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VIRUS-CELL INTERACTIONS

Human Herpesvirus 6A Tegument Protein U14 Induces NF-κB Signaling by Interacting with p65

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ABSTRACT Viral infection induces host cells to mount a variety of immune responses, which may either limit viral propagation or create conditions conducive to virus replication in some instances. In this regard, activation of the NF-κB transcription factor is known to modulate virus replication. Human herpesvirus 6A (HHV-6A), which belongs to the *Betaherpesvirinae* subfamily, is frequently found in patients with neuroinflammatory diseases, although its role in disease pathogenesis has not been elucidated. In this study, we found that the HHV-6A-encoded U14 protein activates NF-κB signaling following interaction with the NF-κB complex protein, p65. Through induction of nuclear translocation of p65, U14 increases the expression of interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein 1 transcripts. We also demonstrated that activation of NF-κB signaling is important for HHV-6A replication, since inhibition of this pathway reduced virus protein accumulation and viral genome copy number. Taken together, our results suggest that HHV-6A infection activates the NF-κB pathway and promotes viral gene expression via late gene products, including U14.

IMPORTANCE Human herpesvirus 6A (HHV-6A) is frequently found in patients with neuro-inflammation, although its role in the pathogenesis of this disease has not been elucidated. Most viral infections activate the NF-κB pathway, which causes the transactivation of various genes, including those encoding proinflammatory cytokines. Our results indicate that HHV-6A U14 activates the NF-κB pathway, leading to upregulation of proinflammatory cytokines. We also found that activation of the NF-κB transcription factor is important for efficient viral replication. This study provides new insight into HHV-6A U14 function in host cell signaling and identifies potential cellular targets involved in HHV-6A pathogenesis and replication.

KEYWORDS HHV-6, NF-κB, gene expression, herpes, tegument

Human herpesvirus 6A (HHV-6A) is a ubiquitous virus that, together with the closely related HHV-6B virus, belongs to the *Roseolovirus* genus within the *Betaherpesvirinae* subfamily (1–5). HHV-6A is frequently found in patients with neuroinflammatory diseases such as multiple sclerosis and has been associated with Alzheimer's disease, although whether primary infection with HHV-6A has a causal role in these or other illnesses has not yet been determined (6–8).

The protein encoded by the HHV-6A U14 gene is a tegument protein that is essential for viral replication (9). HHV-6A U14 belongs to the pp85 superfamily that is shared among betaherpesviruses and has no homologs in alpha- or gammaherpesviruses (5, 10, 11). The U14 genes of HHV-6A, HHV-6B, and HHV-7 share relatively high sequence homology. Other members of the *Betaherpesvirinae*, including human cytomegalovirus (HCMV), encode two tegument proteins (UL25 and UL35) that belong to the pp85 superfamily (12–14); their sequence identities with U14 are less than 20%. The HHV-6 U14 protein has physical and functional interactions with host cellular machineries. U14 of HHV-6A and HHV-6B associate with the tumor suppressor protein p53 in the nucleus and cytoplasm and is incorporated into virions with p53 (15). HHV-6A U14 induces cell cycle

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arrest in G_2/M phase by associating with the host cell protein, EDD, during the early phase of infection (16). Furthermore, HHV-6A U14 is important for virion maturation since deletion of three amino acids in the HHV-6A U14 sequence severely compromises viral growth and virion maturation (9). These results indicate that HHV-6 U14 is a multifunctional protein, although the precise molecular mechanisms that underlie its role in the infection cycle and disease pathogenesis remain poorly understood.

The transcription factor NF-κB plays a pivotal role in many cellular events such as apoptosis, cell proliferation, inflammation, and immunity (17–19). The two components of NF-κB, p65 and p50, are retained in the cytoplasm and are bound to the inhibitor of NF-κB proteins, IκBα. The canonical NF-κB pathway is regulated by a cascade of events that initiates upon receptor stimulation and activation of the IκB kinase (IKK) complex. The activated IKK complex phosphorylates IκBα, which is then polyubiquitinated, resulting in its proteasomal degradation (20). Subsequently, the NF-κB p65/p50 heterodimer is released from cytoplasmic sequestration and translocates into the nucleus, where it binds to DNA and initiates expression of downstream genes, including those that encode cytokines (18, 21–23).

While many viruses have evolved mechanisms that block the NF-κB pathway to evade host immune responses and allow viral replication, some viruses actually utilize this pathway for their propagation (17, 18, 23). For example, human immunodeficiency virus type 1 (HIV-1) replication is enhanced in activated T cells, which are driven by the NF-κB pathway (24). Indeed, HIV-1-encoded gp120 and Tat can trigger NF-κB activation (25–28). The induction of NF-κB signaling by the X protein of hepatitis B virus (HBx) probably promotes hepatocarcinogenesis (29, 30). Epstein-Barr virus (EBV), through its latency membrane protein 1, activates NF-κB signaling pathways to promote the growth of B lymphoblastoid cells, in which EBV establishes latent infection (31, 32). As with EBV, infection with other herpesviruses, including herpes simplex virus 1 (HSV-1) and human cytomegalovirus (HCMV), leads to a biphasic induction of NF-κB signaling (33, 34). Immediately after infection, HSV-1 envelope protein gD and tegument protein UL37, as well as HCMV envelope proteins gB and gH, activate NF-κB; a second wave of NF-κB activation then occurs after viral genome replication (34–36).

HHV-6A is also an important member of the herpesvirus family that establishes lifelong latent infection in humans. However, the relationship between NF-κB signaling and HHV-6A replication has not been analyzed in detail. Here, we focus on the role of the HHV-6A tegument protein, U14, in NF-κB signaling. We report that NF-κB-responsive promoters are activated in cells expressing exogenous HHV-6A U14. We also find that HHV-6A U14 interacts with the NF-κB subunit p65, which may contribute to enhanced DNA binding and transcriptional activity of the NF-κB complex. Our results also indicate that NF-κB pathway activation is important for HHV-6A gene expression. Collectively, the results of our study elucidate a novel interaction between HHV-6A and the NF-κB pathway that might be important for viral replication.

RESULTS

HHV-6A U14 activates NF-κB signaling. Some herpesvirus tegument proteins can modulate intracellular signaling pathways. We thus determined whether the HHV-6A tegument protein, U14, would elicit similar effects. HEK293T cells were cotransfected with hemagglutinin-tagged HHV-6A U14 (HA-U14) and a series of firefly luciferase reporter plasmids harboring an NF-κB-responsive element (NF-κB-luc), the beta interferon promoter (IFN-β-luc), an antioxidant response element (ARE-luc), or a cyclic AMP response element (cAMP-luc). As shown in Fig. 1A, ectopic expression of HA-tagged U14 significantly stimulated the activity of NF-κB-luc. This was selective, since none of the other reporters were activated by HA-U14 significantly (Fig. 1A and B).

