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Modulation of Human Herpesvirus 8/Kaposi's Sarcoma-Associated Herpesvirus Replication and Transcription Activator Transactivation by Interferon Regulatory Factor 7

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Human herpesvirus 8 (HHV-8)/Kaposi's sarcoma-associated herpesvirus infection goes through lytic and latent phases that are regulated by viral gene products, but very little is known about the involvement of host proteins. The replication and transcription activator (RTA) is a viral protein sufficient to initiate lytic replication by activating downstream genes, including the viral early gene open reading frame 57 (ORF 57), which codes for a posttranscriptional activator. In this study, we demonstrate that cellular interferon regulatory factor 7 (IRF-7) negatively regulates this process by competing with RTA for binding to the RTA response element in the ORF 57 promoter to down-regulate RTA-induced gene expression. We also show that alpha interferon represses RTA-mediated transactivation and that repression involves IRF-7. Our study indicates that upon HHV-8 infection, the host responds by suppression of lytic gene expression through binding of IRF-7 to the lytic viral gene promoter. These findings suggest that HHV-8 has developed a novel mechanism to induce but then subvert the innate antiviral response, specifically the interferon-signaling pathway, to regulate RTA activity and ultimately the viral latent/lytic replicative cycle.

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is a recently discovered human gammaherpesvirus, which was first identified in AIDS-associated Kaposi's sarcoma (KS) tissues (5). HHV-8 is the etiological agent of KS and is associated with two other lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease (4, 8). Like other herpesviruses, HHV-8 exhibits two distinct phases of infection: lytic and latent. During latency, viral gene expression is limited to a few tightly controlled genes. These genes are thought to maintain the viral episome, promote immune evasion, and provide a growth advantage to the infected cells (16, 40). Latency enables the virus to establish persistent infection and plays a major role in tumorigenesis (34). The expression of the full set of viral genes occurs only during lytic replication, when virus progeny are produced and the host cell is destroyed (39). Lytic reactivation enables the spread of viruses from the lymphoid compartment to endothelial cells, which plays a role in the development of KS (12, 17).

HHV-8-infected PEL cells harbor the virus in a latent state from which it can be activated to enter lytic replication by treatment with sodium butyrate or 12-*O*-tetradecoylphorbol 13-acetate (TPA). Control of latent-lytic switching is maintained by the HHV-8 *r*eplication and *t*ranscription *a*ctivator (RTA), produced from HHV-8 open reading frame 50 (ORF 50). Expression of HHV-8 RTA is necessary and sufficient to disrupt viral latency and induce lytic replication (10, 27, 43). RTA contains an amino-terminal basic DNA-binding domain and a carboxy-terminal acidic activation domain (28, 45). The activation is mediated by interaction with different viral gene promoters via a sequence-specific motif know as the RTA response element (RRE) (9, 29, 42). It was recently suggested that the subunits of an RTA oligomer may make multiple contacts with a tandem repeat of the core sequences (25).

RTA activates the expression of a number of viral immediate-early and early genes, including its own and several others, such as an early gene, ORF 57 (3, 7, 9, 29). ORF 57 is a homologue of Epstein-Barr virus (EBV) MTA (the M transactivator, BMLF1) and encodes a posttranscriptional activator, which acts as an mRNA export factor. The ORF 57 gene product is thought to bind viral RNA to facilitate mRNA transport from the nucleus to the cytoplasm (1, 19). The result of this transport is modulation of viral gene expression, especially of the intronless late structural genes. For EBV and herpesvirus saimiri, the ORF 57 homologue has been shown to be essential for production of infectious virions (11, 49). Thus, the regulation of ORF 57 expression by RTA plays a critical role in viral infection and maintenance of the balance between latency and lytic reactivation.

Viral infection induces the expression of interferons (IFNs) and other cytokines. The effects of IFN on cells are mediated by IFN-stimulated genes (ISGs), including a family of IFN regulatory factors (IRFs). To date, 10 cellular IRFs have been identified (30, 32, 44). The hallmark of cellular IRFs is an N-terminal DNA binding domain, which contains a tryptophan-rich region and forms a helix-turn-helix motif that interacts with IFN-stimulation response elements (ISREs) in target gene promoters (44). IRFs play a critical role in the activation of other cellular genes, such as those encoding alpha interferon

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(IFN-α) and IFN-β, IFN-stimulated genes, and genes encoding several chemokines (30, 53). Addition of IFN-α inhibits HHV-8 reactivation in PEL cells and reduces the HHV-8 load in cultured peripheral blood mononuclear cells (33); this effect is likely to be mediated by a number of cellular factors including IRFs. One IRF implicated in HHV-8 infection is IRF-7, since it is strongly up-regulated during the early lytic cycle of HHV-8 in human endothelial cells, fibroblast cells, and B cells including the BCBL-1 PEL cell line (36, 38). The IRF-7 gene is induced by IFN through the IFN-stimulated gene factor 3, after which IRF-7 undergoes nuclear translocation, where it activates both IFN-α and IFN-β expression (32, 44). Since IRF-7 is induced early in HHV-8 infection, its role in augmenting viral replication and its effects on immediately-early viral gene expression warrant investigation.

Our previous studies have shown that HHV-8 RTA activates viral ORF 57 gene expression (45). This activation is mediated by the binding of RTA to a 40-bp region within the ORF 57 promoter. The activation of ORF 57 by RTA then contributes to lytic viral replication. In this study, we demonstrate that RTA-mediated transactivation of ORF 57 gene expression can be negatively regulated by IRF-7. This level of control occurs via competitive binding of IRF-7 to the RRE in the ORF 57 promoter. The present study suggests that HHV-8 has adopted a strategy for utilizing a component of the host innate immune response to facilitate suppression of early lytic gene expression and to promote latency and persistent infection.

MATERIALS AND METHODS

Plasmids. Expression plasmids pcDNA-ORF50, which encodes the full-length HHV-8 RTA, and pCMV-Tag50, which encodes a Flag-tagged RTA, were described previously (45). The p57Pluc1 reporter, containing the 453-bp HHV-8 ORF 57 promoter region (nucleotides (nt) 81556 to 82008), was also described previously (45). IRF-7 expression plasmids pcDNA-IRF-7A, pcDNA-IRF-7C, and pcDNA-IRF-7DN, expressing different spliced isoforms of IRF-7, were described elsewhere (50, 51). pCMV-Tag7A and pET28a-IRF7A, which encode the Flag tag- and His tag-fused IRF-7, were created by amplifying the full-length IRF-7 coding region from pcDNA-IRF-7A using 5′ primer (5′ AGGAT CCAT GGCCTTGGCTCCTGAG 3′) and 3′ primer (5′ TGGGCTGCTCGAGCTTT CTGGAGTTC 3′) and inserting the PCR products into pCMV-Tag2B (Stratagene, La Jolla, Calif.) or pET-28a (Novagen, Madison, Wis.) at the BamHI and XhoI sites. The RTA coding sequence of pCMV-Tag50 was inserted into pET-28b (Novagen) to generate pET28b-RTA for expression of His-tagged RTA in *Escherichia coli*. p57Pluc1m, with mutation in the potential IRF-7 binding site, was generated by using a site-directed mutagenesis kit (Stratagene). An IRF-7 RNA interference construct, pGE1-IRF-7i, was obtained by inserting the annealed product of 5′ GATCCCGCTGTGACACCCCCATCTTCGACTTCAGA GGAA GCTTGCTCTGAAGTCGAAGATGGGGGTGTCACAGTTTTTT 3′ and its complementary strand, 5′ CTAGAAAAAACTGTGACACCCCCATCT TCGACTTCAGAGCAAGCTTCCTCTGAAGTCGAAGATGGGGGTGTCA CAGCGG 3′, into the pGE-1 vector (Stratagene) at the BamHI and XbaI sites. The β-galactosidase expression plasmid pCMV-β was purchased from Clontech (BD Biosciences, Palo Alto, Calif.). All PCR generated plasmids, mutations, and fusion protein expression plasmids were verified by sequence analysis.

