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KSHV RTA induces a transcriptional repressor, HEY1 that represses *rta* promoter

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is a γ-herpesvirus that is frequently associated with the formation of tumors like Kaposi's sarcoma and primary effusion lymphomas. KSHV RTA is required for lytic replication/reactivation in KSHV-infected cells. We developed an inducible RTA expression cell line to search for RTA-inducible genes. Using DNA chip technology, we showed that *hey1*, a basic helix-loop-helix-containing transcription factor, increased three- to fourfold in this line. In a luciferase reporter system, RTA activated the *hey1* promoter in several cell lines; conversely, HEY1 repressed the *rta* promoter. Chromatin immunoprecipitation assays indicated that HEY did not bind the *rta* promoter directly, although it may repress *rta* expression as part of a complex. Thus, *hey1* may help to downregulate RTA after its burst of expression during lytic replication/reactivation. Similar repression mechanisms may downregulate immediate-early gene expression in other herpesviruses, although different transcription factors are probably involved.

Keywords: Kaposi's sarcoma-associated herpesvirus; RTA; HEY1; Transcriptional repressor; Lytic replication; Reactivation

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV8), is the most recently discovered human herpesvirus [1]. KSHV is associated etiologically with Kaposi's sarcoma (KS), a vascular spindle cell tumor frequently occurring in patients with AIDS [2], and it is associated with several other malignancies, including primary effusion lymphomas (PEL) [3] and a subtype of multicentric Castleman's disease (MCD) [4].

Upon infection, the expression of herpes genes is tightly regulated, usually at the level of the first cascade of gene expression [5]. Generally, immediate-early (IE) genes, most of which are regulatory factors, upregulate the early (E) genes' expression, and the IE genes' expression then declines. The late (L) genes are expressed last, and the mature virions subsequently form and are released. The IE genes do not continue to be transcribed, although some protein expression remains throughout this process. Thus, IE gene expression is regulated, and some repression mechanism could be involved in the regulation.

The viral gene *ORF50* (*rta*) plays a key role in inducing lytic replication in KSHV-infected cells. Its product, the replication and transcription activator (RTA) is necessary and sufficient to trigger the lytic reactivation of KSHV in the cells [6]. RTA activates a variety of viral and cellular promoters through several different mechanisms [7]. To activate the transcription of the viral lytic genes ORF57 and K8, RTA directly binds to their promoters at two palindromic sequences, and deletion of these sequences prevents the activation by RTA [8]. RTA uses different response elements to activate the transcription of PAN and K12 [9,10]. Other reports show that RTA transactivates *cis* elements without directly binding to the K9 (vIRF) response element (RE) [11], and it upregulates its own transcription by binding to a cellular factor [12]. Liang et al. reported that RTA acts as a functional homologue of

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the cellular Notch intracellular domain (NICD), which interacts with RBP-Jκ through co-repressor molecules [13].

To find RTA-inducible genes, we developed an ecdysone/glucocorticoid-inducible RTA cell line and used it with the DNA microarray to identify genes that are activated by RTA. We found that some cellular genes, chemokine receptor 4 (*cxcr4*), neurofilament light polypeptide (*nefl*), carbonic anhydrase II (*ca2*), myomesin 2 (*myom2*), and *hey1*, were induced by RTA. Here, we focus on *Hairy/E(spl)-related with YRPW (Hey1)*. HEY1 is a transcriptional repressor, a member of the basic helix-loop-helix (bHLH) protein family [14,15], and is upregulated by Notch ligand binding without de novo protein synthesis [16]. Using a luciferase reporter assay, we confirmed that RTA increased the transcription from the *hey1* promoter by a factor of about ten, and in contrast, HEY1 repressed *rta* promoter activity. Thus, HEY1 may be involved in the cessation of IE gene expression, which occurs in the early phase of infection.

Materials and methods

Cells. Human embryonic kidney cell lines, 293 and 293T, an African green monkey kidney cell line, COS7, and a mouse fibroblast cell line, NIH3T3, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS [Invitrogen, Carlsbad, CA]), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan). 293/ECR cells (Invitrogen, Carlsbad, CA) were transfected with the RTA expression vector, pIND-RTA (see below), and several 293pIND/RTA1 cell lines were isolated by selection with 0.8 μg/ml G418 (Nacalai Tesque). 293pIND/LZ cells were also established (see below) as a control. RTA expression was induced by the addition of 1 μM ponasterone A (Invitrogen). A KSHV-infected PEL cell line, BC3, was cultured in RPMI 1640 (Nissui) with 20% FBS and 100 U/ml penicillin G and 100 μg/ml streptomycin (Nacalai Tesque). To induce KSHV reactivation, the BC3 cells were treated with 25 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma–Aldrich, St. Louis, MO). All cell lines were maintained in a 5% CO_2 atmosphere at 37 °C.