To determine the specificity of the HA-U14 mediated NF-κB pathway, we carried out another round of reporter assays. HEK293T cells were transfected with NF-κB-luc or CRE-luc reporter plasmid and then stimulated with either TNF-α or colforsin. As previously described (18, 37), tumor necrosis factor alpha (TNF-α) and colforsin

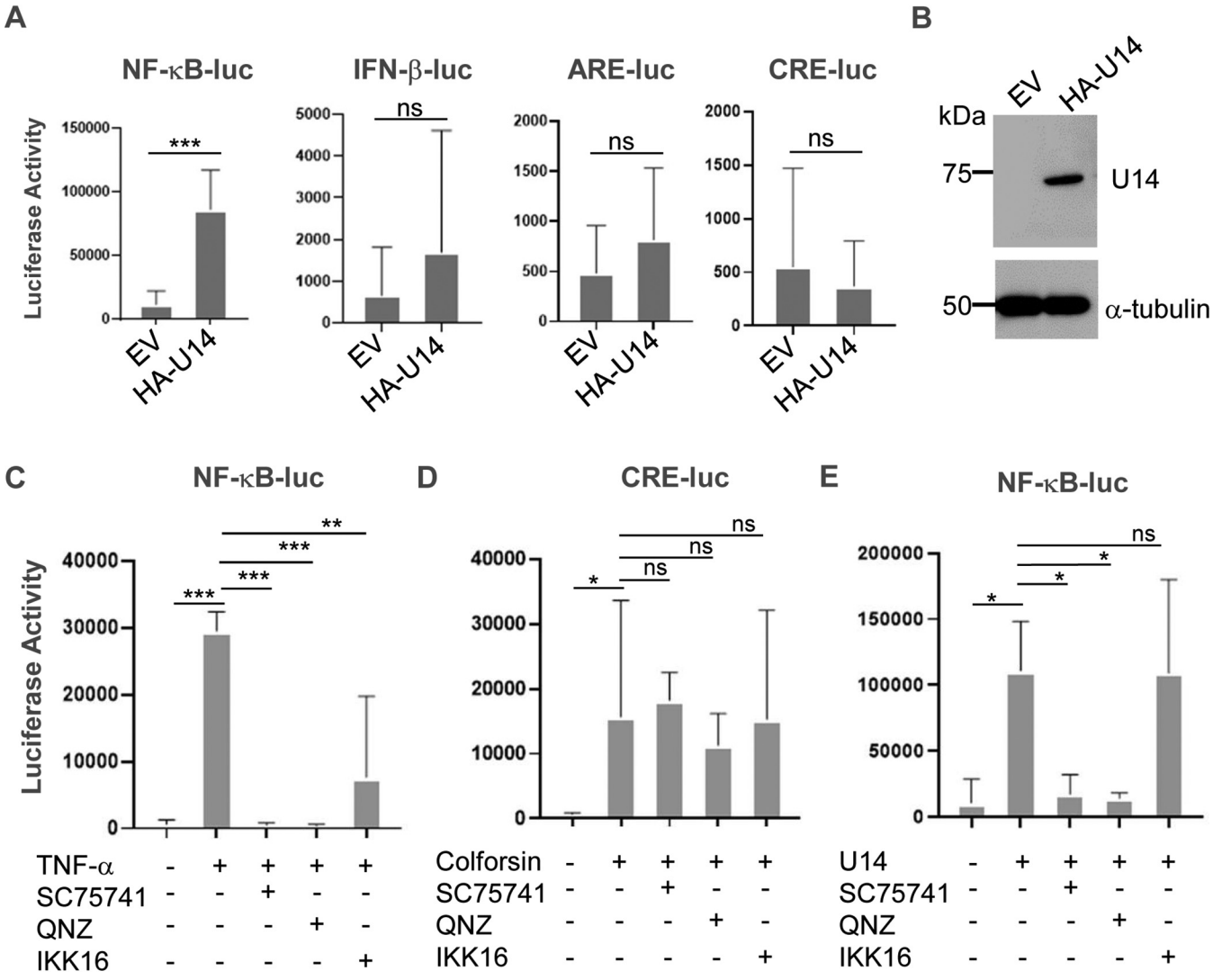

FIG 1 Exogenous expression of HHV-6A U14 induces the activation of NF-κB promoter. (A) HEK293T cells were cotransfected with either a NF-κB-luc, IFN-β-luc, ARE-luc, or CRE-luc reporter plasmid, together with pRL-CMV as an internal control plasmid, along with either an empty vector or plasmid expressing HA-U14. The luciferase activity was measured at 24 h posttransfection. The means and standard deviations for each data set are shown ($n = 7$ for NF-κB, $n = 4$ for IFN-β, ARE and CRE; ***, $P < 0.001$ [unpaired Student t test]). (B) HEK293T cells were transfected with HA-U14 expression plasmid or empty plasmid (EV). At 24 h posttransfection, the cells were analyzed using immunoblot. (C to E) HEK293T cells were cotransfected with NF-κB-luc (C and E) or CRE-luc (D) reporter plasmid with (E) or without (C and D) the plasmid expressing HA-U14. At 24 h after transfection, TNF-α (C) or colforsin (D) was added for 3 h, followed by SC75741, QNZ, or IKK16 treatment for another 2 h prior to determine the firefly luciferase activity. The data are shown as means and standard deviations ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ [Tukey's test]).

significantly stimulated the activity of NF-κB-luc or CRE-luc, respectively (Fig. 1C and D). We then analyzed the effects of the following NF-κB inhibitors. SC75741 impairs DNA binding of the NF-κB subunit p65 (38, 39), whereas QNZ indirectly inhibits NF-κB activity through store-operated calcium entry (40). IKK16 is a selective inhibitor of the IKK protein, which is required for NF-κB signaling (41, 42). SC75741, QNZ, or IKK16 significantly reduced TNF-α-driven NF-κB activation but did not reduce colforsin-dependent cAMP pathway activation (Fig. 1C and D). As shown in Fig. 1E, SC75741 and QNZ impaired HA-U14-dependent stimulation of NF-κB-luc activity, whereas the IKK inhibitor, IKK16, had no effect. These observations suggest that HHV-6A U14 specifically stimulates the NF-κB pathway in an IKK-independent manner.

During canonical NF-κB signaling, a dimeric complex comprised of the NF-κB subunits p65 and p50 translocates to the nucleus and stimulates gene transcription (18). To determine whether HHV-6A U14 induces this translocation, we performed subcellular fractionation experiments. As shown in Fig. 2A, p65 was detected in both nuclear and

