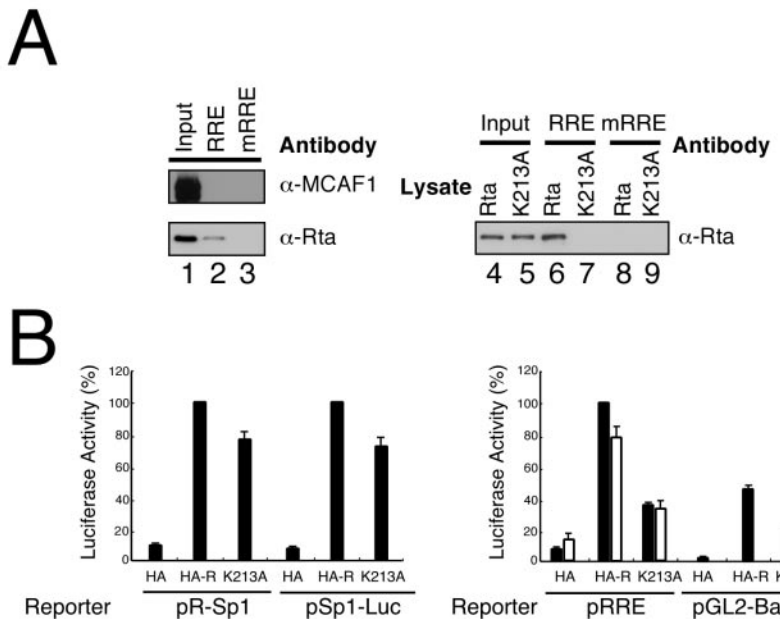

Figure 10. Transcriptional activation of Rp, an Sp1 promoter and an RRE promoter by Rta. (**A**) 293T cells were transfected with pCMV-R and the lysate was mixed with biotin-labeled probes, RRE and mRRE. Proteins bound to the probe were captured using streptavidin magnetic beads. Proteins extracted from the beads were analyzed by IB with anti-Rta and anti-MCAF1 antibody. A similar study was performed with 293T cells transfected with plasmids that expressed HA-Rta (HA-R) and Rta(K213A). (**B**) An empty vector (HA) and plasmids that expressed HA-Rta (HA-R) and Rta(K213A) (K213A) were cotransfected with reporter plasmids pR-Spl, pSp1-luc, pRRE and pGL2-Basic (closed rectangle). Meanwhile, pcDNA-MCAF1 was also cotransfected to examine whether MCAF1 influenced the transactivation activity of pRRE (open rectangle). The nonspecific activation of the transcription of the luciferase gene in pGL2-Basic by Rta and Rta(K213A) was probably attributed to a GGGAGG sequence, which resembles an Sp1-binding site, near the cloning site of the plasmid. Luciferase activity was determined at 24 h after transfection. Each transfection experiment was performed at least three times, and each sample in the experiments was prepared in duplicate.

(Figure 10A, lane 3), indicating that Rta binds to RRE. However, although Rta binds to MCAF1 (Figure 2), MCAF1 was not retained by the RRE probe (Figure 10A, lane 2). Additionally, by testing a panel of mutations in the DNA-binding domain in Rta, we found that the K213 residue in Rta was critical to its binding to RRE; a lysine to alanine substitution of this residue (K213A) eliminated the binding capacity of Rta to RRE (Figure 10A, lane 7). This site was outside the MCAF1-binding domain, so this mutant protein could be used to establish that activating an Sp1 promoter was independent of DNA binding. In a transient transfection study, both Rta and Rta(K213A) activated pR-Sp1 and pSp1-luc in 293T cells (Figure 10B). However, Rta(K213A) activated an RRE-reporter plasmid at a level ~60% lower than that activated by Rta (Figure 10B). Furthermore, cotransfecting pcDNA-MCAF1 did not affect the ability of HA, HA-R and Rta(K213A) to activate the pRRE promoter (Figure 10B).

Activation of Sp1-mediated transcription by Rta

We also tested whether Rta activates cellular promoters that are activated by Sp1, including the promoters of *p16*, *p21* and *SNRPN*, in an epithelial cell line, 293T. Transfecting pTA (Figure 11) alone, which contained a luciferase gene transcribed from a promoter with only a TATA sequence, expressed a low background value of luciferase activity. However, the activity was activated 6.9-fold by cotransfection of pCMV-R. In parallel experiments, the *p16* promoter was activated by pCMV-R 14.8-fold; the *p21* promoter 8.2-fold and the SNRPN promoter 11.1-fold (Figure 11).