Plasmids. The ORF50/RTA cDNA from pcDNA3.1MycHis-RTA [11] was inserted into the pIND vector (Invitrogen). The resulting construct contained the full 691 amino acids of ORF50/RTA with MycHis at the C-terminus and was named pIND/RTA. pIND/LZ contained a β-galactosidase gene under the same promoter. The reporter construct, pGL3Rp (−914), as described previously [12], was used in the reporter assay shown in Fig. 4. Other reporter constructs (pGL3Rp [−1543], pGL3Rp [−914], pGL3Rp [−395], pGL3Rp [−338], pGL3Rp [−311], pGL3Rp [−264], pGL3Rp [−259], pGL3Rp [−238], and pGL3Rp [−163]) were made, each containing a PCR-amplified DNA fragment. These reporter constructs included the transcription start site of RTA [17]. The putative HEY1 REs corresponded to the RTA-REs in the *rta* promoter, as described in our previous report [12]. The REs were inserted into the *Nhe*I site of pe1bTATA-Luc, and these inserted reporter plasmids (pe1b-RE) contained three copies of the RE in tandem. The starting and ending positions of the REs are shown in Table 2. The *hey1* promoter region, from −898 to +140, was amplified by PCR, with the following primers: HEY1-S, 5′-AAGCTCGAGCGTCCCCTGAGCCCACAGTTG-3′ and HEY1-AS, 5′-AGCTCGCTCCATGGTGGCTCCCTGG-3′. Genomic DNA (100 ng) extracted from BC3 cells was the template. The amplified fragment was digested with *Xho*I and *Nco*I, inserted into pGL3-Basic (Promega, Madison, WI), and named pHEY1. All fragments amplified by PCR were confirmed by sequencing with an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA). The HEY1 ORF was amplified with the primers HEY1NBAM30 (5′-CCCAGGGATCCAGCATGAAGCGAGCTCACC-3′) and HEY1 CCLA31 (5′-CTACATCGATTCTTTAAAAAGCTCCGATCTG-3′) and digested with *Bam*HI and *Cla*I. The digested fragment was inserted into pFLAGCMV6c (Sigma–Aldrich), named pFHEY1, and expressed FLAG-tagged HEY1. The murine HDAC1 expression vector, pGST-mHDAC1, which was inserted into pGEX-2TK, was kindly provided by C. Seiser [18].

Transfection. For transfection, we used the Superfect® transfection reagent (Qiagen, Germany), according to the manufacturer's protocol. For the reporter assay, the day before transfection, 10^5 cells/well of 293 cells were seeded onto a 12-well culture plate (Iwaki, Chiba, Japan). One microgram of each effector plasmid (either pFHEY1 or pCMVFLAG2 for the *rta* promoter assay and either pcDNA-RTA or pcDNA3.1-MycHis for the *hey1* promoter assay) and 0.1 μg of each reporter were introduced into the cells. Forty-eight hours post-transfection, the cells were harvested and the luciferase assay was performed.

Cell lysate was prepared in 50 μl of the reporter lysis buffer (Glo® Lysis buffer 1×, Promega). Ten microliters of lysate and 50 μl luciferase substrate buffer (Bright-Glo® Luciferase Assay System, Promega) were mixed in a measuring tube, and the luciferase activity was measured immediately with a luminometer (LUMAT® LB 9507:EG & Berthhold, Bag Wildbad, Germany). The β-galactosidase activity was measured colorimetrically with 0.5 mg/ml chlorophenol red-β-D-galactopyranoside (CPRG) in Z buffer (0.1 M sodium phosphate, pH 7.5; 10 mM KCl; 1 mM $MgSO_4$; 50 mM of 2-mercaptoethampl) using a microplate reader (Benchmark®, Bio-Rad Laboratories, Hercules, CA) at 570 nm.

DNA microarray. Synthetic polynucleotides (80-mers) representing 22,656 human genes (MicroDiagnostic, Tokyo, Japan) were arrayed with a custom-made arrayer. Poly(A)+ RNA was prepared from cells with the TRIzol reagent (Invitrogen) and the poly(A) Purist® Kit (Ambion, Austin, TX). Two micrograms of poly(A)+ RNA was labeled with Cyanine 5-dUTP or Cyanine 3-dUTP. Hybridization and subsequent washes of the arrays were performed with a Labeling and Hybridization Kit (MicroDiagnostic, Tokyo, Japan). Hybridization signals were measured with a GenePix 400A scanner (Axon Instruments, Union City, CA), and then processed into primary expression ratios (ratios of Cyanine 5-labeled to Cyanine 3-labeled samples) by the GenePix Pro software (Axon Instruments). A secondary expression ratio for each gene was calculated by averaging the primary expression ratio, obtained from an experiment with Cyanine 5-labeled 293pIND/RTA1 and Cyanine 3-labeled 293pIND/LZ samples, and the reciprocal of the primary expression ratio obtained from an experiment with Cyanine 3-labeled 293pIND/RTA1 and Cyanine 5-labeled 293pIND/LZ. The secondary expression ratios calculated from this pair of experiments were converted into log2 values as the final expression ratios.

Quantitative PCR. Total RNA was prepared with the SV® Total RNA Isolation System (Promega) according to the manufacturer's protocol. The cDNA was made from 5 μg total RNA using 1 μl (200 U) SuperScript™ RNase H^- Reverse Transcriptase and 1 μl random primers (200 μM) (Invitrogen). Ten percent of the synthesized cDNA reaction was subjected to reaction in a LightCycler® with FastStart DNA Master SYBER Green I (Roche Diagnostics, Inc., Huntsvile, AL). The gene-specific primers were: 5′-GAGCCAGCATGAAGCGAGCTCACCCCGAGT-3′ (NHEY1F) and 5′-TGCAGCATTTTCAGGTGATCCACGGTCATC-3′ (NHEY1R) for HEY1, 5′-CCGAATTCAAATGGCGCAAGATGAC AAG-3′ (ORF50S2) and 5′-AAGAATTCAGGCTCACCCCGCTGCAT GCGGC-3′ (ORF50AS) for RTA, 5′-CGGTTACCATGGAGGGGA TC-3′ (CXCR4NOTIRTS) and 5′-GAAGTCGGGAATAGTCAGCA-3′ (CXCR4RT2AS) for CXCR4, and 5′-CATTGACCTCAACTACATG G-3′ (GAPDHFW) and 5′-CTCCATGGTGGTGAAGACGCC-3′ (GAPDHRV) for GAPDH. The amplified fragments from these primer sets were about 300, 400, 580, and 200 bp, respectively. The primers 5′-GG GAGGCCAGCGTATTCAGGAC-3′ (RpChIPS) and 5′-GTAGCTG GGTCCTATGGGGGTTGG-3′ (RpChIPAS) for nt 71192 to 71425 for ChIP DNA amplification matched the RTA promoter region [12]. We used the nucleotide positions in the KSHV genome sequence of NCBI Accession No. U75698.