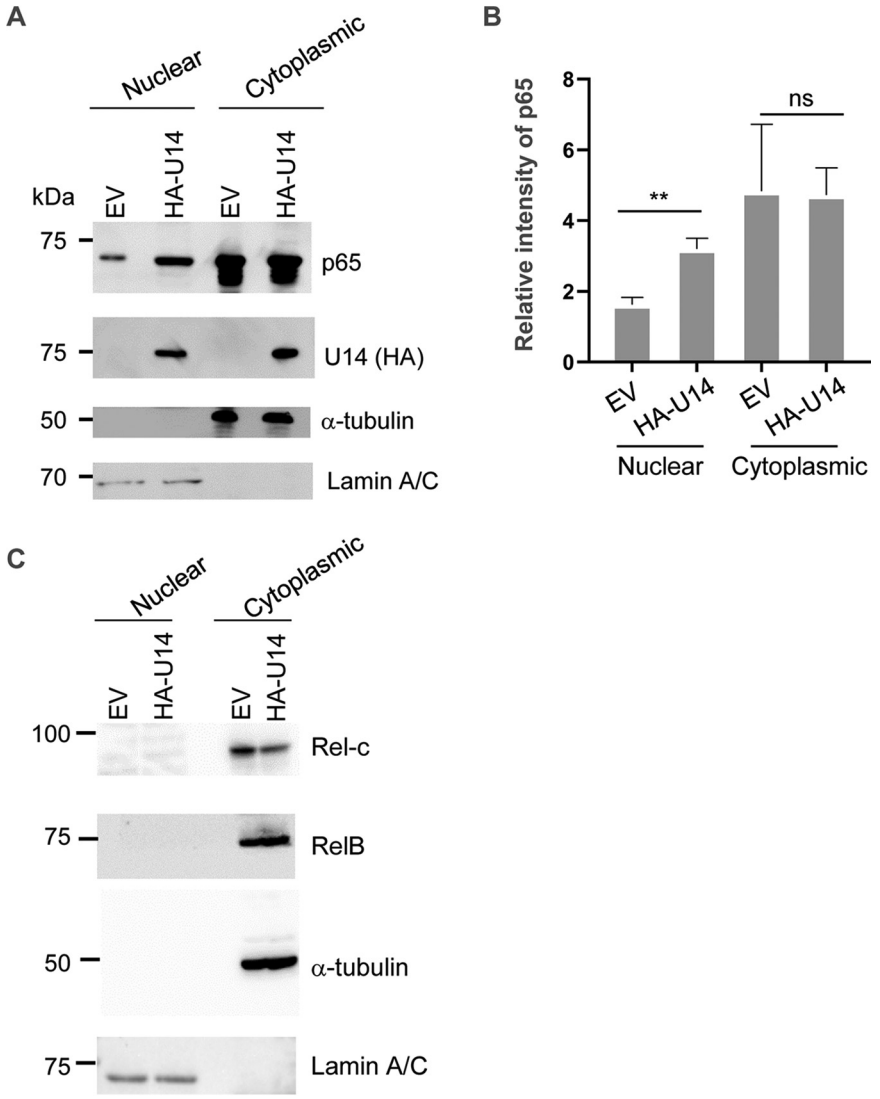

FIG 2 Exogenous expression of HHV-6A U14 induces the nuclear translocation of NF-κB protein p65. (A) HEK293T cells were transfected with the HA-U14 expression plasmid or empty plasmid (EV). At 24 h posttransfection, the cells were fractionated into cytoplasmic and nuclear fractions. (B) The intensities of p65 in each fraction, normalized to those of Lamin A/C or α-tubulin, respectively, are shown as means $\pm$ the standard deviations (n = 3; **, $P < 0.01$ [unpaired Student t test]). (C) The noncanonical RelB subunit and the other canonical NF-κB proteins, c-Rel are also shown.

cytoplasmic fractions. However, the amount of p65 in the nuclear fraction was significantly increased in cells transfected with HA-U14 expression plasmid compared to those transfected with the empty plasmid (Fig. 2A and B). In both conditions, α-tubulin and Lamin A/C were detected only in the cytoplasmic and nuclear fractions, respectively (Fig. 2A). On the other hand, the noncanonical NF-κB subunit RelB was undetected in the nuclei of HA-U14-expressing cells despite its abundance in the cytoplasm (Fig. 2C). Another canonical NF-κB protein, c-Rel, was also absent from the nuclei of cells expressing HA-U14 (Fig. 2C). Together, these results suggest that HHV-6A U14 expression selectively induces the nuclear translocation of the p65 NF-κB protein.

HHV-6A U14 increases the expression of NF-κB-regulated genes. Activation of NF-κB induces the expression of genes encoding growth factors, cytokines, and chemokines and their receptors; this results in modulation of host cell proliferation and the innate immune response (17, 18). To determine whether HHV-6A U14 modulates the expression of NF-κB-regulated genes, we analyzed the levels of NF-κB target

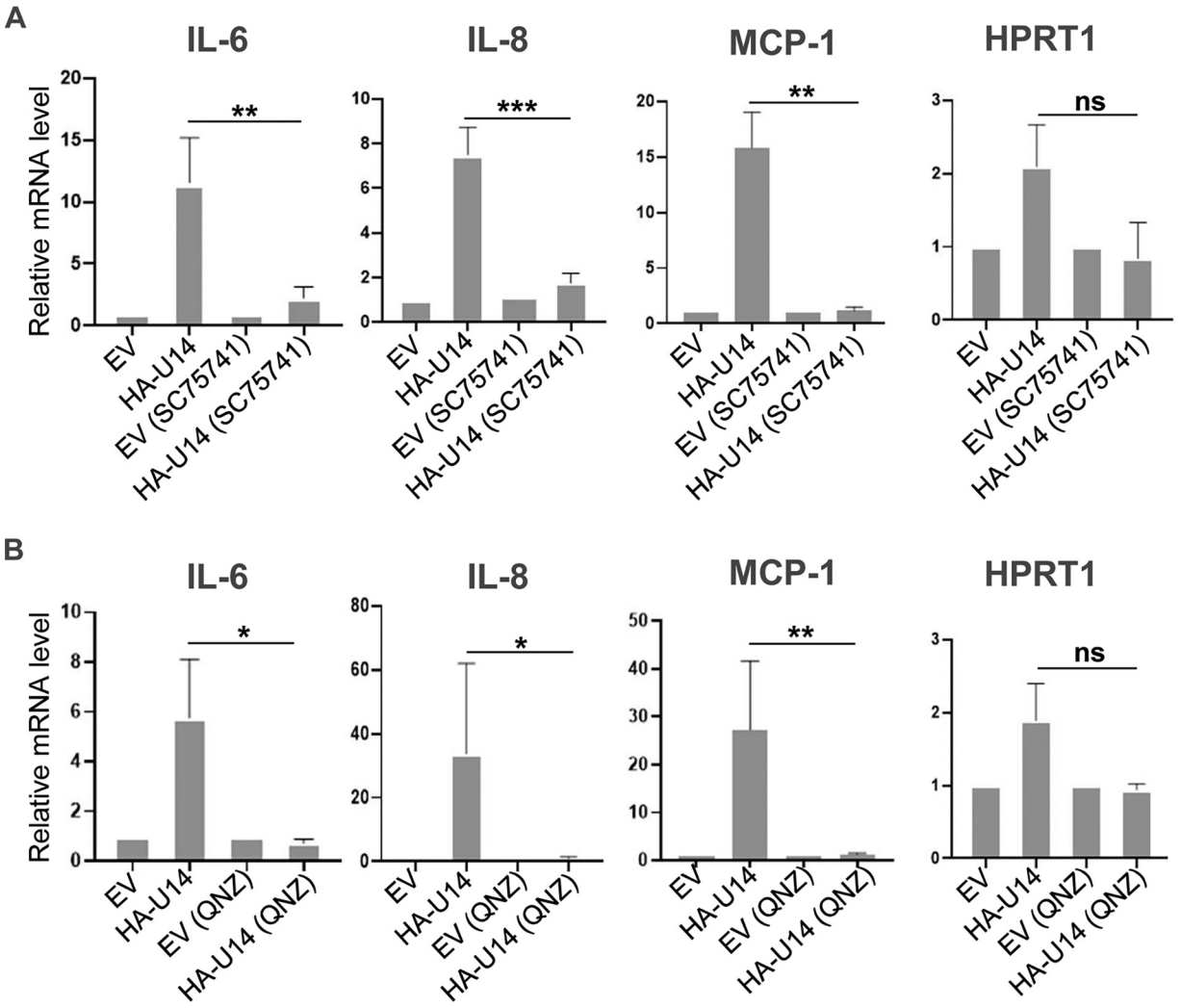

FIG 3 Exogenous expression of HHV-6A U14 increases the expression of NF-κB- regulated genes. HEK293T cells were transfected with HA-U14 expression plasmid or empty plasmid (EV). At 24 h posttransfection, cells were either treated with 5 μM SC75741 or 10 μM QNZ, while another set of cells was left untreated. Medium was then changed after 10 min to remove the drug, and RNA was extracted from cells at 1 h after treatment. The expression of *IL-6*, *IL-8*, *MCP1*, and *HPRT1* mRNA was quantified using quantitative real-time PCR (qRT-PCR) for those treated with SC75741 (A) or QNZ (B). Relative mRNA amounts were normalized to those of β-actin. The data are shown as means ± the standard deviations ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ [unpaired Student *t* test]).

genes in the cells expressing HHV-6A U14. Quantitative PCR (qPCR) revealed that transfection of cells with HA-U14 significantly increased transcription of *IL-6*, *IL-8*, and *MCP-1*, all of which are NF-κB-regulated genes (Fig. 3). However, the level of *HPRT1*, which is not an NF-κB target, was unaffected (Fig. 3). Incubation with the NF-κB inhibitors SC75741 (Fig. 3A) or QNZ (Fig. 3B) for 60 min blocked the HHV-6A U14-dependent increases in gene expression.