Cell culture, transfection, and luciferase assays. The human embryonic kidney fibroblast cell line 293T was cultured in Dulbecco's modified Eagle medium (Mediatech, Herndon, Va.) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2. Transfection was carried out with LipofectAMINE reagent (Invitrogen, Carlsbad, Calif.) as described previously (6). The total amount of transfected DNA in each well was kept constant by the addition of control vector plasmid. Transfected cells were harvested 48 h posttransfection. The luciferase activity was measured with a luciferase assay system (Promega, Madison, Wis.). Transfection efficiency was normalized by using a pCMV-β-gal expression plasmid as an internal control. The mean luciferase values were derived from at least three independent transfection assays.

BCBL-1 is an HHV-8-positive PEL cell line. TRE×BCBL-1 is a BCBL-1 cell line carrying a gene-inducible system, and TRE×BCBL-1 RTA is a TRE×BCBL-1 cell line carrying a tetracycline-inducible RTA gene (35). BJAB and DG75 are HHV-8- and EBV-negative Burkitt's lymphoma cell lines (2, 31). These cell lines were maintained in RPMI 1640 (Mediatech) plus 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO_2. B cells were transfected by electroporation at 250 V and 960 μF in 0.4 ml of RMPI 1640 medium without FBS as described previously (52). The total amount of electroporated DNA in each cuvette was kept constant by the addition of control vector plasmid. Cells were collected at 72 h postelectroporation. Luciferase activities were measured with a luciferase assay system (Promega). Electroporation efficiency was normalized by using a pCMV-β-gal expression plasmid. Each result was obtained from an average of at least three independent experiments.

Expression and purification of recombinant proteins. *E. coli* BL21(DE3) cells harboring the pET28b-RTA or pET28a-IRF7A plasmid were cultured overnight at 37°C in 5 ml of Luria-Bertani broth containing 50 μ of kanamycin/ml. Each 500 ml of Luria-Bertani broth containing 50-μg/ml kanamycin was inoculated with 5 ml of an overnight culture and grown for 3 to 4 h at 37°C until the culture reached an A_{600} of 0.8. Protein expression was induced for 4 h with 0.1 to 0.5 mM isopropylthio-β-D-galactoside at 28°C. The cells were then harvested by centrifugation, resuspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 10 mM imidazole [pH 8.0]) with 0.5 mM phenylmethylsulfonyl fluoride. After sonication and centrifugation, the His-tagged RTA or His-tagged IRF-7 proteins in the supernatant were purified with Ni^{2+}-nitrilotriacetic acid agarose (QIAGEN, Valencia, Calif.) under native conditions. Proteins in each fraction were analyzed by Western blotting with an anti-His_6 monoclonal antibody–horseradish peroxidase conjugate (BD Biosciences). Fractions containing RTA or IRF-7 were dialyzed against phosphate-buffered saline (pH 7.4), divided into aliquots, and stored at −70°C.

Mammalian-derived recombinant IRF-7 protein was purified from transfected 293T cell extract. 293T cells in 100-mm-diameter dishes were transfected with 5 μg of pCMV-Tag7A. Cells were collected, washed twice with 1× TBS (50 mM Tris-HCl, 150 mM NaCl [pH 7.4]), and then lysed in 2 ml of 1× lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 [pH 7.4]). Flag-tagged IRF-7 was bound to anti-Flag M2 affinity gel (Sigma, St. Louis, Mo.), washed with 1× TBS, eluted with 20 mM glycine-HCl at pH 3.0, and then immediately neutralized with 1 M Tris-HCl (pH 8.0). Eluted fractions were analyzed by Western blotting with anti-Flag M2 antibody (Stratagene). Flag-IRF-7 proteins were divided into aliquots and stored at −70°C.

Antibodies and Western blot analysis. Anti-Flag M2 antibody was purchased from Stratagene; anti-His_6 monoclonal antibody–horseradish peroxidase conjugate was purchased from BD Biosciences; anti-β-tubulin antibody was purchased from Sigma. Anti-RTA, anti-ORF 57, and anti-IRF-7 antibodies were described previously (37, 46, 50). For Western blot analysis, the Hybond-P membrane and ECL detection reagents (Amersham, Piscataway, N.J.) were used.

EMSA. The probes were obtained by annealing complementary oligonucleotides and then labeling with [α-^{32}P]dATP (Amersham), using DNA polymerase I Klenow fragment (Fermentas, Hanover, Md.). The 57I, 57R, and 57IM probes are as described in Fig. 1. The AP1 probe was purchased from Promega. The consensus ISRE probe from ISG-15 and the PAN probe were synthesized according to previous reports (18, 41). The Tap-2 ISRE and Tap2m ISRE probes were described earlier (52). The electrophoretic mobility shift assay (EMSA) was performed essentially as described previously (52). Purified RTA protein or IRF-7 protein was incubated with 20,000 to 50,000 cpm of labeled probe in a volume of 12.5 μl containing 20 mM HEPES-KOH, 1 mM $MgCl_2$, 0.1 mM EGTA, 2 μg of poly(dIdC), and 4% Ficoll 400 (pH 7.9) for 20 min at room temperature. For competition assays, a 50-fold excess of cold probe was added to the binding mixture. For super-shift assay, 0.1 μl of rabbit anti-IRF-7 antibody was added 10 min before the addition of the probe. The binding mixtures were loaded onto a 4.5% polyacrylamide gel in 0.25× TBE buffer (22.5 mM Tris-borate, 0.5 mM EDTA), and the gel was run at 4°C and then dried onto a piece of Whatman 3MM paper (Whatman, Clifton, N.J.) followed by autoradiography.

RESULTS

The HHV-8 ORF 57 promoter has a functional IRF-7 binding site. The expression of ORF 57 is regulated by RTA and is mediated by the binding of RTA to RREs located within the ORF 57 promoter. Analysis of the RRE_{ORF57} sequence revealed a potential IRF-7 binding site, 5′-ATTTTTCGTTT*G*-3′, at nt 81930 to 81941 in the HHV-8 BC-1 genome (Fig. 1). The complementary sequence is identical to the consensus

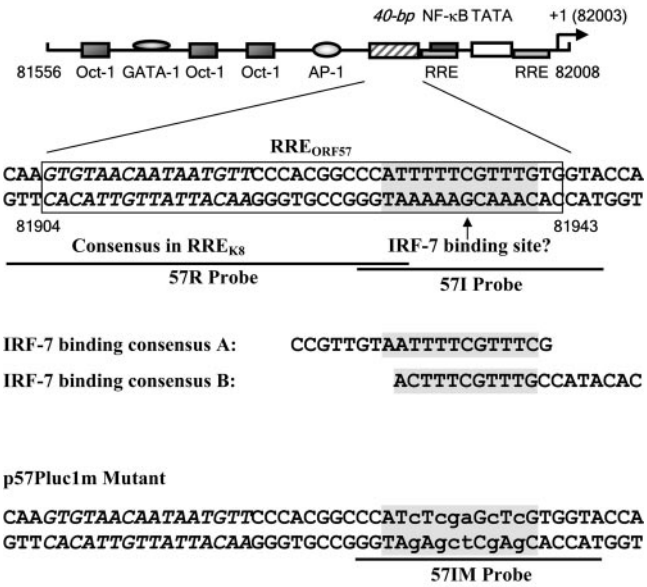


FIG. 1. RTA response element and putative IRF-7 binding site in the HHV-8 ORF 57 promoter. The locations of various putative promoter-regulatory elements and the EBV RRE-like sequences are indicated on the promoter segment. The sequence of 40-bp RRE region is boxed, the identical nucleotides shared between ORF 57 RRE and ORF K8 RRE are italicized, and the putative IRF-7 binding site is shadowed. Two IRF-7 binding consensus sequences are also shown (complementary sequences of cloned IRF-7 binding sites by Lin et al. [26]). The p57Pluc1m mutant plasmid with mutations in the potential IRF-7 binding site was identical to the p57Pluc1 reporter construct except for the highlighted region, with changes shown in lowercase. The bold lines indicate the region of probes corresponding to the RRE_{ORF57} sequence.