Immunofluorescence assay (IFA). Cells were washed with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4,

and 1.5 mM KH_2PO_4), spotted on a 24-well slide glass, dried, and then fixed with 4% paraformaldehyde-PBS for 30 min at 4 °C. After the fixation, the cells were washed with PBS and incubated in 0.5% Triton X-100–PBS for 30 min at room temperature. The slide was washed for 5 min in each of the following: distilled water, 70%, 80%, 100% ethanol, and 100% ethanol again. Rabbit anti-HEY1 antibodies were a kind gift from Dr. Kageyama. The mouse anti-RTA monoclonal antibody was generated in our laboratory [19]. The secondary antibodies were goat anti-mouse immunoglobulin G (IgG) antibodies conjugated with Alexa 488 and goat anti-rabbit IgG antibodies conjugated with Alexa 546 (Molecular Probes, Eugene, OR). After incubation with the first and the second antibodies, the cells were washed with PBS containing 0.1% Tween® 20 (PBS-T) three times. The cells were then counterstained with 0.1 μg/ml of 4′,6′-diamidino-2-phenylaliondole, dilactate (DAPI) (Molecular Probes) in PBS-T and mounted in 90% glycerol-PBS. The fluorescence signals were observed with a laser confocal microscope (LSM 510, Carl Zeiss Microimaging Inc., Thornwood, NY.).

Electrophoretic mobility shift assay (EMSA). The procedures for EMSA were as described previously [11]. Briefly, 293T cells were transfected with FLAG or FLAG-tagged HEY1 and harvested after two days. BC3 cells with or without with TPA (25 ng/ml) treatment were also harvested. The harvested cells were washed twice with PBS and resuspended in NE A buffer (10 mM Hepes [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and a protease inhibitor cocktail [Cat No. P8340, Sigma–Aldrich]) and allowed to incubate on ice for 10 min. After centrifugation (1000*g*, 5 min, 4 °C), the cells were lysed in NE A buffer containing 0.05% NP-40 and suspended to isolate the nuclei. The nuclei were collected and resuspended in NE C buffer (5 mM Hepes [pH 7.9], 1.5 mM $MgCl_2$, 0.1 mM EDTA, 300 mM NaCl, 20% [vol/vol] glycerol, 0.5 mM dithiothreitol, and a protease inhibitor cocktail [Cat No. P8340, Sigma–Aldrich]). The solution was rocked on ice for 30 min and spun at 15,000*g* for 10 min. The soluble fraction, i.e., the nuclear extract (NE) was diluted with NaCl-free NE C buffer to adjust the NaCl concentration to 150 mM. The concentration of the NE was colorimetrically determined using a Bio-Rad protein assay Kit.

To prepare 5′ end-labeled probes for EMSA, the RE3 fragment was purified from 6% polyacrylamide–bispolyacrylamide (29:1) gel electrophoresis after filling-in reaction with the Klenow fragment and substrates (Takara Shuzo). The fragment was treated with shrimp alkaline phosphatase (Roche Diagnostics) according to the manufacturer's protocol. The RE3 fragment was labeled in 1-pmol aliquots with [γ-^{32}P]ATP (5000 mCi/mmol; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and 10U T4 polynucleotide kinase (New England Biolabs), followed by spin column gel filtration through 1 ml Sephadex® G25 (Amersham) and ethanol precipitation. The total incorporation of ^{32}P in the purified probes was counted. The probe was then dissolved in distilled water at approximately 10^5 cpm/μl.

The binding reaction of the probe (10^5 cpm) to the NE (5 μg of protein per reaction) was performed in 20 μl binding buffer (25 mM Hepes [pH 7.5], 20% glycerol, 0.1% NP-40, 1 mM dithiothreitol, and 1 μg/ml poly(dI/dC)) for 30 min at room temperature. The salt concentration was adjusted by adding KCl to a final concentration of 50 mM. Unlabeled probe was added in the competition analysis at a 50-fold molar excess. In some cases, 1 μg mouse anti-FLAG antibody (Sigma–Aldrich) was incubated with the EMSA. One microgram of mouse immunoglobulin (DAKO, Copenhagen, Denmark) was used as a negative control.