Interaction of HHV-6A U14 with NF-κB regulatory proteins. To determine the step in the signaling pathway at which HHV-6A U14 induced NF-κB activation, we performed coprecipitation experiments in cells transfected with Strep-tagged HHV-6A U14 (Strep-U14) or control plasmid. As shown in Fig. 4, the NF-κB component p65 was specifically coprecipitated with Strep-U14. Of note, we could not detect precipitation of the inhibitory factor IκBα with Strep-U14. The NF-κB signaling pathway is regulated by the ubiquitin-proteasome system, and IκBα degradation leads to nuclear entry of NF-κB and its subsequent binding to DNA. However, we did not detect reduction of IκBα in HHV-6A-U14-expressing cells in our hands (Fig. 4).

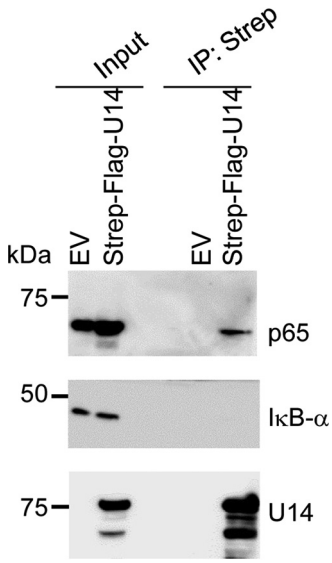

FIG 4 HHV-6A U14 interaction with the NF-κB proteins. HEK293T cells were transfected with Strep-Flag-U14 expression plasmid or empty plasmid (EV). At 24 h posttransfection, the cells were lysed, and the extracts were subjected to precipitation using Strep-Tactin beads, followed by immunoblotting.

To further confirm the interaction of HHV6-A U14 with the NF-κB components, we analyzed their subcellular localization using confocal microscopy. In agreement with our previous observation, p65 was cytoplasmic in cells transfected with control plasmid (Fig. 5). After transfection with HA-U14, p65 was colocalized with HA-U14 (Fig. 5). Approximately 60% of cells showed p65 and HA-U14 colocalization in the nucleus and the cytoplasm; p65 and HA-U14 were only detected in the cytoplasm in the remaining 40% of cells (Fig. 5). In both cases, fluorescence line scanning revealed a partial colocalization of HA-U14 and p65. Taken together, these results suggest that there is a physical and functional interaction between HHV-6A and the NF-κB component, p65.

NF-κB is activated in HHV-6A-infected cells. Next, we focused on whether HHV-6A U14-dependent activation of NF-κB is also observed in HHV-6A-infected cells. To do this, first we examined the nuclear localization pattern of p65 in infected cells. The HHV-6A permissive $CD4^+$ T cell line, JJhan, was either mock infected or infected with HHV-6A U1102, and cells were subsequently fractionated into cytoplasmic and nuclear compartments. As shown in Fig. 6, p65 was detected in the nuclear fraction of HHV-6A-infected cells, whereas p65 was detected to a lesser extent in this fraction in mock-infected cells. This increasement of nuclear p65 in infected cells was statistically significant (Fig. 6B). Consistent with the transfection data from Fig. 2, HHV-6A U14 was detected in both nuclear and cytoplasmic fractions following infection.

Then, we analyzed the expression of NF-κB-regulated cytokines in HHV-6A-infected JJhan cells in the presence or absence of SC75741. As shown in Fig. 7, the levels of *IL-6*, *IL-8*, and *MCP-1* mRNA, as well as the viral IE1 and U14 mRNA, were significantly increased in HHV-6A-infected cells compared to mock-infected cells. Furthermore, treatment with NF-κB inhibitor SC75741 reduced the levels of these mRNAs, although the level of IE1 reduction by SC75741 was not statistically significant (Fig. 7). The expression of *HPRT1*, which is not regulated by NF-κB, was almost unchanged following HHV-6A infection (Fig. 7). These results suggest that NF-κB signaling is activated in HHV-6A-infected JJhan cells.

Since an interaction between HHV-6A U14 and NF-κB proteins was observed following transient expression of HHV-6A U14 (Fig. 4), we next determined whether this interaction also occurred in infected cells. As expected, anti-HHV-6A U14 antibody coprecipitated the NF-κB component p65 from lysates of HHV-6A-infected cells but not from mock-infected cells (Fig. 8). Consistent with the transient expression of HHV-