IRF-7 binding sequence, 5′-GAA(A/T)N(C/T)GAAAN (T/C) -3′ (26), except where the first G residue in the consensus is replaced by a C residue and the last T/C residue is replaced by an A residue (Fig. 1). Similar substitutions have been described previously (26). The presence of a potential IRF-7 binding sequence in the ORF 57 promoter suggested that IRF-7 might bind and regulate ORF 57 expression.

To determine whether IRF-7 binds to this consensus sequence, EMSAs were performed by using a labeled double-stranded oligonucleotide, 57I, from nt 81928 to 81946 in the ORF 57 promoter sequence (Fig. 1). Binding was detected by using bacterially expressed, His-tagged IRF-7 protein (Fig. 2A, lane 2). Only very weak binding was detected with 57IM, a probe containing a mutant IRF-7 binding site, and no binding was detected with 57R, a probe that contains the 5′ region of the RRE_{ORF57} but not the putative IRF binding site (Fig. 2A, lanes 4 and 6). The addition of the specific competitor, 57I, significantly decreased binding of IRF-7 to the labeled probe (Fig. 2B, lane 3), the mutated competitor 57IM only had minor inhibitory effect, and 57R did not compete (Fig. 2B, lanes 4 and 5). The addition of either an IRF-7 consensus binding target, ISRE oligonucleotide (Fig. 2B, lane 6), or another known IRF-7 binding site, Tap2 oligonucleotide (Fig. 2B, lane 7), inhibited IRF-7 binding to the 57I probe, with Tap2 inhibiting more effectively. A mutant Tap2 probe, Tap2m, did not inhibit complex formation (Fig. 2B, lane 8). An unrelated oligonucleotide probe, AP1, did not inhibit binding (Fig. 2B, lane 9). Addition of an anti-IRF-7 antibody supershifted the IRF-7-DNA complex (Fig. 2B, lane 10), but an antibody to another HHV-8 gene product, anti-K15, did not (Fig. 2B, lane 11).

Given the specific binding of IRF-7 to the ORF 57 promoter, it seemed plausible that IRF-7 might regulate ORF 57 gene expression. To identify the effect of IRF-7 on ORF 57 expression, we measured the activity of an ORF 57 promoter-reporter construct in the presence of exogenous IRF-7 in 293T cells which expressed very little endogenous IRF-7. The expression of Flag-tagged IRF-7 was monitored by Western blotting, using anti-Flag antibody. Increasing amounts of IRF-7 expression plasmid (0 to 1 μg) resulted in dose-dependent increases of ORF 57 promoter activity, but only 10-fold activation was observed with the highest concentration of IRF-7 added (Fig. 2C). To confirm the specificity of the interaction between IRF-7 and the ORF 57 promoter, the p57Pluc1m mutant plasmid, identical to the p57Pluc1 reporter construct except for mutations in the potential IRF-7 binding site, was generated (Fig. 1). Mutation of the IRF-7 binding site within the ORF 57 promoter abolished its responsiveness to IRF-7 (Fig. 2C). Based on our observation that IRF-7 binds to the consensus IRF-7 binding motif in the ORF 57 promoter and results in some enhancement of ORF 57 promoter-mediated gene expression, we hypothesized that the ORF 57 promoter has a functional IRF-7 binding site, the binding of which may regulate ORF 57 expression.

IRF-7 suppresses RTA activation of ORF 57 gene expression. Since the IRF-7 binding site in the ORF 57 promoter overlaps with the RRE and the binding of IRF-7 enhances ORF 57 expression, it was important to investigate the interplay between IRF-7 and RTA on the RTA-responsive ORF 57 promoter. To examine this potential interaction, the p57Pluc1 reporter plasmid was transfected into 293T cells with a fixed amount of pCMV-Tag50, an RTA expression plasmid, and with increasing amounts of pCMV-Tag7A. The expression levels of Flag-tagged RTA and IRF-7 proteins were detected by Western blotting with anti-Flag antibody. As expected, the ORF 57 promoter was strongly activated by HHV-8 RTA; however, when increasing amounts of the IRF-7 expression plasmid pCMV-Tag7A were added, the RTA-mediated ORF 57 activation was repressed in a dose-dependent manner (Fig. 3A). RTA activation was reduced by 77% when 2 μg of IRF-7 expression plasmid was added. The reduction of RTA activity, however, was not due to an IRF-7-mediated diminution in RTA expression, since similar levels of RTA were expressed (Fig. 3A).

To confirm that the repression of RTA by IRF-7 is specific, the p57Pluc1 reporter plasmid was transfected into 293T cells with a fixed amount of pCMV-Tag7A and with increasing amounts of pCMV-Tag50 (Fig. 3B). As expected, the activation of ORF 57 expression by RTA was inhibited by IRF-7, but the inhibition was less effective with increasing amounts of RTA. Activation was repressed by 40% by IRF-7 when 25 ng of RTA expression plasmid was added but only by 27% when 0.5 μg of RTA was added.

IRF-7 repression of RTA transactivation was also observed in B cells. In BJAB and DG75, two EBV/HHV-8-negative cell lines, IRF-7 reduced RTA activation by 84 and 67%, respectively (Fig. 3C). To determine whether the suppression of RTA activation of the ORF 57 promoter was specifically due to