Chromatin immunoprecipitation (ChIP) assay. pFLAG- or pFHEY1-transfected 293 cells were washed with PBS and cross-linked with 1% formaldehyde at 37 °C for 10 min. The cross-linked cells were washed with PBS twice and resuspended in 1 ml buffer A (10 mM Tris–HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Triton X-100, 1 mM DTT, 0.5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail [Sigma–Aldrich]). The extracted nuclei were pelleted by low-speed centrifugation and resuspended in 800 μl buffer B (10 mM Tris–HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, and 1 mM $CaCl_2$) and 50 U micrococcal endonuclease (Takara Shuzo) was added. After the incubation, 3 mM EGTA (pH 7.8) (final concentration) was added to terminate the reaction and the solution was spun at 10,000*g* for 5 min at 4 °C. The supernatants (SP1) were collected, and the pellet was resuspended in 800 μl LS buffer (10 mM Tris–HCl, 10 mM NaCl, and 1 mM EDTA) and spun again. The supernatants from this spin were mixed with SP1 and divided into 200, 700, and 700-μl aliquots for immunoprecipitation. The 200 μl was used to detect the input DNA fragment. The 700-μl supernatant samples were subjected to immunoprecipitation either with 0.5 μg of mouse anti-FLAG antibody or 0.5 μg control mouse immunoglobulin and 10 μl protein G-Sepharose (Amersham), which was preincubated in a binding buffer containing 0.2 mg salmon sperm DNA per ml and 0.05 mg of poly(dI/dC)/ml for 30 min, in a 1.5-ml siliconized tube (Assist, Tokyo, Japan). After an overnight incubation at 4 °C, the precipitates were washed five times with immunoprecipitation assay buffer (50 mM Tris–HCl [pH 7.6], 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, and 1% NP-40), four times with LiCl buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, and 150 mM LiCl), and once with TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA).

To extract the DNA fragment, TE with 1% SDS, 0.2 M NaCl, and proteinase K (Roche Diagnostics) was added to the washed precipitates. After incubation at 65 °C for 8 h, the eluted solution was subjected to phenol–$CHCl_3$ extraction and ethanol precipitation. The procedures for PCR and quantitative PCR are described above.

Results and discussion

Hey1 expression is dependent on RTA

To search for genes induced by KSHV RTA, RTA-inducible cell lines were established. In this system, the addition of ponasteron A (Pon A), an insect hormone, initiates de novo expression of the introduced gene through its REs via an artificial nuclear receptor (a retinoid × receptor) (Invitrogen). From the DNA array analysis of the RTA-inducible cell lines (293pIND/RTA1) compared with that of the LacZ-inducible ones (293pIND/LZ), we found that the *hey1* mRNA increased in the presence of Pon A (Table 1). The other host mRNAs induced in this system included CXC chemokine receptor 4 (*cxcr4*), neurofilament light polypeptide (*nefl*), carbonic anhydrase II (*ca2*), and myomesin 2 (*myom2*) (Table 1). To confirm the increase in mRNA, quantitative PCR was performed for *hey1* and *cxcr4* in a KSHV-infected cell line, BC3, with or without TPA (25 ng/ml) treatment and in the RTA-inducible cell line, 293pIND/RTA1 (Fig. 1). As shown in Fig. 1A, after Pon A treatment, the *rta* mRNA level increased by 2.5-fold by 24 h and had nearly returned to baseline by 48 h. In contrast, the *hey1* and *cxcr4* mRNAs increased about 2- and 2.5-fold, respectively, with the highest amount observed at the last, 48 h, time point. Similarly, in the TPA-induced BC3 cells, the *rta* mRNA level was highest at 12 h, but *hey1* and *cxcr4* appeared to be continuing to increase at 48 h (Fig. 1B). From these results, it appeared that *hey1* and *cxcr4* mRNA expression followed RTA expression, which was consistent with the DNA microarray data.

Next, we used IFA to follow the expression patterns of these molecules, and we confirmed that HEY1 was co-expressed with RTA in Pon A-induced 293pIND/RTA1, but not in the 293pIND/LZ control cell line (Fig. 2A and B). In addition, HEY1 was expressed in TPA-treated BC3 cells, but not in the untreated BC3 cells or in the KHSV-negative cell line, BJAB (Fig. 2C and

Table 1
The RTA-inducible genes

RTA-inducible	Genes	Log_2 value
Hairy/enhancer-of-split related with YRPW motif (HEY1)	NM 012258	1.9946
Chemokine receptor 4 (CXCR4)	NM 003467	2.1372
Neurofilament, light polypeptide (NEFL)	NM 006158	2.1112
Carbonic anhydrase II	NM 000067	2.9723
Myomesin 2	NM 003970	3.0093

Table 2
Nucleotide sequences of the RE1, RE2, and RE3 fragments [12]

Name	Sequence	Start/end positions position
RE1	GATGTGGTACCGAATGCCACAATCTGTGCCCTCCAGCTC CTACACCATGGCTTACGGTGTTAGACACGGGAGGTCGAG	−264/−226
RE2	TCTCACAATTTTCATCTCCAATACCCGGAATTGGGATA AGAGTGTTAAAAGTAGAGGTTATGGGCCTTAACCCTAT	−227/−190
RE3	GAATTGGGATACACACCTCCATGTTCAGTCACATGTACGCT CTTAACCCTATGTGTGGAGGTACAAGTCAGTGTACATGCGA	−200/−160