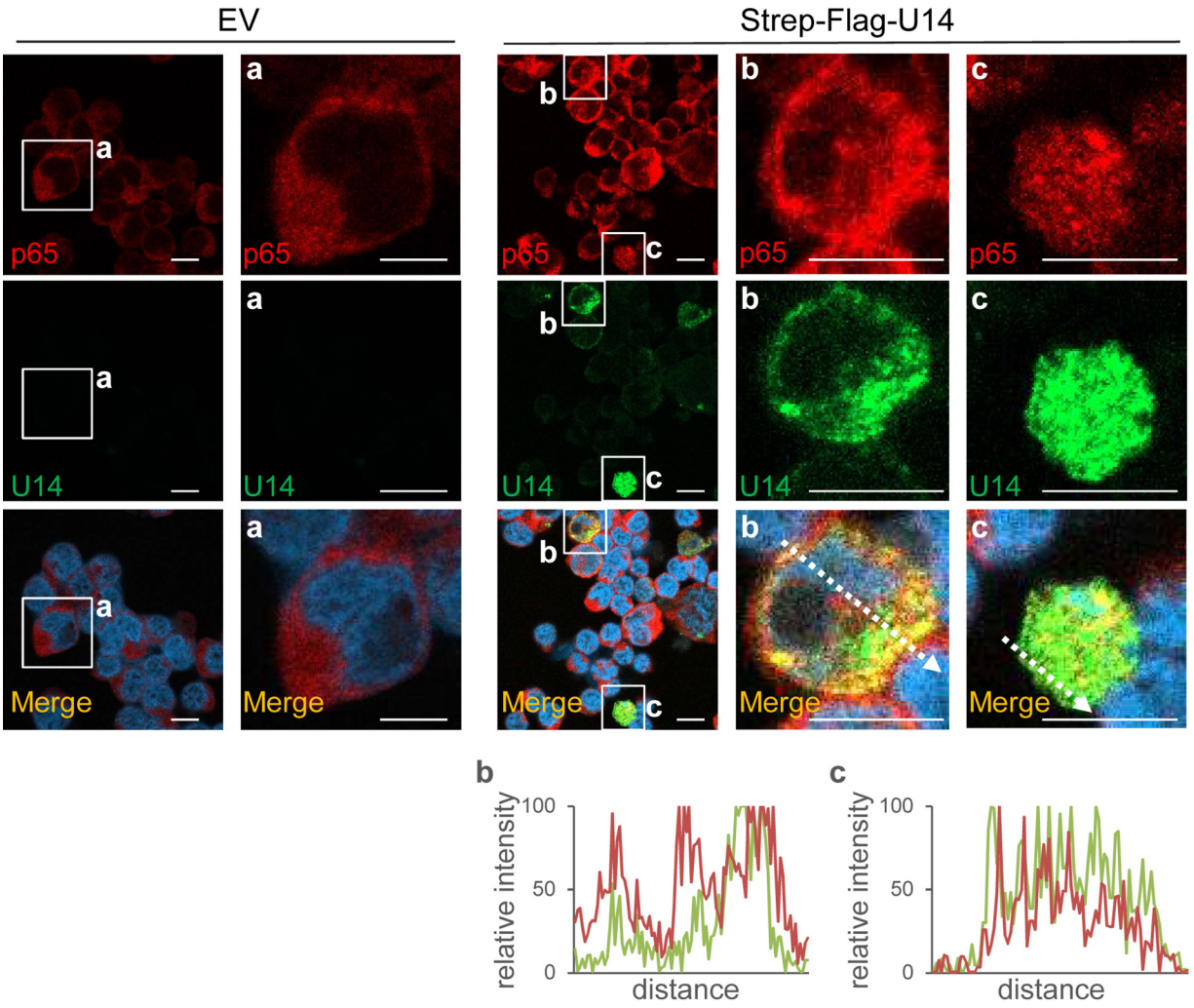

FIG 5 HHV-6A U14 colocalizes with p65. HEK293T cells were transfected with Strep-Flag-U14 expression plasmid or empty plasmid (EV). At 24 h posttransfection, the cells were observed under a confocal microscope after costaining with anti-FLAG and anti-p65 antibody. Nuclear DNA was stained with Hoechst 33342. The boxed area (a, b, and c) in each left panel is shown in the corresponding panel to the right. Scale bars, 10 μm. Fluorescence line scans along the dotted lines of the merged images are shown beneath the image.

6A U14 (Fig. 4), IκBα was not efficiently coprecipitated from either lysate with anti-HHV-6A U14 antibody (Fig. 8). As shown in Fig. 8, the amount of IκBα was slightly reduced in U1102-infected cells compared to the mock-infected cells. However, this was not consistently observed, and we are therefore unable to conclude whether HHV-6A infection leads to IκBα degradation.

Furthermore, U14 and p65 colocalized in cells infected with HHV-6A U1102 (Fig. 9). As we observed for transient transfection (Fig. 5), the majority of HHV-6A U14 and p65 was cytoplasmic in HHV-6A-infected cells, although both proteins could also be detected in the nucleus in up to 70% of infected cells (Fig. 9). Fluorescence line scanning showed that HA-U14 and p65 partially colocalized in infected cells. Taken together, these results demonstrate that HHV-6A U14 can interact with NF-κB proteins and induce the expression of NF-κB-regulated genes in infected cells.

Importance of the NF-κB pathway in HHV-6A gene expression. As described above, NF-κB promoted the expression of inflammatory cytokines in HHV-6A-infected cells, which possibly accelerates the pathology induced by HHV-6A infection. We next investigated whether NF-κB enhanced viral gene expression and replication. JJhan cells were mock infected or infected with HHV-6A U1102 for 24 h and then left untreated or treated with either SC75741 or QNZ. Cells were incubated for a further

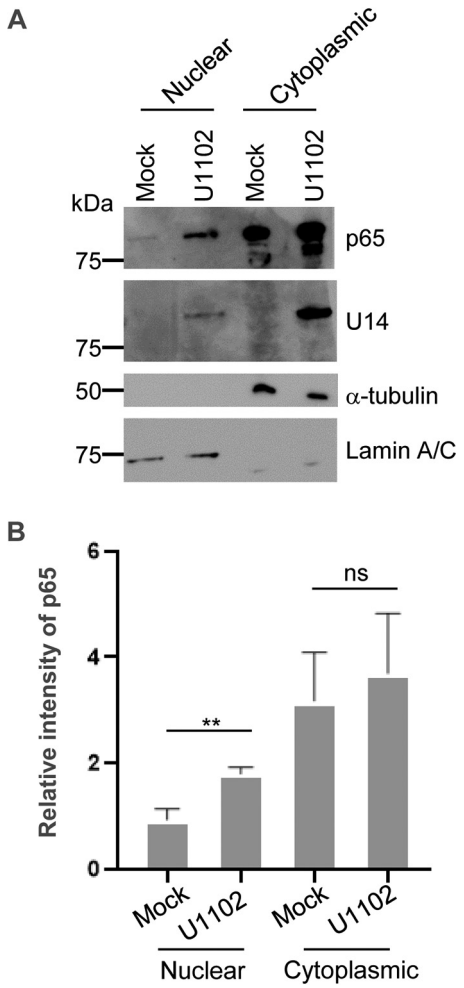

FIG 6 HHV-6A infection induces the nuclear translocation of p65. (A) JJhan cells were either mock infected or infected with U1102. At 72 h postinfection, cells were fractionated into cytoplasmic and nuclear fractions and analyzed by immunoblotting with the indicated antibodies. (B) The intensities of p65 in each fraction were normalized to those of Lamin A/C or α-tubulin, respectively, and are shown as means $\pm$ the standard deviations ($n = 3$; **, $P < 0.01$ [unpaired Student t test]).

48 h to allow HHV-6A propagation before analyze. As shown in Fig. 10, SC75741 or QNZ treatment slightly reduced viability in both mock- and HHV-6A-infected cells, although not to a statistically significant level. These cells were also subjected to immunofluorescence and immunoblot analysis. In the majority of infected cells that were not treated with the NF-κB inhibitor, expression of the immediate early protein IE2, as well as the late proteins U14 and gQ1, could be detected by fluorescence microscopy. However, these proteins were barely expressed in infected cells that were treated with either SC75741 or QNZ (Fig. 11A). In this experiment, we did not determine the infectivity at time of treatment since we could not detect these viral proteins at 24 h after infection. Immunoblot analysis revealed that both of the inhibitors attenuated the accumulation of U14 and gQ1 (Fig. 11B). Moreover, qPCR analysis showed that both of the inhibitors significantly reduced the number of HHV-6A genome copy in the supernatant of the infected cells (Fig. 11C). These observations suggest that NF-κB signaling is important for viral gene expression and progeny viral yields in JJhan cells.