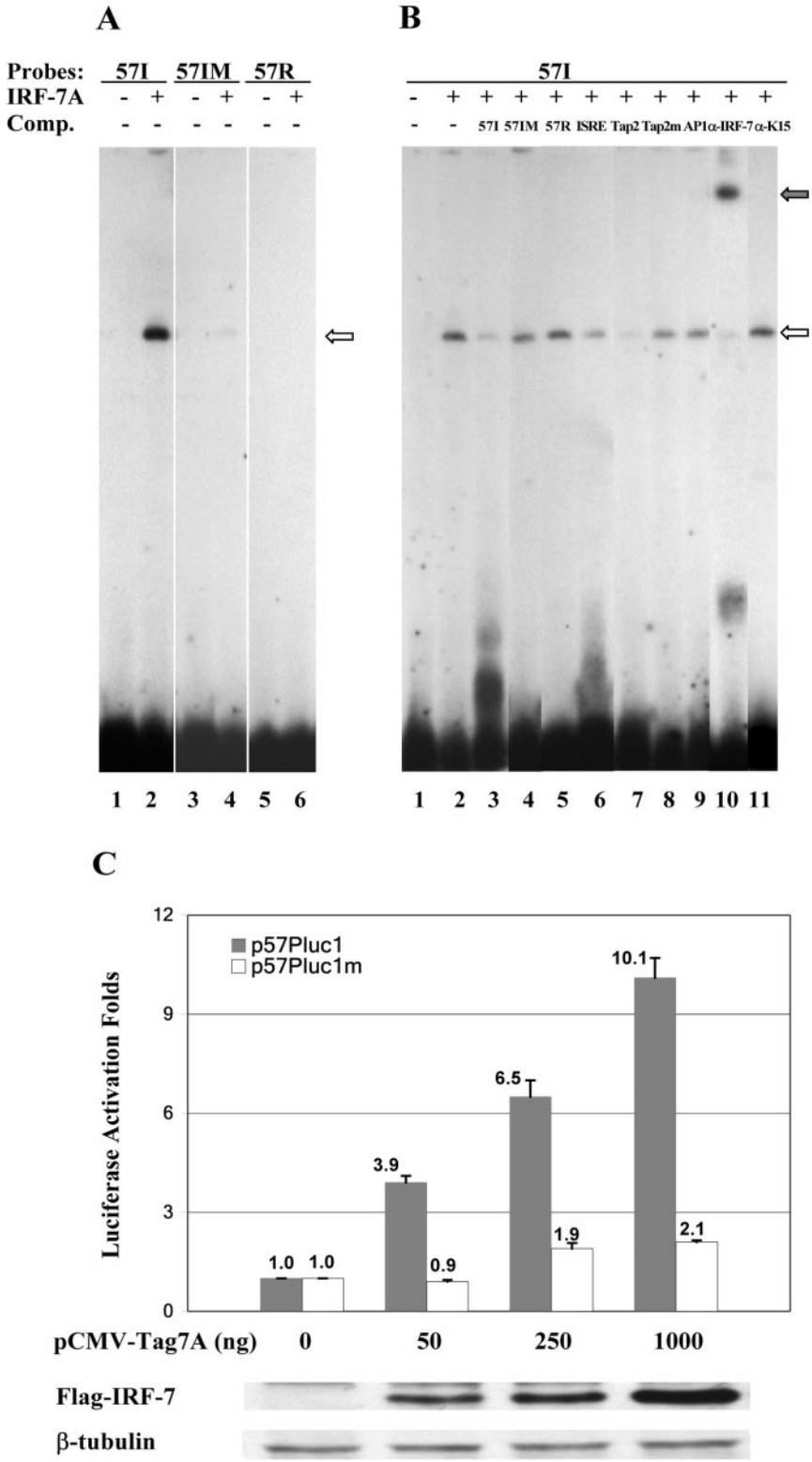


FIG. 2. IRF-7 binds to the putative IRF-7 binding site of the ORF 57 promoter. (A) EMSA of the 57I, 57IM, and 57R probes with IRF-7 protein. Partially purified His-tagged IRF-7 was incubated with labeled probes: 57I (lanes 1 and 2), 57IM (lanes 3 and 4), or 57R (lanes 5 and 6). The open arrow indicates specific binding of IRF-7. (B) Competitive binding and super-shift assays of the IRF-7–57I complex. A 50-fold excess of specific competitors (57I in lane 3, ISRE in lane 6, and Tap2 in lane 7) and nonspecific competitors (57IM in lane 4, 57R in lane 5, Tap2m in lane 8, and AP1 in lane 9) were used. Rabbit anti-IRF-7 (lane 10) and anti-K15 (lane 11) antibodies were also used. The open arrow indicates the specific binding of IRF-7. The shaded arrow indicates the super-shift of IRF-7. (C) IRF-7 binding site in the ORF 57 promoter is critical for IRF-7 response. Twenty nanograms of p57Pluc1 or p57Pluc1m was cotransfected into 293T cells with various amounts of IRF-7 expression plasmid pCMV-Tag7A, and luciferase activities were tested. In parallel, protein expression was detected by Western blotting using anti-Flag and anti-β-tubulin monoclonal antibodies. The numbers above each bar represent *n*-fold activation and are normalized to 1 in the absence of IRF-7. Results are averages from at least three independent experiments, and the standard deviations are shown.

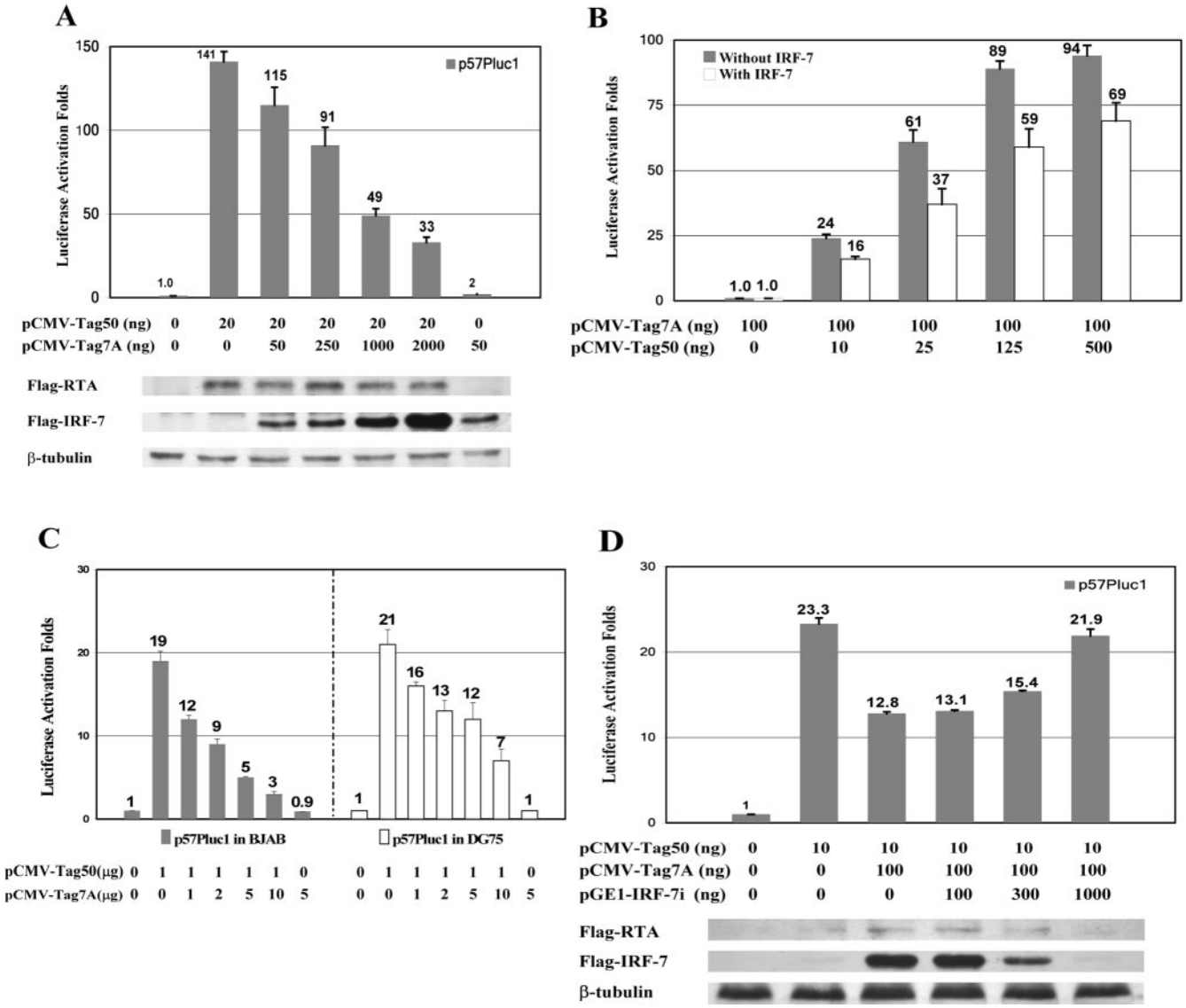


FIG. 3. IRF-7 represses RTA-mediated transactivation. (A) Transactivation of p57Pluc1 by RTA is repressed by IRF-7 in 293T cells. The cells were cotransfected with pCMV-Tag50 and pCMV-Tag7A. Luciferase activity and Western blot analysis were carried out. (B) Repression of RTA-mediated transactivation by IRF-7 at different RTA-to-IRF-7 plasmid DNA ratios. DNA transfection was carried out as described for panel A, with a fixed amount of pCMV-Tag7A and increasing amounts of pCMV-Tag50. (C) Repression of RTA transactivation by IRF-7 in B cells. BJAB or DG75 cells were electroporated with pCMV-Tag50 and pCMV-Tag7A. The luciferase activities were measured. (D) Repression of RTA-mediated transactivation can be prevented by addition of IRF-7i. 293T cells were cotransfected with pCMV-Tag50, pCMV-Tag7A, and various amounts of IRF-7i construct. Luciferase activities were measured, and Western blot analysis was carried out.