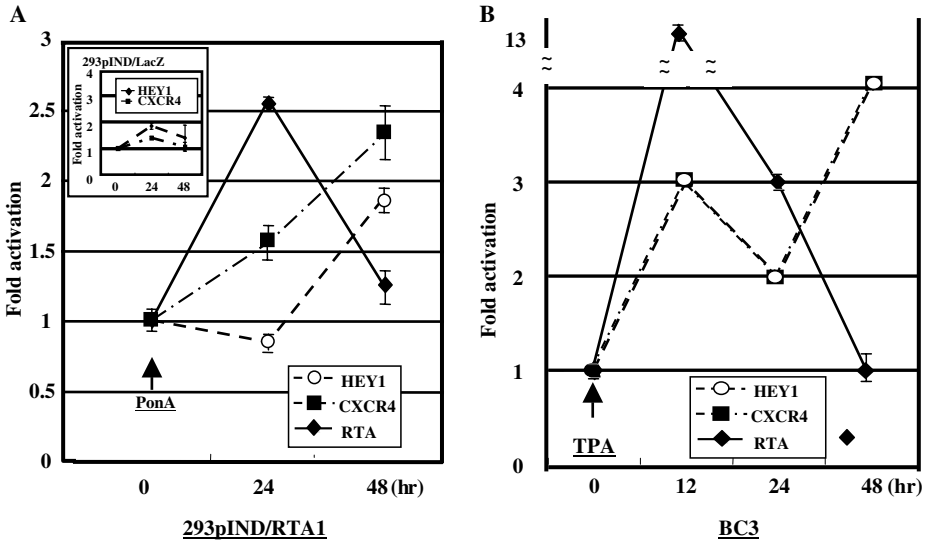

Fig. 1. *Hey1* and *CXCR4* expressions increase with *rta* mRNA expression. The gene expression kinetics were determined in (A) 293pIND/RTA1 cells and (B) BC3 cells. Total RNA was extracted at the indicated time from 293pIND/RTA1 and BC3 cells after treatment with Pon A and TPA, respectively. The cDNAs were subjected to quantitative PCR. The copy number of RTA, HEY1, and CXCR4 were quantified using a standard curve that was drawn by measuring serial dilutions of each cDNA-containing plasmid. GAPDH gene expression was also quantified for normalization. The target/GAPDH at time 0 was set to 1. The inset in (A) shows the gene expression kinetics in 293pIND/LacZ cells.

D). These results strongly suggested that *hey1* expression is dependent on *rta* expression.

RTA activates hey1 expression

Liang, et al. reported that RTA directly interacted with RPB-Jκ [13] and that RBP-Jκ interacting with RTA was required for the lytic reactivation [17]. RBP-Jκ usually recruits corepressor complexes to repress target genes when the Notch signaling pathway is off [20,21]. The *hey1* promoter region has three RTA REs: two RBP-Jκ sites and one Oct-1-binding site (Fig. 3A) [22]. To confirm the transcriptional activation of the *hey1* promoter by RTA, a luciferase reporter assay was performed. We prepared two reporter constructs, pGL3B and pHEY1, in which pHEY1 contained the region ~1 kbp upstream of the *hey1* transcription start site. As shown in Fig. 3B, compared with the control promoter activity, the activity of the *hey1* promoter was clearly upregulated by RTA expression in BC3, COS7, and NIH3T3 cells (Fig. 3B). Recently, Chang et al. reported that RTA induces B-cell activation markers such as CD21 and CD23a through RBP-Jκ [9]. RTA is very likely to use a similar mechanism to activate cellular genes, such as *Hey1*. Thus, RTA activated the