DISCUSSION

NF-κB is a crucial element of immunity associated with the regulation of antiviral response. Although host cells use NF-κB as a defense mechanism against invading

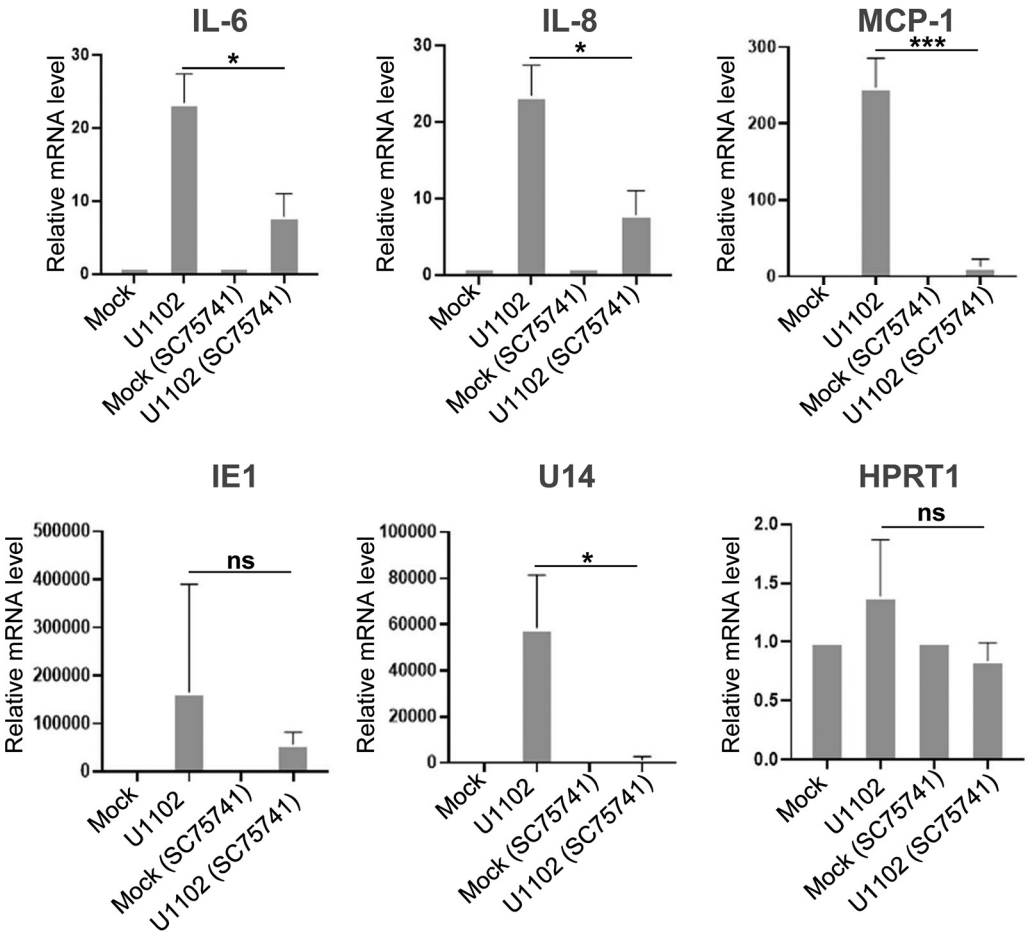

FIG 7 mRNA expression level of different genes in HHV-6A-infected cells. JJhan cells were mock infected or infected with U1102. At 72 h postinfection, the cells were either treated with 5 μM SC75741 (NF-κB inhibitor) or left untreated for 10 min. The medium was changed 10 min later to remove SC75741, and RNA was extracted from the cell at 1 h after treatment. The expression of mRNA of IL-6, IL-8, MCP1, IE1, U14, and HPRT1 was quantified using qRT-PCR. Relative amounts of these mRNA were normalized to that of β-actin. The data are shown as means $\pm$ the standard deviations ($n = 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ [unpaired Student t test]).

pathogens, the pathogens can in some instances develop alternative mechanisms to "hijack" NF-κB-driven antagonism and exploit it for their benefit. Here, we report a novel function of HHV-6A U14 as an activator of the NF-κB pathway. We found that NF-κB-responsive promoters can be activated in the presence of HHV-6A U14. Also, transfection or viral infection led to an HHV-6A U14-dependent redistribution of the p65 subunit of NF-κB to the nucleus. Moreover, we showed that interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1) transcripts were upregulated in the presence of HHV-6A U14 in an NF-κB-dependent manner. Since NF-κB signaling is also important for HHV-6A gene expression, our results suggest that HHV-6A U14 plays an important role in viral replication.

The tegument proteins are specific components of the herpesvirus virion that are located between the capsid and the envelope (43). In addition to acting as structural components, tegument proteins have additional functions (43, 44). Following membrane fusion, many of these tegument proteins are released into the cytoplasm, where they are thought to play important roles in viral replication by activating signaling pathways, altering the host immune response, and initiating viral gene expression (43, 44). The HSV-1 UL37 protein, which is conserved among *Herpesviridae*, activates NF-κB signaling following envelope-membrane fusion (36). Similarly, tegument-derived HHV-6A U14 is delivered to the cytoplasm after membrane fusion, and HHV-6A U14 is

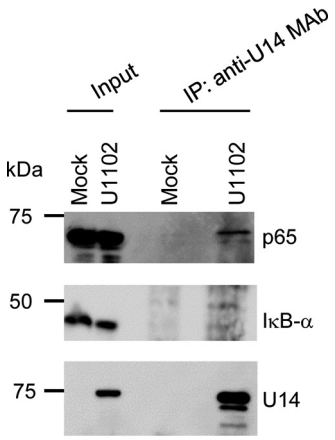

FIG 8 HHV-6A U14 interacts with the NF-κB protein p65 in HHV-6A-infected cells. JJhan cells were mock infected or infected with U1102. At 72 h postinfection, these cells were lysed, and the extracts were subjected to immunoprecipitation with anti-U14 mouse monoclonal antibody (MAb), followed by immunoblotting.