IRF-7, an IRF-7 RNA interference plasmid, pGE1-IRF-7i, was used to inhibit IRF-7 expression. Fewer RTA and IRF-7 expression plasmids were used in the study, since RNA interference assays required a high ratio of IRF-7i construct to IRF-7 plasmid to give maximum efficiencies in silencing. For RTA expression, twofold less plasmid used in the experiment resulted in fourfold less RTA protein expressed and fivefold less transactivation activity (compare Fig. 3A and D, lanes 2), and the transactivation was inhibited by about 50% upon cotransfection with 100 ng of IRF-7 expression plasmid (Fig. 3D, lane 3). With increasing amounts of pGE1-IRF-7i added, IRF-7 expression levels were reduced, and the inhibitory effects of IRF-7 on RTA transactivation were proportionally reversed (Fig. 3D).

Interaction between the IRF-7 binding site in RRE_{ORF57} and the DNA binding domain of IRF-7 mediates the repression of RTA transactivation. Since it appeared that IRF-7 repression was mediated through binding to the promoter region, we sought to investigate the role of this IRF-7 binding site in IRF-7 interference with RTA activation of a mutant ORF 57 promoter construct, p57Pluc1m (Fig. 1). RTA transactivated the mutant p57Pluc1m much less than wild-type p57P1-luc. The addition of IRF-7 failed to inhibit the activation of p57Pluc1m by RTA (Fig. 4A), suggesting that IRF-7 inhibited

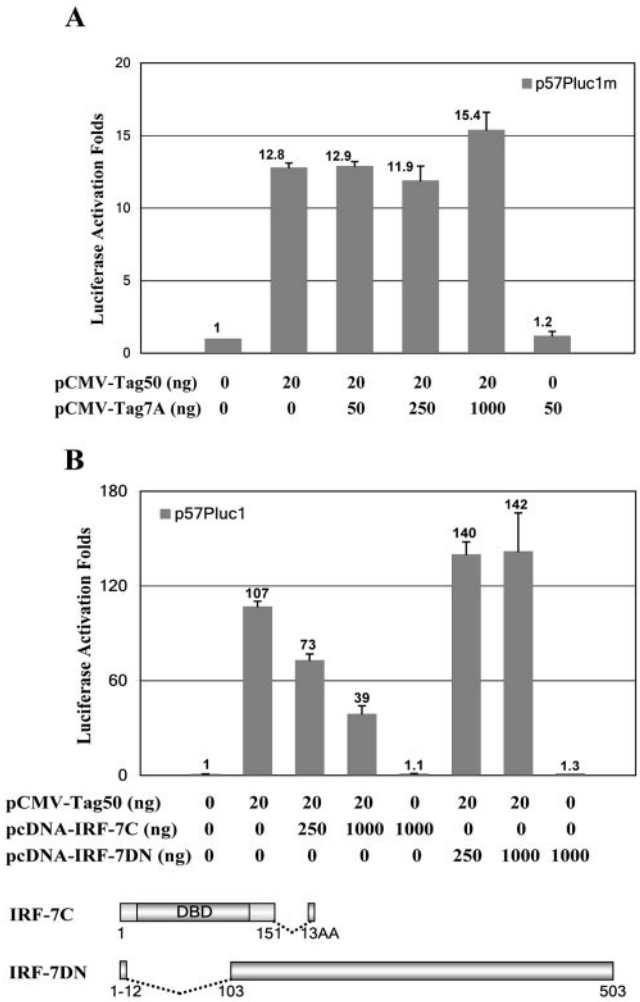


FIG. 4. The IRF-7 binding site in the RRE of the ORF 57 promoter and the binding domain of IRF-7 are required for repression of RTA-mediated transactivation. (A) The IRF-7 binding site in the ORF 57 promoter is critical for repression of RTA transactivation by IRF-7. 293T cells were cotransfected with the mutant ORF 57 promoter construct p57Pluc1m, pCMV-Tag50, and various amounts of pCMV-Tag7A. Luciferase activities analysis was carried out. (B) IRF-7C represses RTA transactivation of the ORF 57 promoter. Transfections were performed with IRF-7C and IRF-7DN expression plasmids. The DBD of IRF-7C is indicated.

RTA activation of the ORF 57 promoter by binding to the IRF-7 target site in the promoter region or by occupying or occluding the RRE.

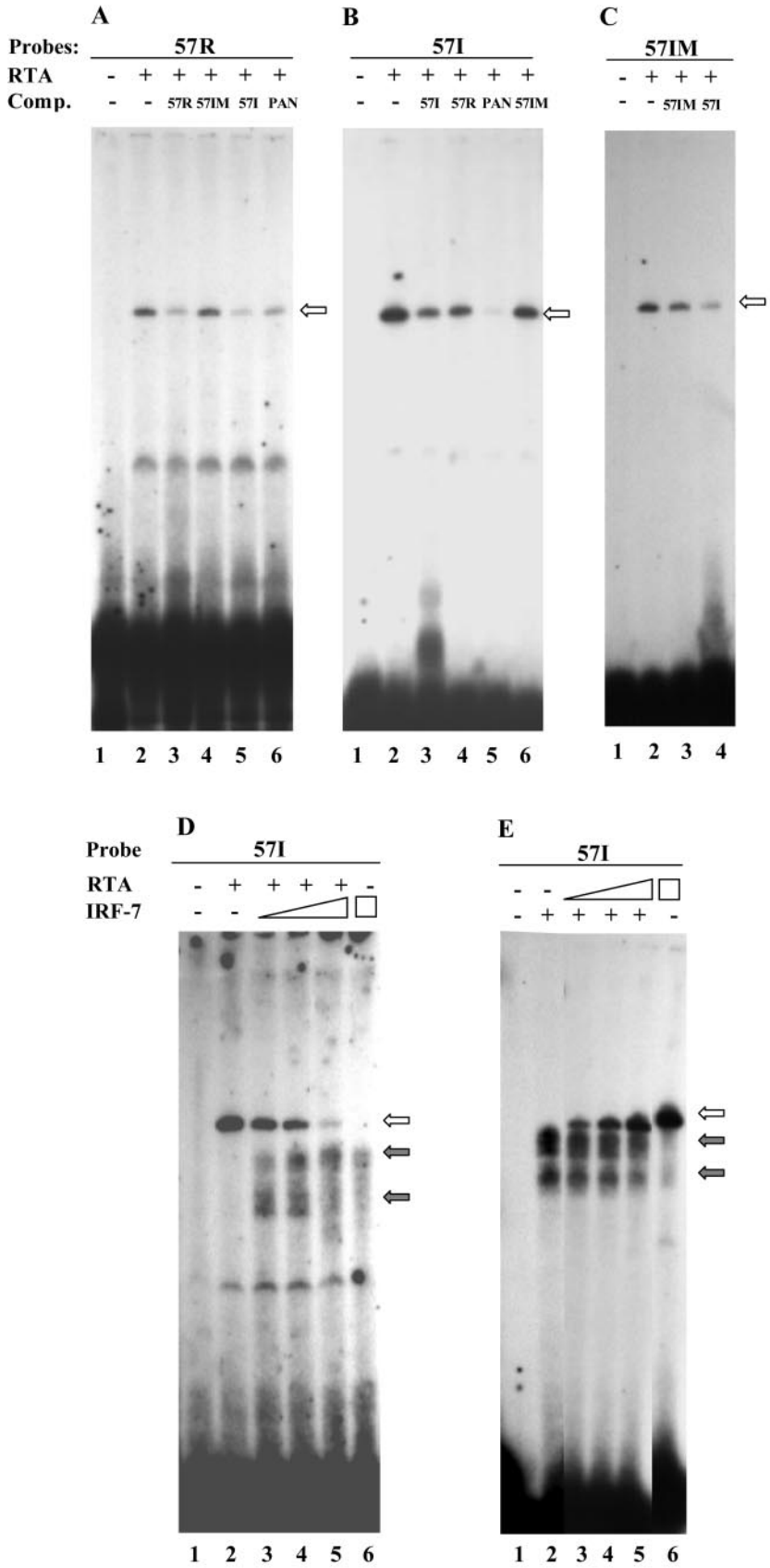
To identify the IRF-7 domains that are involved, two IRF-7 mutants were tested (Fig. 4B). IRF-7C retains the N-terminal 151-amino-acid DNA binding domain (DBD), and IRF-7DN has lost the DBD but retains the activation domain. These two IRF-7 variants were tested for their effects on ORF 57 promoter expression in the presence and absence of RTA. As shown in Fig. 4B, IRF-7C was found to repress RTA-mediated ORF 57 gene expression to the same level as full-length IRF-7, whereas IRF-7DN failed to repress. Both IRF-7C and IRF-7DN had no obvious effect on ORF 57 promoter activity when transfected alone in the absence of RTA (Fig. 4B). These results suggest that IRF-7 represses RTA transactivation of the ORF 57 promoter through the N-terminal DBD, whereas the activation and regulation domains are not required.