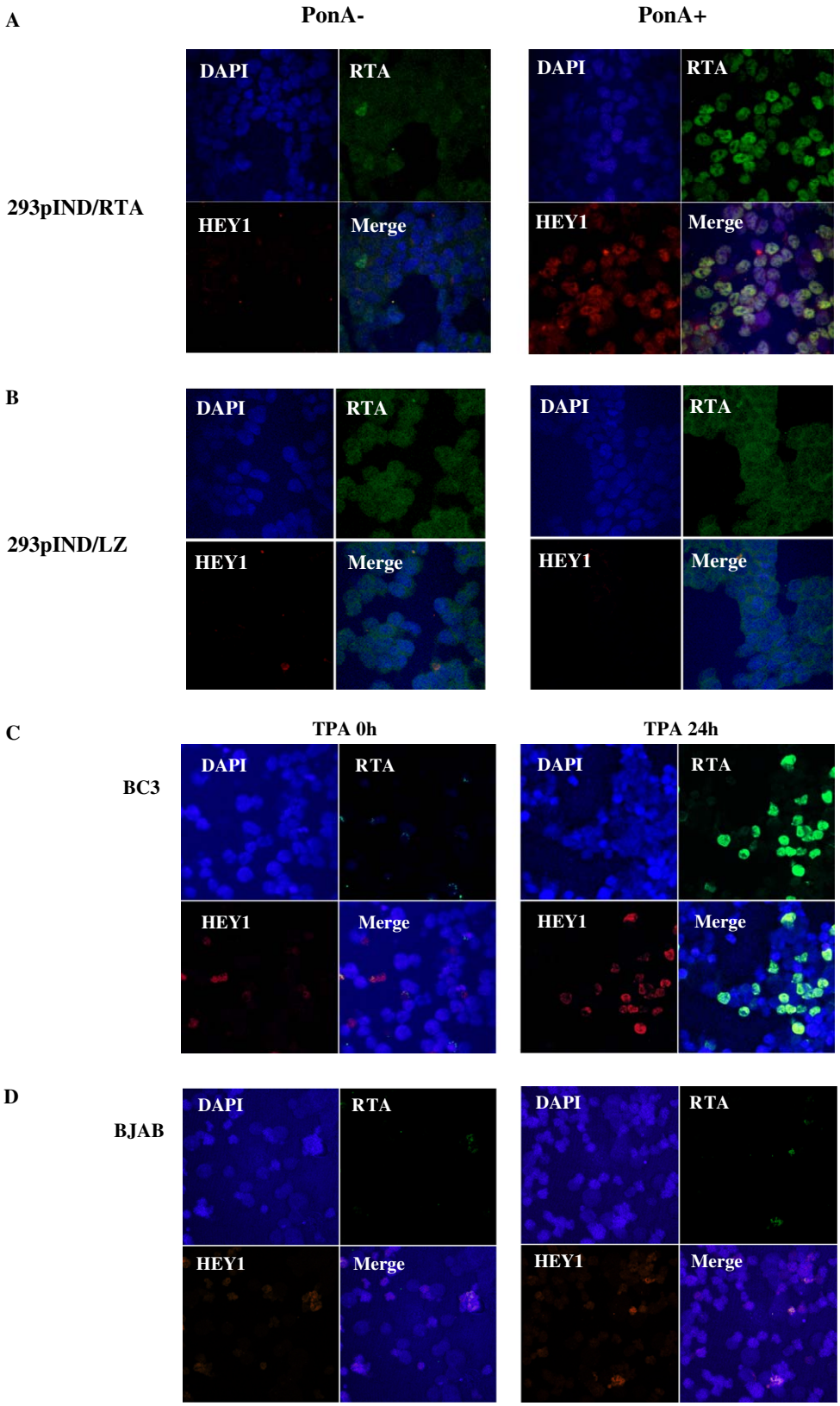

Fig. 2. Immunofluorescence assay for HEY1 expression in RTA-inducible cell lines. (A,B) 293 pIND/RTA and pIND/LZ cells were stimulated with PonA (1 μM) or (C,D) BC3 and BJAB were treated with TPA (25 ng/ml), and the RTA and HEY1 expression was observed with a confocal microscope (LSM510 Carl Zeiss Microimaging). RTA was labeled with goat anti-mouse IgG antibodies conjugated with Alexa 488 (green), and HEY1 was labeled with goat anti-rabbit IgG antibodies conjugated with Alexa 546 (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

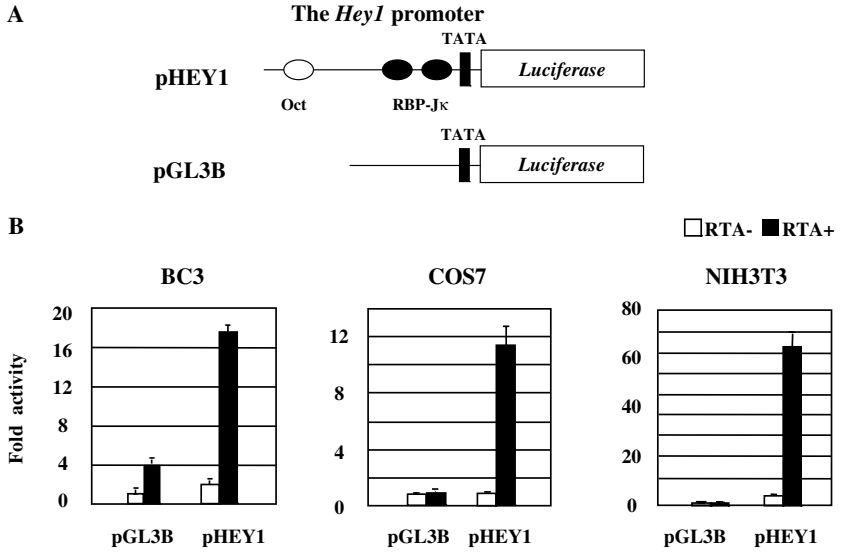

Fig. 3. The *hey1* promoter is activated by RTA. (A) Schematic diagrams of the reporter constructs for pHEY1 and pGL3B are shown, and putative characteristic elements on the pHEY1 promoter are indicated. RBP-Jκ and Oct-1 are RTA-REs. (B) Transient cotransfection assays were performed using BC3, COS7, and NIH3T3 cells. Forty-eight hours after transfection, the cells were harvested and assayed. The cells were co-transfected with pCMV-β-gal for normalization of the transfection efficiency. The results are presented as fold activity compared to basal activity (pGL3B with pcDNA empty vector), which was set to a value of 1.

hey1 promoter, and—although we did not perform a detailed analysis for this report—it probably activated the *hey1* promoter through the Oct1- and the two RBP-Jκ-binding sites, as reported previously [12,13].

HEY1 represses rta expression

Because the *hey* family genes act as transcriptional repressors, we also examined the negative regulation of the *rta* promoter by HEY1 in the reporter assay. The activity of the full-length promoter (pGL3Rp [−1543]) was about half of the control, in the presence of pFHEY1 (Fig. 4A). To learn which part of the promoter contained the HEY1 response element, we constructed a deletion series of reporter plasmids (Fig. 4A and some of these were described elsewhere [12]). Like the full-length promoter activity, these deletion mutant constructs, through −238, were also repressed by about 50% under the HEY1 expression condition (Fig. 4A). In contrast, further deletion to −163 resulted in little repression by HEY1 (Fig. 4A). From these results, it appeared that the HEY1 RE for repression was within the −238- to −163-bp region in the *rta* promoter [8].

For a more detailed analysis, we divided this region into three overlapping parts, termed RE1, RE2, and RE3. Constructs were made with each part inserted as 3 tandem copies and inserted into the pe1bTATA-luc plasmid (Fig. 4B). This reporter assay showed that the RE3 region was required for the repression by HEY1. The other regions, RE1 and RE2, were neither enhanced nor repressed by HEY1. Thus, the HEY1 RE for repression may be in the RE3 region; note, however, that in the absence of HEY1, this promoter region had higher activity than the basal construct (pe1b).