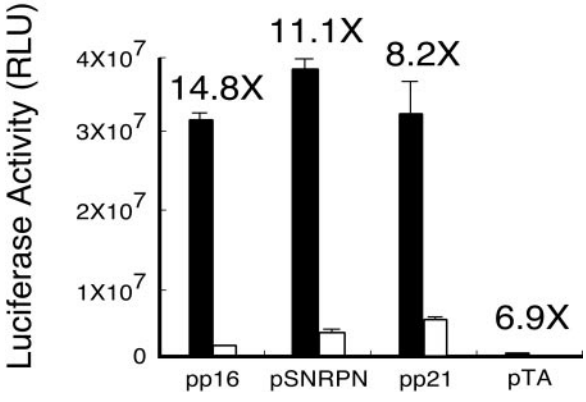

Figure 11. Activating the promoters of *p16*, *p21* and *SNRPN* by Rta. 293T cells were cotransfected with a reporter plasmid and pCMV-R (closed rectangle) or an empty vector (open rectangle). The reporter plasmids used herein contained a luciferase gene transcribed from the promoter of *p16* (pp16), *SNRPN* (pSNRPN) and *p21* (pp21). Plasmid pTA was a reporter plasmid that contained the TATA sequence of BRLF1. Activation of pTA by Rta was probably attributed to an Sp1-binding sequence, GGGAGG, in the multiple cloning site of the cloning vector, pGL2-Basic, that was used to construct pTA. Luciferase activity was determined at 24 h after transfection. Each transfection experiment was performed at least three times, and each sample in the experiment was prepared in duplicate.

DISCUSSION

Rta is an EBV-encoded transcription factor, which is crucial in regulating the expression of EBV lytic gene. However, as well as regulating the EBV genes, Rta binds to Rb to release E2F1

from Rb to affect the cell cycle progression (24). Rta also modulates the phosphorylation of p38 and c-Jun-N-terminal kinases, ultimately affecting gene expression (11), indicating that Rta affects not only the expression of EBV but also the functions of cellular genes. Expression of Rta may be especially important to epithelial cells since Rta is constitutively expressed by EBV in these cells (25). Ragoczy *et al*. (10) established already that Rta activates the transcription of BRLF1 through two Sp1 sites in Rp. Sp1 is a crucial transcription factor that regulates many cellular genes, including those involved in housekeeping functions (26), cell differentiation (27), methylation (28) and oncogenesis (29). Hence, if Rta influences Sp1-mediated transcription, then the expression of Rta may profoundly affect the expression of cellular genes. Accordingly, the ultimate goals of this work are to explore whether and how Rta affects the transcription of the genes that are activated by Sp1.

A yeast two-hybrid screen was performed and revealed that an Sp1-binding protein, MCAF1, interacts with Rta. The interaction was then verified *in vitro* with bacterially expressed proteins (Figure 2) and *in vivo* by immunoprecipitation (Figure 3). Additionally, CHIP analysis revealed the formation of an Sp1–MCAF1–Rta complex on an Sp1 site in cells (Figure 6). This work also verifies the results of Ragoczy *et al*. (10), who noted that binding of Rta to Sp1 on an Sp1 probe could not be detected by electrophoretic mobility shift assay, perhaps because the protein complex is large. The putative protein–DNA complex might not enter the gel or might have dissociated during electrophoresis. We therefore used DAPA to search for binding of the protein complex to a biotin-labeled Sp1 probe (Figure 5). Additionally, a CHIP assay was performed to verify the binding of these proteins to the Sp1 site in Rp *in vivo*. This investigation was originally conducted in P3HR1 cells following EBV lytic induction. The results indicated that MCAF1, Rta and Sp1 bind to Rp (data not shown). However, our subsequent investigations showed that MCAF1 and Rta also form a complex on ZRE (L.-K. Chang and S.-T. Liu, unpublished data). Only 7 nt separate the Sp1 site and ZRE in Rp, making it extremely difficult to distinguish the binding of MCAF1 to the Sp1 site or ZRE by CHIP analysis. Therefore, CHIP assay was conducted by immunoprecipitating the proteins at the Sp1 site in Rp in pR-Sp1 (Figure 4), in which the ZRE was mutated (Figure 1).

We mapped the Rta-binding domain on MCAF1 in the region between amino acids 650 and 981 (Figure 7B). An earlier work revealed that the domains in MCAF1 that interact with Sp1 are located in D1 and D2 (Figure 7A) (14). We determined that excess Rta does not affect the binding of MCAF1 to Sp1 (Figure 2E), indicating that although the Sp1- and Rta-binding regions overlap on MCAF1, Rta does not interfere with the binding of Sp1 to MCAF1. A lysine to alanine substitution on the K213 residue eliminated the capacity of Rta to bind to RRE (Figure 10A) and reduced the capacity of Rta to activate an RRE-reporter plasmid (Figure 10B). The fact that K213 is located outside of the MCAF1-binding domain and the K213A mutation transactivates pR-Sp1 and pSp1-luc (Figure 10B) verifies that the transactivation of Rp by Rta is independent of RRE binding.