IRF-7 can compete for RTA binding to the ORF 57 promoter. The expression of ORF 57 can be stimulated by RTA, and the target site for RTA transactivation has been mapped to a 40-bp sequence encompassing nt 81904 to 81943 in the ORF 57 promoter (9, 29, 46, 47). The core element required for RTA responsiveness consists of a conserved 12-bp palindromic sequence (Fig. 1). It was reported that RTA directly binds to sequences within the palindrome, and both the palindrome and flanking sequences contribute to DNA binding and transactivation by RTA (29).

We show here that IRF-7 binds to the 40-bp region in the ORF 57 promoter and suppresses RTA-mediated activation; therefore, it is possible that IRF-7 competes with RTA for binding to this region and that such competition inhibits RTA activation. To determine the role of the palindrome and flanking sequences in mediating DNA binding by RTA and by IRF-7, two subfragments of the ORF 57 40-bp region, 57R and 57I (Fig. 1), were tested for binding to RTA and to IRF-7. Both the 57R and 57I probes were found to bind RTA, as indicated by the arrows in Fig. 5A and B. Interestingly, RTA protein appears to bind to 57I better than the 57R probe when the same amounts of probe and protein were added.

To confirm the binding of RTA to 57R and 57I, a competition assay was carried out with a 50-fold excess of an unlabeled 57I, 57R, PAN, or mutant (57IM) probe. The PAN probe was tested because it also contains an RRE known to bind RTA. The binding of RTA to both 57R and 57I probes was competed by its homologous unlabeled probe and by the PAN probe (Fig. 5A and B). Addition of the mutant 57IM probe (Fig. 5A, lane 4) had little effect on RTA binding to 57R but could reduce RTA binding to 57I by about 50% (Fig. 5B, lane 6), suggesting that the mutations in the 57I probe did not abolish RTA binding even though the binding of IRF-7 was affected (Fig. 2B, lane 4). Indeed, RTA was found to bind to the 57IM in EMSA (Fig. 5C, lane 2) where the addition of unlabeled 57IM and 57I competed for RTA binding. However, 57I appears to be more effective than 57IM (Fig. 5C, lanes 3 and 4), confirming that RTA binds to 57I with higher affinity than 57IM.

Since IRF-7 and RTA bound the same 19-nt sequence in the ORF 57 promoter (Fig. 2A and 5B), IRF-7 might compete with RTA for binding to this site and thereby repress RTA-mediated transactivation of the ORF 57 promoter. In order to test this possibility, EMSA was carried out in the presence of both RTA and IRF-7. Mammalian Flag-IRF-7 was used in these experiments due to the similarity in size between *E. coli*-expressed IRF-7 and RTA. As expected, in the absence of IRF-7, RTA binds to the 19-nt 57I probe (Fig. 5D, lane 2; Fig. 5E, lane 6), but RTA binding was decreased as increasing amounts of IRF-7 were added (Fig. 5D, lanes 3 to 5). In parallel, IRF-7 binding decreased as increasing amounts of RTA were added (Fig. 5E, lanes 3 to 5). No novel complex was observed in the presence of both RTA and IRF-7, suggesting that no stable protein-protein interaction between RTA and IRF-7 forms in this assay system. This failure of IRF-7 and RTA to directly interact was supported by coimmunoprecipitation and pull-down analysis (data not shown). Our results


A
Probes: 57R
RTA - + + + + +
Comp. - - 57R 57IM 57I PAN
1 2 3 4 5 6
B
57I
- + + + + +
- - 57I 57R PAN 57IM
1 2 3 4 5 6
C
57IM
- + + +
- - 57IM 57I
1 2 3 4
D
Probe 57I
RTA - + + + + -
IRF-7 - -
1 2 3 4 5 6
E
57I
- - - +
- + + + + -
1 2 3 4 5 6

also indicate that the 19-nt 57I probe can accommodate either IRF-7 or RTA but not both simultaneously. Therefore, IRF-7 can compete with RTA for binding to the IRF-7 binding site in the ORF 57 promoter and vice versa.

Induction of IRF-7 by IFN-α is involved in the repression of RTA-mediated transactivation of ORF 57 in HHV-8 infected Cells. Previous reports have shown that IFN-α can inhibit the expression of several viral genes and the accumulation of HHV-8 DNA in BCBL-1 cells subsequent to TPA induction (33). It is likely that this inhibition is mediated through IFN response genes, such as IRFs. To determine whether IRF-7 is involved in the suppression of lytic viral genes, such as ORF 57, TRE×BCBL-1 RTA cells were cultured in the presence or absence of IFN-α and then induced with tetracycline (Fig. 6A). In this cell line, the RTA gene is integrated into the BCBL-1 genome, and RTA expression is tightly controlled by tetracycline (35). The tetracycline-induced His-tagged RTA protein can be detected by anti-His_6 antibody, and the expression of viral RTA and ORF 57 can be detected specifically with anti-RTA and anti-ORF 57 antibodies. IFN-α treatment induced the expression of IRF-7 but did not alter the accumulation of RTA. Concomitant with the increase in IRF-7, ORF 57 expression was reduced by more than 50% (Fig. 6A, lanes 2 and 3). In control TRE×BCBL-1 cells without integrated RTA, IFN-α also induced the expression of IRF-7 as expected, but the RTA expression was not stimulated by tetracycline, and only very little ORF 57 expression was detected (Fig. 6A, lanes 5 to 8).

To confirm that IRF-7, but not other IRFs induced by IFN-α, is directly involved in repression of RTA-mediated activation of the ORF 57 promoter, an RNA interference construct, pGE1-IRF-7i, was used to specifically suppress IRF-7 expression subsequent to IFN-α stimulation. BJAB or DG75 cells were transfected with p57Pluc1 reporter plasmid, pCMV-Tag50, and various amounts of IRF-7i construct and then treated with IFN-α. In the absence of IRF-7i, IRF-7 was induced by IFN-α, and the RTA-mediated activation of ORF 57 promoter activity was repressed by 83% in BJAB cells and by 63% in DG75 cell (Fig. 6B). However, when pGE1-IRF-7i was transfected, the IFN-α-induced IRF-7 expression was reduced to an undetectable level. In parallel, the inhibitory effect of IRF-7 on RTA transactivation was reversed in a dose-dependent manner (Fig. 6B). These results suggest that IRF-7, but not other IRFs or other cellular genes, is responsible for the repression of RTA-mediated transactivation subsequent to IFN-α treatment.