HEY1 is associated with a complex on the rta promoter region

HEY1 has a bHLH domain, which might be able to bind directly with an RE to repress transcription. Therefore, we performed EMSA using BC3 nuclear extract (NE) or NE from 293 cells transfected with the HEY1 expression vector. HEY1 was expressed well (data not shown), and we observed several specific shifted bands (data not shown). However, when TPA-induced or non-induced BC3 NE was incubated with the RE3 DNA, the anti-HEY1 antibodies had no effect. Even when we used the NE from 293 cells transfected with the HEY1 expression vector, we did not find any evidence that HEY1 directly bound the element (data not shown). These results indicated that HEY1 might not directly bind RE3, even though it has a bHLH domain.

Our data clearly showed that HEY1 repressed *rta* promoter activity (Fig. 4). The HEY1 repressive effect was reported to be necessary and sufficient for the recruitment of a co-repressor complex [23]. EMSA might not be suitable to detect weak or non-direct binding with DNA. Therefore, to address whether HEY1 interacted with this region as a component of a repressor complex, we performed a chromatin immunoprecipitation (ChIP) assay (as shown in Fig. 5), which is more useful for detecting a component of a complex binding to DNA.

The immunoprecipitates were amplified by quantitative-PCR using specific primers set around the RE3 region. The

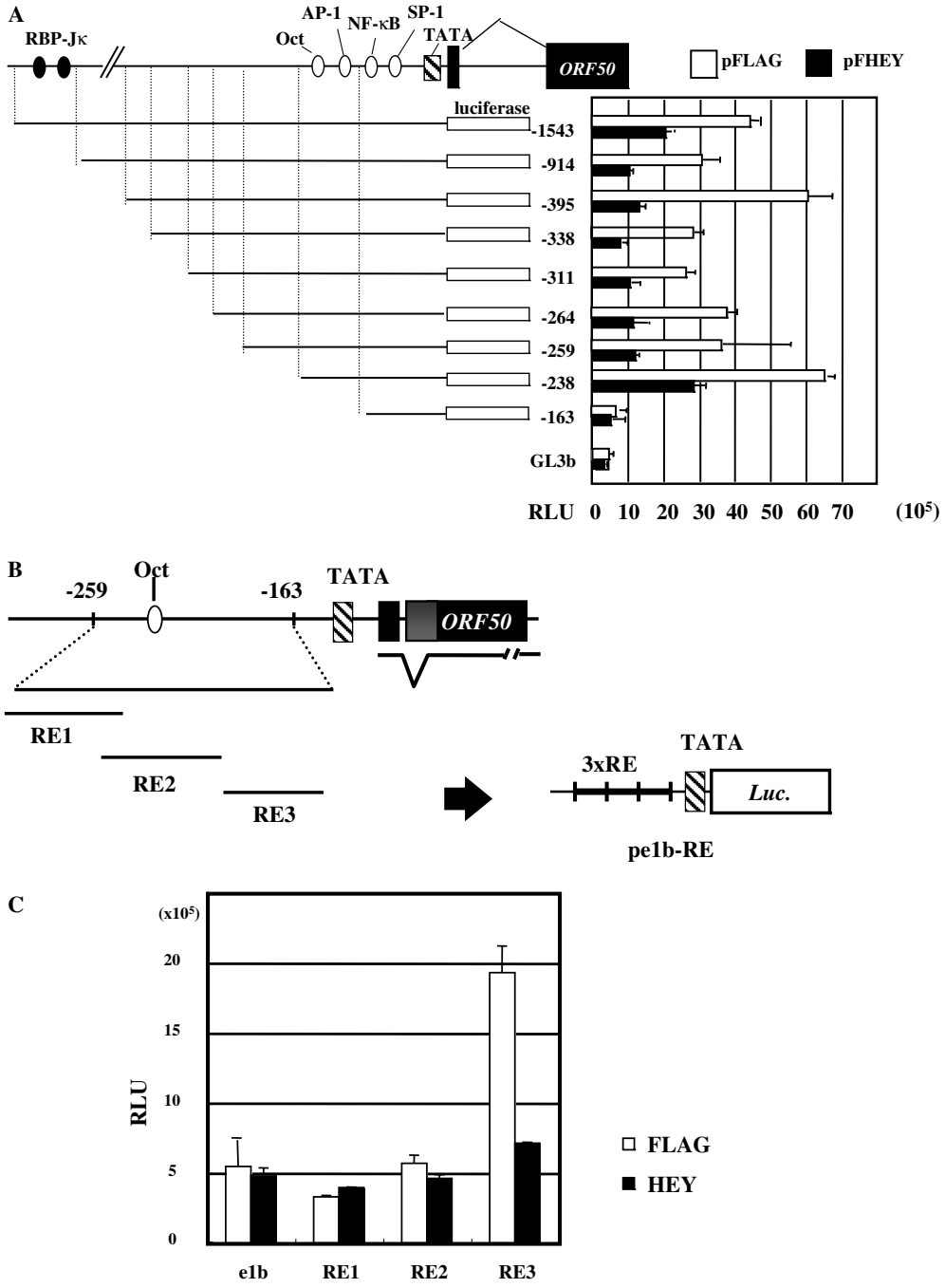

Fig. 4. Effect of HEY1 on the activity of the *rta* promoter. (A) Schematic representation of the *rta* promoter region and luciferase activity of the deletion mutants. The luciferase activity of the REs was normalized to the activity of β-galactosidase. 293T cells were co-transfected with one of the various truncated *rta* reporter constructs or the REs with either pFLAG or pFHEY1. Forty-eight hours post-transfection, the cells were harvested and each luciferase activity was measured. The luciferase activity was normalized to the activity of β-galactosidase. The data were obtained in three independent experiments and the average and standard deviation are shown. (B) Schematic representations of the REs of the schematic HEY1 repressive element. Each was inserted into pe1bTATA-Luc as three tandem repeats and the resulting vector was named pe1b-RE. (C) The relative luciferase activity of the putative HEY1 repressive elements is shown as above.

specificity was evaluated by comparing the amplification level of HEY1-specific immunoprecipitates to control immunoprecipitates. The results suggest that HEY1 could be involved in binding this element (Fig. 5).