Rta contains an RRE-binding domain located between amino acids 1 and 320 (Figure 7), so the Rta–MCAF1–Sp1 complex might also bind to an RRE. However, our DAPA study indicates that this is not the case (Figure 10A). Additionally, transfecting pcDNA-MCAF1 has little effect on the activation of an RRE-reporter plasmid by Rta (Figure 8C), further indicating that MCAF1 does not participate in that activation. The lack of activation may be attributed to the fact that the RRE-binding domain and the MCAF1-binding domain overlap on Rta (Figure 7); the binding of Rta to RRE may prevent the binding of Rta to MCAF1.

We find that Rta activates Rp through Sp1, suggesting that the expression of Rta may also influence the transcription of the cellular genes that are regulated by Sp1. We chose three important regulating genes, *p16*, *p21* and *SNRPN*, that contain Sp1 sites to study the capacity of Rta to activate Sp1-mediated transcription. *p16* is a tumor suppressor gene that contains aberrantly hypermethylated CpG islands in its promoter in cancer cells (30). *p21* is known to regulate cell cycle progression (31); *SNRPN* is an imprinted gene, typically unmethylated and heavily methylated on the active paternal and inactive maternal alleles, respectively (32). pCMV-R activates the promoters of these three cellular genes 10- to 15-fold in transfection experiments (Figure 11). A reporter plasmid that contains four of the six Sp1 sites in the *p21* promoter, pSp1-luc (Figure 1), is also strongly activated by Rta (Figure 10B). The BNLF1 promoters contain several Sp1 sites (33,34); the transcription activation of this gene by Rta probably involves the binding of the Sp1–MCAF1–Rta complex to these sites. Furthermore, Rta activates the transcription of *c-myc* proto-oncogene (35). The promoter of this gene lacks an RRE but contains several Sp1 sites near the TATA sequence (36). These findings indicate the crucial role of Rta in regulating Sp1-mediated transcription. Moreover, EBV lytic promoters are typically hypermethylated during viral latency (37,38). Additionally, MCAF1 binds to MBD1 on CpG sequences to repress gene transcription, so this laboratory is currently studying how the interaction between Rta and MCAF1 affects CpG methylation during EBV latent-lytic switching.

One of the important questions addressed herein concerns the effect of the interactions among Sp1, MCAF1 and Rta on the activation of the EBV lytic cycle. As is generally known, BRLF1 is not transcribed during EBV latency in B cells but is transcribed in epithelial cells (25). Therefore, if MCAF1 participates in BRLF1 autoregulation, then transfecting pcDNA-MCAF1 to epithelial cells that are infected by EBV may promote expression of Rta from the virus. This, indeed, is the case; the expression of Rta from maxi-EBV in 293 cells was enhanced after the cells were transfected with pcDNA-MCAF1 (Figure 9B, lanes 5 and 6). Additionally, the transfection also led to the expression of Zta (Figure 9A), perhaps through the activation of BZLF1 transcription by Rta (11). These observations indicate that MCAF1 participates in EBV lytic activation in 293 cells. However, MCAF1 did not activate Rta expression in P3HR1 cells (Figure 9A). The expression of Rta was only evident when a little pCMV-R was cotransfected with pcDNA-MCAF1 (Figure 9A, lanes 6 and 7), suggesting that the expression of Rta is a prerequisite for EBV lytic activation by MCAF1. Moreover, an earlier work demonstrated that MCAF1 is abundant in the cell, which may explain why transfecting >10 μg of pcDNA-MCAF1 is required to detect the

enhancement of the expression of EBV immediate-early genes (Figure 9).

This work demonstrates that Rta can activate gene transcription by two mechanisms, by which Rta activates not only EBV but also cellular genes, which may ultimately affect cell cycle progression and oncogenesis. EBV in epithelial cells is generally known to express Rta constitutively (25). Therefore, activation of cellular genes and especially an EBV oncogene, BNLF1, may be crucial to the oncogenesis of the cells. However, Rta expression causes the expression of Zta and activates the EBV lytic cycle (11). Therefore, the cells die by the lytic production of EBV virions. Accordingly, the activation of host genes may only be possible if the lytic productive cycle is abortive. However, our work seems to indicate that although Rta is constitutively expressed from maxi-EBV in 293 cells, the extent of Rta expression may not sufficiently activate the EBV lytic cycle. Not only was Rta expressed at a low level, but also the expression of Zta by the cells was undetected by immunoblot analysis (Figure 9B, lane 2). This low level of Rta expression did not cause the expression of EA-D (data not shown). Additionally, maxi-EBV in 293 cells released only few virions except when the cells were treated with TPA and sodium butyrate, or transfected with a plasmid that expressed Zta (Y.-F. Chiu and S.-T. Liu, unpublished data). These results suggest that a low level of Rta expression in 293 cells may not activate the EBV lytic cycle. This continuously low level of Rta expression may influence the physiology of cells and importantly determine the pathogenicity of the virus.

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Conflict of interest statement. None declared.

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