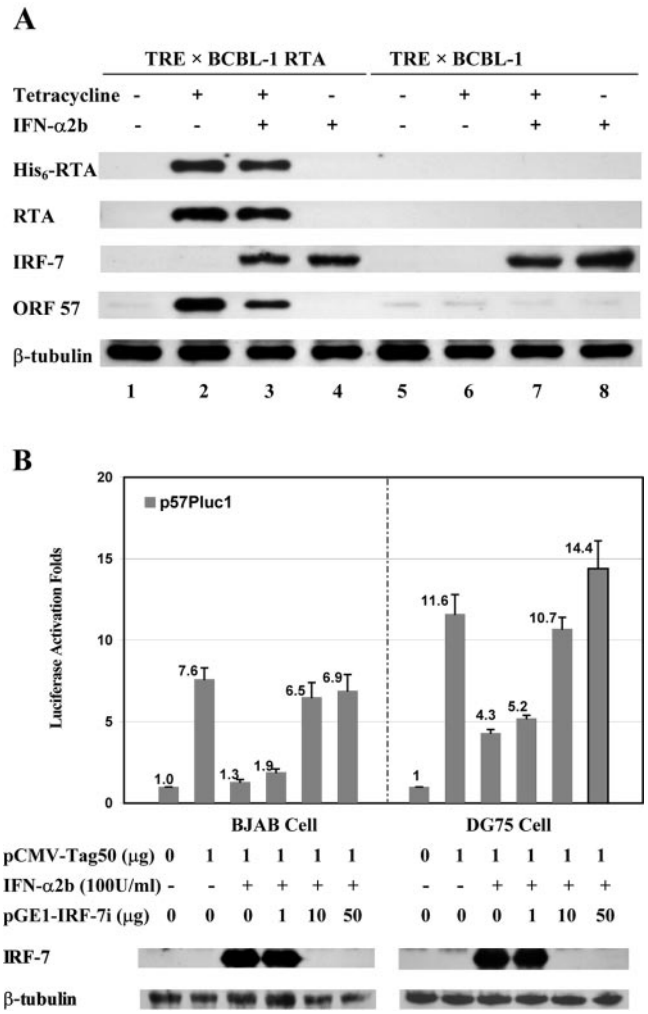


FIG. 6. IRF-7 is involved in repression of HHV-8 RTA-mediated transactivation upon treatment by IFN-α. (A) TRE×BCBL-1 RTA cells were cultured for 24 h in the presence or absence of IFN-α2b and subsequently induced with 1 μg of tetracycline/ml. At 8 h after induction, the cells were harvested and total protein (10 μg) was analyzed by Western blotting with the anti-His_6, anti-RTA, anti-IRF-7, anti-ORF 57, and anti-β-tubulin antibodies. The TRE×BCBL1 cells were treated with tetracycline and used as negative controls. (B) BJAB or DG75 cells were electroporated with pCMV-Tag50 and various amounts of IRF-7i and subsequently cultured in the presence or absence of 100 U of IFN-α2b/ml. Luciferase activity was measured. The expression levels of IRF-7 and β-tubulin in cell lysate (10 μg of total protein) were analyzed by Western blotting using anti-IRF-7 and anti-β-tubulin antibodies.

FIG. 5. Competitive binding to the ORF 57 RRE by RTA and IRF-7. (A) RTA binds to the consensus region of the ORF 57 RRE. Partially purified His-tagged RTA was incubated with labeled 57R probe. A 50-fold excess of specific competitors (57R in lane 3, 57I in lane 5, PAN in lane 6) and a mutated competitor (57IM in lane 4) were used. The arrow indicates the specific binding of RTA to the 57R probes. (B) RTA binds to the IRF-7 binding site of the ORF 57 promoter. His-tagged RTA was incubated with labeled 57I probe. The arrow indicates the specific binding of RTA to the 57I probe. A 50-fold excess of specific competitors (57I in lane 3, 57R in lane 4, PAN in lane 5) and a mutated competitor (57IM in lane 6) were used. (C) EMSA of the 57IM probe with RTA. His-tagged RTA protein was incubated with labeled 57IM probe. The arrow indicates the specific binding of RTA to the 57IM probe. A 50-fold excess of specific competitors (57IM in lane 3 and 57I in lane 4) was used. (D) Competition of RTA binding to the 57I probe by IRF-7. His-tagged RTA (0.1 μg) (lanes 2 to 5) was incubated with labeled 57I probe and increasing amounts of Flag-IRF-7 (50 ng in lane 3, 0.15 μg in lane 4, or 0.45 μg in lanes 5 and 6). The open and shaded arrows indicate the binding of RTA and IRF-7 to the 57I probe, respectively. (E) Competition of IRF-7 binding by RTA. Flag-IRF-7A protein (0.5 μg) (lanes 2 to 5) was incubated with labeled 57I probe and increasing amounts of His-tagged RTA (20 ng in lane 3, 60 ng in lane 4, or 0.2 μg in lanes 5 and 6). Open and shaded arrows indicate the specific binding of RTA and IRF-7 to 57I probe, respectively.

DISCUSSION

Two key steps in the life cycle of all herpesviruses, including HHV-8, are the establishment of latency and the ability to reactivate from latency to lytic replication (34). For HHV-8, ORF 50/RTA has been found to be sufficient to initiate lytic gene expression in latently infected PEL cells (10, 27, 28, 43). One of the genes affected by RTA is ORF 57, which is an important viral lytic gene. ORF 57 is conserved, and its homologues can be found throughout the herpesvirus family members. These homologues include BMLF1 of EBV, IE63 of herpes simplex virus type 1 (HSV-1), UL69 of human cytomegalovirus, ORF 57 of herpesvirus saimiri, and gene 4 of varicella-zoster virus. For HSV-1, the ORF 57 homologue was found to bind RNA in vivo, enhance RNA 3′ processing, inhibit pre-mRNA splicing, and perhaps facilitate the nuclear export of HSV-1 transcripts. HHV-8 ORF 57 has *trans*-regulatory functions that appear to be mediated at the posttranscriptional level and are essential for production of infectious virus (1). Thus, down-regulation of RTA-mediated ORF 57 expression is likely to have an effect on HHV-8 lytic replication. Even though we have found an IRF-7 binding site in the HHV-8 ORF 57 promoter, it is not clear whether similar IRF-7 binding sites can be located within other ORF 57 homologous genes. It is also not clear whether there are IRF-7 binding sites located in other HHV-8 genes. A preliminary analysis of the HHV-8 genome revealed a potential IRF-7 binding site located in the promoter of the K14 gene. Whether this is a functional IRF-7 binding site and whether the binding of IRF-7 modulates K14 gene expression need to be determined.

RTA not only is essential for viral reactivation but also plays an important role in the pathogenesis of HHV-8 infection (8, 48). A number of previous studies have focused on elucidating the mechanism whereby RTA induces lytic gene expression (25, 29, 42, 48). The direct binding of RTA to specific promoter RRE sequences was shown to contribute to the activation of various early and late viral genes during lytic replication, and the expression of these genes is modulated by the DNA binding affinity of RTA for the various target sites (42). A number of cellular and viral coactivators have been described as playing a role in RTA-mediated transcription activity, where most were found to enhance lytic gene expression in the presence of RTA (13, 14, 23, 24, 46, 47). These RTA-interacting factors may also play a role in modulating the binding affinity of RTA to its target sites.

In spite of what is known about the induction of lytic gene expression by RTA, very little is known as to how RTA activities can be down-modulated in order for the virus to establish or maintain latency. Whether down-regulation is mediated by the repression of RTA expression or by the repression of RTA transactivation function needs to be clarified. It was previously reported that RTA-mediated gene expression can be down-regulated by its interaction with histone deacetylase and latent nuclear antigen and by the inhibitory effects of phosphorylation by kinases such as PARP-1 and hKFC (13, 15, 22). In this study, we have further explored the mechanism whereby RTA-mediated gene expression is modulated and found that IRF-7 can act as a competitor of RTA in regulation of ORF 57 gene expression. The up-regulation of ORF 57 promoter-mediated gene expression by RTA was suppressed in the presence of IRF-7 (Fig. 3) even though IRF-7 by itself is a weak transactivator of ORF 57 (Fig. 2). This inhibition is likely due to competitive binding between IRF-7 and RTA, resulting in the suppression of RTA recruitment onto the viral lytic ORF57 gene promoter region by IRF-7, followed by the inhibition of RTA-mediated transcriptional activation and viral lytic replication.