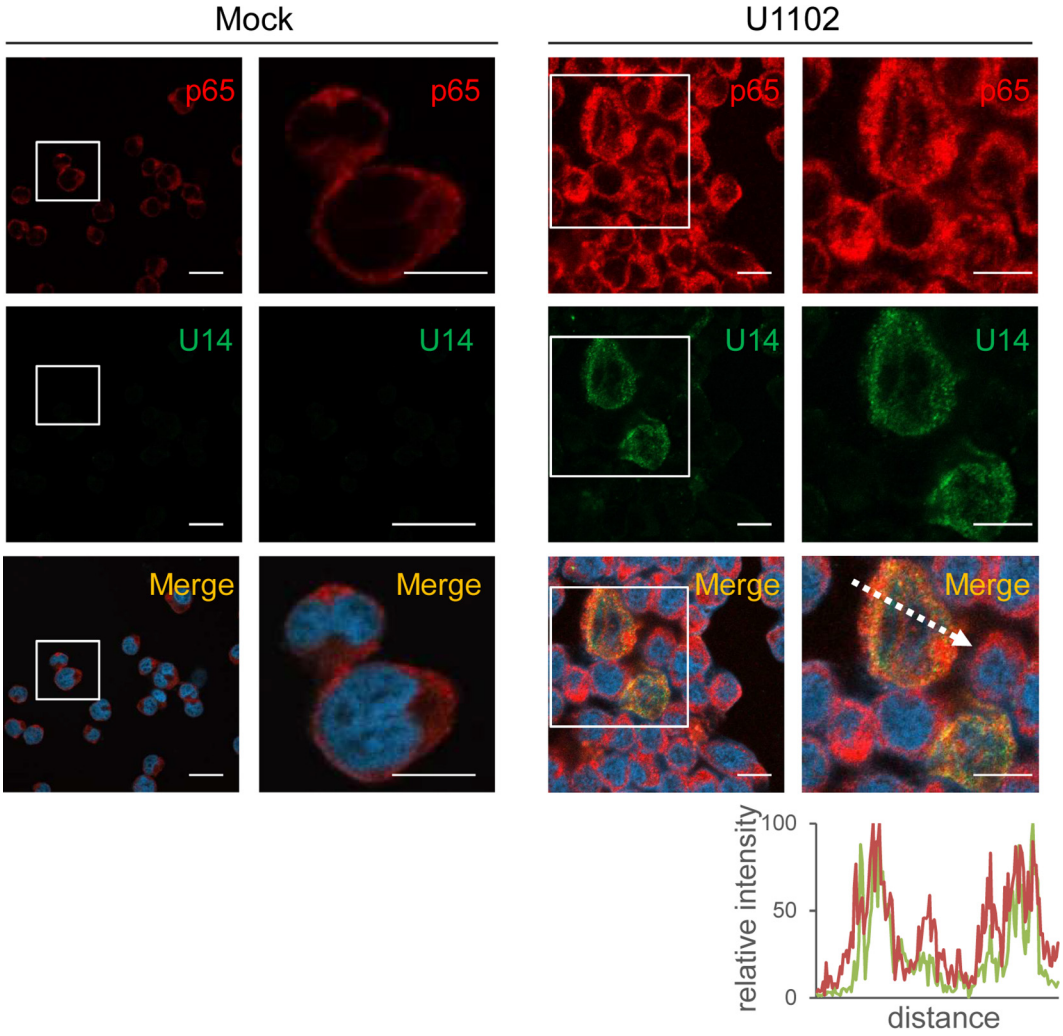

FIG 9 HHV-6A U14 colocalizes with p65 in HHV-6A-infected cells. JJhan cells were mock infected or infected with U1102. At 72 h postinfection, the cells were analyzed using immunofluorescence with the indicated antibodies and Hoechst 33342. The boxed area in each left panel is shown in the corresponding panel to the right. Scale bars, 10 μm. Fluorescence line scans along the dotted lines of the merge image are shown beneath the image.

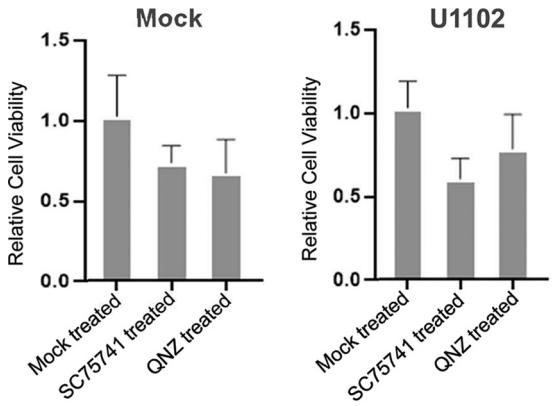

FIG 10 Effect of NF-κB inhibitors on the viability of JJhan cells. JJhan cells were either mock infected or infected with U1102. At 24 h after infection, these cells were left untreated or treated with either 5 μM SC75741 or 10 μM QNZ. After 48 h, cell viability was measured. The data are shown as means ± the standard deviations (n = 4 for mock-infected cells and n = 3 for U1102-infected cells [Tukey's test]).

detected as dot-like structures in both the nucleus and the cytoplasm immediately after infection (15), indicating its roles early after infection. In the present study, however, we did not detect induction of NF-κB signaling early after infection (data not shown).

We cannot rule out the possibility that other viral proteins contribute to NF-κB activation. The other late proteins of HHV-6A could also enhance NF-κB signaling and viral gene expression. Furthermore, HHV-6A U14 is a multifunctional protein that is important for virion morphogenesis, and it interacts with p53 and induces cell cycle arrest (15, 16). Of note, the NF-κB inhibitors used in this study possibly impaired viral gene expression and replication through their effects on cell viability. To reveal the significance of the interaction between HHV-6A U14 and NF-κB, the p65 binding site on HHV-6A U14 must be determined in future studies. The crystal structure of the N-terminal half of HHV-6B U14 has revealed potentially functional sites (45). These sites must be evaluated with regard to their potential role in NF-κB activation.

Herpesvirus gene expression during productive infection represents a classic regulatory cascade (43). Immediate early genes require no new protein synthesis for their expression. Immediate early proteins activate the promoter of early genes whose transcription is totally independent of viral DNA synthesis, whereas the expression of late genes is augmented by (or in some cases totally dependent on) viral DNA synthesis (43). Thus, HHV-6A U14 is a late gene, since its expression is blocked in the presence of a DNA polymerase inhibitor (15). The expression of immediate early and early genes is regulated by NF-κB, since promoters of these genes contain the consensus sequence for NF-κB binding (46–51). Because NF-κB is also important for HHV-6A gene expression and viral propagation at later times after infection, HHV-6A U14 might help to maintain the constitutive expression of immediate early and early genes and/or activate cellular genes responsible for viral replication through NF-κB signaling at later times after infection (Fig. 12). NF-κB is a master regulator of the antiviral response and it is thus not surprising that many viral proteins have evolved to inhibit NF-κB signaling (17, 18). This is also true in the case of herpesviruses (52–55). It had been reported that HSV-1 UL24 and HSV-1 UL42, both of which are conserved among *Herpesviridae*, can inhibit NF-κB (53, 56). As NF-κB enhances expression of viral immediate early and early genes (18, 48, 57), the functional interaction between NF-κB and herpesviral infection is not simple, and it must be tightly regulated. In the case of betaherpesviruses, survival of the infected cells must be ensured for at least 72 h due to the long viral replication cycle. In this case, maintenance of prosurvival NF-κB signaling might be important to resist cell death. Our findings provide important insight into the novel interaction between viruses and NF-κB signaling and might suggest new targets for antiviral therapy.