HEY1 has been reported to form a complex with HDAC1, mSin3A, and N-CoR, whose complexes act as repressors [23]. We tried to detect the interaction of HEY1 with HDAC1 by GST pull-down assay, using GST-tagged HDAC1, but could not detect an interaction (data not shown). In the case of mSin3A, we performed immunoprecipitation followed by immunoblotting, but could not detect this interaction, either. Taken together,

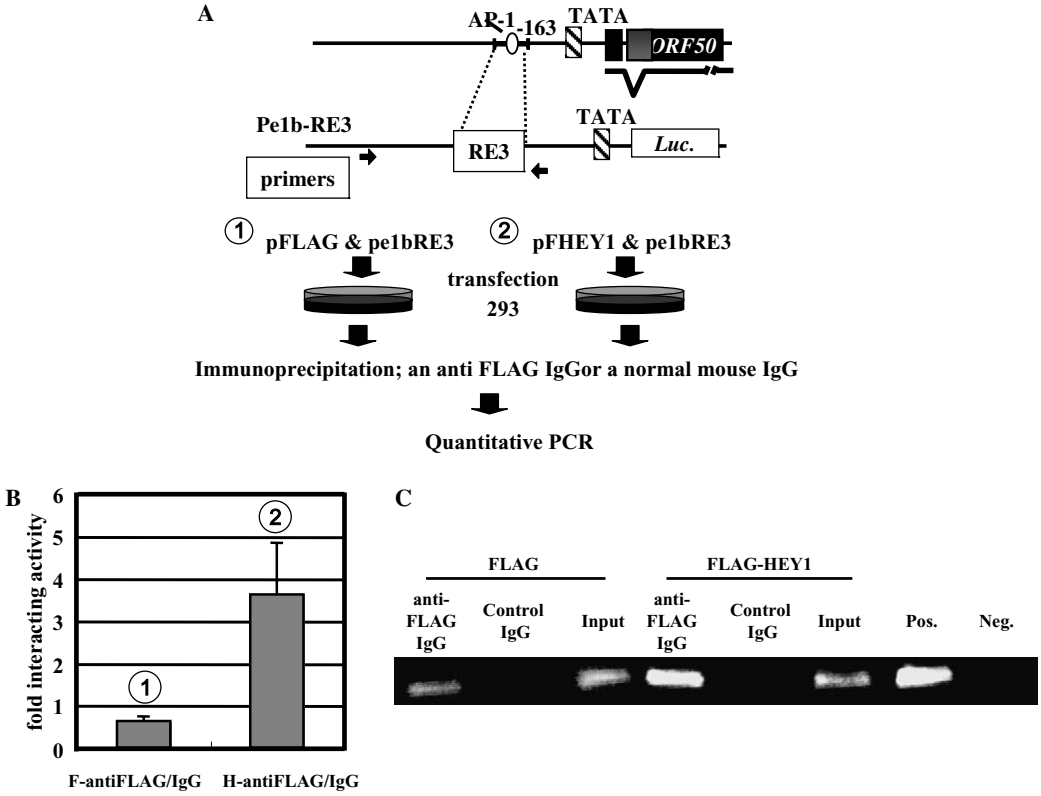

Fig. 5. HEY1 association with the *rta* promoter. A schematic diagram of the chromatin immunoprecipitation (ChIP) assay method is shown (A). Quantitative PCR revealed that HEY1 could be associated with the *rta* promoter (B). The fold interacting activity refers to the amplification level using quantitative PCR of immunoprecipitates brought down with anti-FLAG against those brought down with control IgG for each case. The photograph shows PCR products amplified from ChIP samples. Input, pre-immunoprecipitation DNA fragments. Pos, amplified plasmid pe1b-RE3 only. Neg, double distilled water (C).

our results indicate that HEY1 represses *rta* expression as a component in a complex on the RE3 region, but the mechanism of the repression remains to be elucidated. Some insight into the mechanism may come from studies that the repressive action of HEY1 is due to not only the recruitment of HDAC1, mSin3A, and N-CoR, but also to a negative or unknown function of HEY1 itself [22,24–27]. HEY1 interacts with some transcription activating factors, thereby abolishing their transcription activation activity [25,26].

When KSHV reactivation is induced by TPA treatment, RTA expression is induced, leading to subsequent lytic viral gene expression. RTA expression subsequently declines around the early-late phase. Our data suggest that this decline in immediate-early (IE) gene expression could be regulated through the expression of repressor genes like *hey1*. In addition, Curry and colleagues demonstrated that activated Notch-1, -2, and -4 induced HES, Hey1, and Hey2 to block apoptosis, which were involved in establishment of primary and immortalized KS tumor cell [27]. Although we used PEL cell line, induced HEY1 might functionally interfere in cellular apoptosis during primary lytic replication/reactivation. Viewed from another perspective, the repression of IE gene expression may increase the possibility of aborting the lytic replication/reactivation, thus maintaining viral latency.

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