The RRE in the HHV-8 ORF 57 promoter contains two RTA binding sites, and IRF-7 acts as an RTA competitor for one of the sites. The RRE of ORF 57 was previously characterized and was found to be a target for either direct RTA binding or indirect binding mediated by cellular factors, such as RBPjκ (23, 24, 28, 42). The RRE has been mapped to a 40-bp region which contains a consensus sequence found in the promoters of other RTA-responsive genes, such as K8 (29, 45). In this study we identified two RTA binding sites in the ORF 57 RRE region. The first is the 31-bp 5′ region which also contains the K8 consensus sequences (Fig. 1), and the second site overlaps with the first site and is located towards the 19-bp 3′ region of the RRE. The second site, but not the first site, can be used by both RTA and IRF-7, and RTA appears to bind with a stronger affinity to the second site based on our mobility shift analysis (Fig. 5A and B).

The presence of two RTA binding sites in the ORF 57 RRE suggests that two RTA molecules can bind to the RRE either as a dimer or as two separate monomers. The two binding sites are distinct; one is the RTA binding site, and the other is the RTA/IRF-7 binding site. It is possible that the binding affinities mediated by these two sites are quite different, and the binding of one or both could even be mediated by a cellular factor, such as RBPjκ (23). We also do not know whether the same binding domain is used by RTA for each site. Thus, the affinity of RTA for each binding site and the mechanism of binding need to be further investigated. In addition, given the effective competition of RTA binding by IRF-7 to the second site and the close proximity of the two sites, it is likely that RTA may bind as a dimer to this ORF 57 RRE region. In our proposed model (Fig. 7), we hypothesize that the binding of RTA to both sites in the ORF 57 promoter RRE region will result in strong activation of ORF 57 expression. In such a model, activation is anticipated to be mediated via RTA dimerization and the recruitment of other transcriptional factors and RNA polymerase to the promoter region. The displacement of at least one RTA from the RRE upon binding of IRF-7 to the proposed IRF-7 binding site would result in weak or no activation. It is possible that HHV-8 uses the inhibitory effect of IRF-7 binding to regulate between lytic and latent infection and that inhibition of ORF 57 expression by IRF-7 will favor latency. Further study will be needed to provide evidence for or against this hypothesis.

IFN inhibits HHV-8 lytic replication, possibly via suppression of RTA activity by IRF-7. IFN-α has been shown to have strong inhibitory effects on HHV-8 gene expression. In addition, blocking of IFN-α with neutralizing antibodies resulted in more-extensive cytopathic effects and more-efficient viral particle release in these cells (33). In vivo, IFN-α inhibits HHV-8 reactivation, reduces HHV-8 viral load in peripheral blood mononuclear cells from patients with KS, and has been successfully used in therapy for KS (21, 33).

The mechanism whereby IFN represses HHV-8 replication

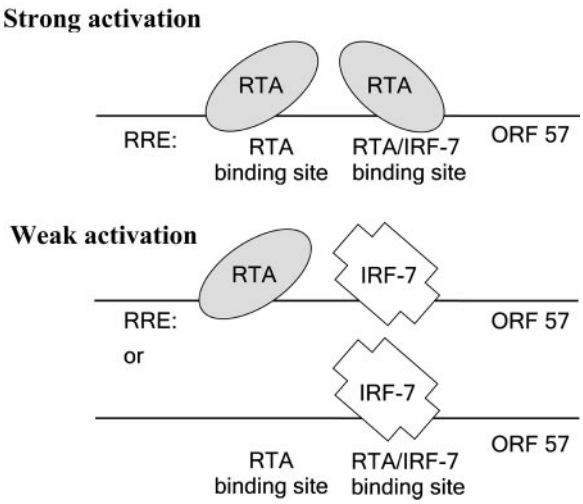


FIG. 7. A proposed model for the repression of HHV-8 RTA-mediated transactivation of ORF 57 by IRF-7. Strong activation represents the binding of RTA to two sites in the ORF 57 promoter RRE region. Weak activation represents the displacement of at least one RTA from the RRE upon binding of IRF-7 to the IRF-7 binding site.

is unclear. The effect could be mediated directly by IFN or could be indirect via other IFN-induced cellular or viral genes. In this study we showed that at least one IFN-induced cellular protein, IRF-7, plays a role in inhibition. IRF-7 is one of the most strongly induced cellular transcripts after de novo HHV-8 infection and TPA-stimulated reactivation (38). Using HHV-8-infected and uninfected cells, we demonstrated that both endogenously expressed and transfected IRF-7 can compete for the RTA binding site within the ORF 57 RRE region and inhibit RTA-mediated activation. This suggests that IFN-α might block HHV-8 reactivation and lytic replication through the competition of IRF-7 with RTA by modulating downstream viral genes, such as ORF 57. Since the activation of the ORF 57 promoter by RTA cannot be completely suppressed even in the presence of excess of IRF-7 (Fig. 3A), it suggests that other viral and/or cellular factors may also be involved in regulating RTA transactivation during infection. Indeed, it has recently been demonstrated that the HHV-8 latent nuclear antigen protein also plays a role in regulating RTA function by directly interacting with RTA (22). It is possible that other IFN-induced genes, in addition to IRF-7, may also be involved in modulating lytic HHV-8 replication. However, our demonstration that RNA interference specific for IRF-7 can eliminate the inhibitory effects of RTA-mediated ORF 57 gene expression upon induction by IFN (Fig. 6B) argues against a role for other cellular proteins or IRFs in mediating the inhibition of ORF 57 expression. At this point, it is premature to assume that there is no role for other IRFs in modulation of the expression of other HHV-8 genes. The roles of other IRFs, as well as the role of four HHV-8-encoded IRF homologues, in HHV-8 replication and/or latency are a topic of continuing investigation.

IRF-7 is an important cellular factor involved in modulating viral gene expression upon HHV-8 infection. It also plays a major role in the interferon-mediated gene expression pathway and the antiviral innate immune response. It is likely that IRF-7 could affect viral lytic replication via multiple mechanisms. One mechanism is the suppression of viral replication as suggested by our study. In fact, it has recently been shown that HHV-8 encodes a protein, ORF 45, which is expressed early upon viral infection and blocks the phosphorylation and nuclear accumulation of IRF-7 (54). This countermeasure for IRF-7 could be a mechanism that the virus has developed to overcome the inhibitory effect of IRF-7 and therefore enable the virus to go through lytic replication. Further analysis is required to understand the interplay among IRF-7, RTA, and ORF 45 in the regulation of HHV-8 RTA activity and lytic replication.

Since RTA is sufficient for the induction of the viral lytic cycle, the control of RTA-mediated expression is anticipated to play a pivotal role in the maintenance of viral latency. Certain protein modifications or negative regulatory factors that inactivate RTA transactivation could serve to limit the extent of lytic replication or may even lead to the abortion of lytic replication (20). Our results support the recently published gene array data indicating that during early infection, in the presence of interferon and IRF-7, ORF 50 was expressed, followed by brief expression of a limited number of lytic viral genes, but ORF 57 expression was not detected (20). This could be due to the suppression of RTA-mediated ORF 57 activation by IRF-7, which then favors the establishment of latency and persistent infection. Similarly, during reactivation, the expression of RTA induced lytic replication, which then induced interferon and IRF-7, and subsequently shut down lytic replication to reestablish latency. Our results suggest that IRF-7, one of the IFN-induced products, is usurped by the virus to inhibit viral lytic reactivation, to maintain HHV-8 latency, and to escape from the host immune response. Our study also suggests a complex interplay among RTA activation pathways, the repression of those pathways by IRF-7, and the potential regulation of this system by other viral proteins, such as ORF 45. Such a system provides sensitivity to the cellular environmental conditions that promote latency or lytic replication when appropriate and may provide mechanistic bases for IFN-α therapy of Kaposi's sarcoma and other HHV-8-related diseases. This interplay between the host innate immunity and the ability of the invading virus to make use of the host defense mechanism to regulate its own replication may be a general mechanism not strictly limited to HHV-8.

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