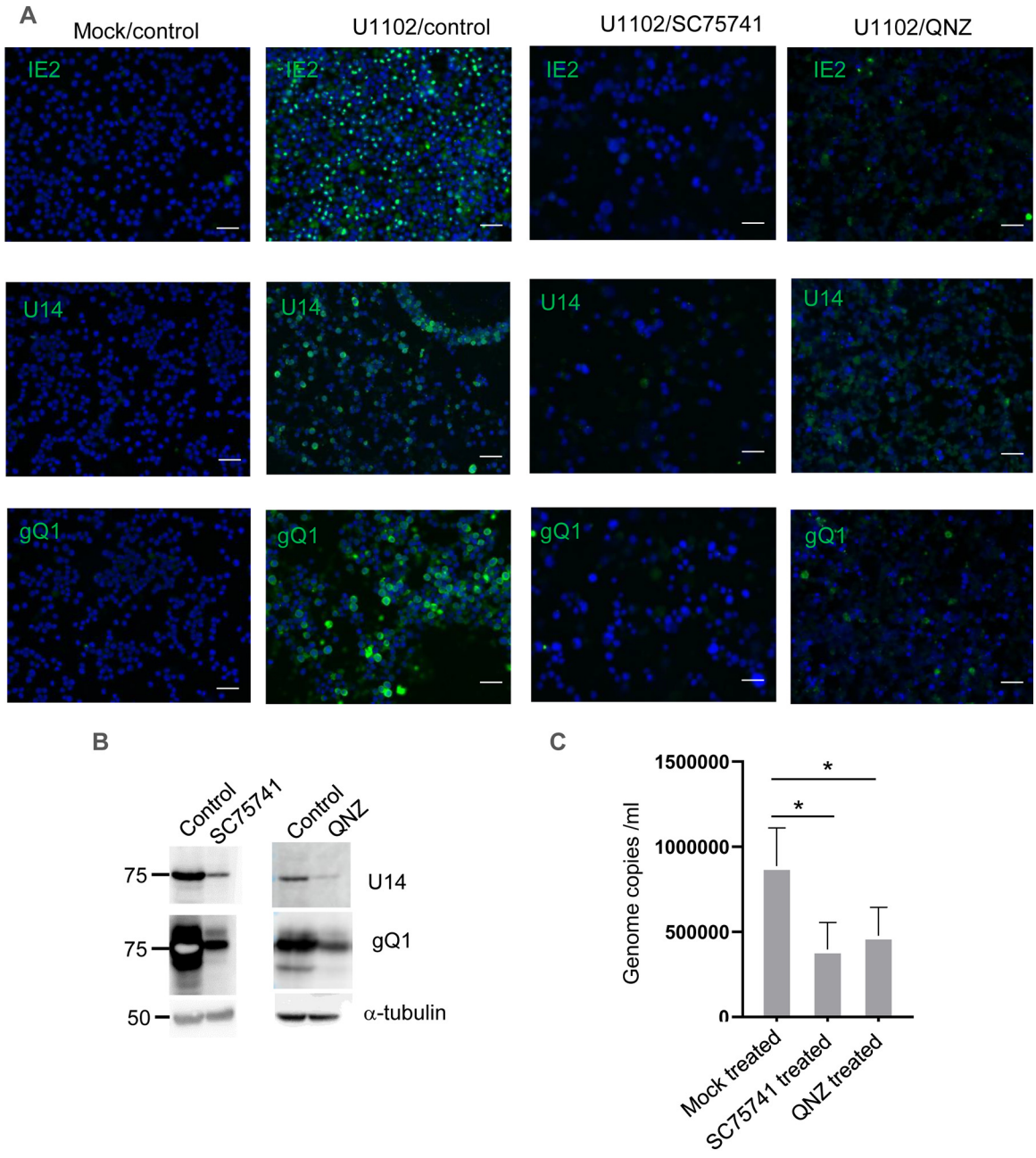

FIG 11 NF-κB inhibitors reduce virus replication. JJhan cells were mock infected or infected with U1102. At 24 h postinfection, the infected cells were treated with SC75741 or QNZ or mock treated for 48 h. At 72 h after infection, these cells were analyzed using fluorescence microscopy (A) and immunoblotting (B) with the indicated antibodies and Hoechst 33342, while genome copy numbers were measured using qPCR (C) following DNA extraction from the supernatant of the infected cells. The data are shown as means ± the standard deviations ($n = 4$; *, $P < 0.05$ [Tukey's test]).

MATERIALS AND METHODS

Cells and viruses. HEK293T cells were cultured in Dulbecco modified Eagle medium supplemented with 8% fetal bovine serum (FBS). The human T-lymphoblastoid cell line, JJhan, was cultured in RPMI 1640 medium containing 8% FBS. Umbilical cord blood mononuclear cells (CBMCs) were cultured as described previously (58). CBMCs were purchased from the Cell Bank of the RIKEN BioResource Center, Tsukuba, Japan. The usage of CBMCs in this study was approved by the ethics committee of Kobe University Graduate School of Medicine. The HHV-6A strain U1102 was used for this study, and HHV-6 cell-free virus was prepared from CBMCs as described previously (15). Transfection experiments were performed using Lipofectamine 3000 (Thermo Fisher Scientific) as previously described (59, 60). Cell viability was evaluated using cell counting kit-8 (Dojindo) according to the manufacturer's instructions.

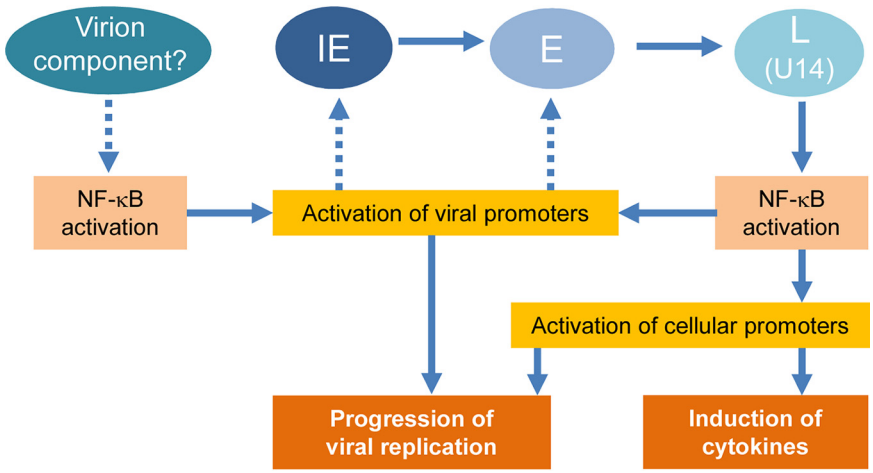

FIG 12 Proposed model of events involved in HHV-6A-mediated NF-κB signaling. After infection, virion components may induce NF-κB signaling to activate viral promoters. HHV-6 gene expression then occurs in a cascade manner (IE→E→L). Expression of late genes (including U14) initiates the activation of NF-κB signaling that can either trigger the activation of cellular genes, as well as viral gene expression, both of which may influence further viral replication. On the other hand, the NF-κB signaling also induces the expression of proinflammatory cytokines (including IL-6 and IL-8).

Plasmids. The entire coding sequence of HHV-6A U14 fused with either HA tag (16) or Strep-Flag tag was cloned into the pCAGGS-MCS expression vector for transient analysis.

Antibodies. For immunoblotting and immunofluorescence analysis, we used mouse monoclonal antibody against α-tubulin (DM1A; Sigma), rabbit monoclonal antibody against p65 (D14E12; Cell Signaling Technology) and RelB (C1E4; Cell Signaling Technology), rabbit polyclonal antibody against c-Rel (Cell Signaling Technology), mouse monoclonal antibody against IkBα (L35A5; Cell Signaling Technology), anti-HA mouse monoclonal antibody (HA-7; Sigma), anti-FLAG mouse monoclonal antibody (M2; Sigma), and mouse anti-Lamin A/C (14/LaminAC; BD Bioscience). Monoclonal antibodies to HHV-6A IE2 (AIE2-1), U14 (AU14), and gQ1 (AgQ1-119) were produced and used as previously described (15, 58, 61).

Luciferase assay. HEK293T cells were cotransfected with either a pGL4.32 (NF-κB-luc; Promega), p125-Luc (IFN-β-luc [62]), pGL4.37 (ARE-luc; Promega), or pGL4.29 (CRE-luc; Promega) reporter plasmid, along with an empty vector (EV) or plasmid carrying HA-U14. As an internal control, pRL-CMV (Promega) containing the *Renilla* luciferase gene driven by the cytomegalovirus (CMV) promoter was cotransfected into the target cells. At 24 h posttransfection, firefly and *Renilla* luciferase activities were independently assayed using the dual-luciferase reporter assay system (Promega). The luciferase activity (Fluc/Rluc) was calculated as follows: (firefly luciferase activity)/(*Renilla* luciferase activity) (59, 63, 64). To analyze the effect of inhibitors, transfected cells were untreated or treated with 10 ng of TNF-α or 10 μM colforsin as an inducer of NF-κB and CRE, respectively, at 24 h after transfection. At 3 h posttreatment, the cells were further treated individually with the NF-κB inhibitors SC75741 (5 μM), QNZ (10 μM), or IKK16 (1 μM) or mock treated. After a further 2 h, firefly luciferase activity was determined.

Quantitative real-time PCR. Total RNA was isolated from the cells with a NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions, and cDNA was synthesized from the isolated RNA with SuperScript III (Thermo Fisher Scientific). The amount of cDNA of specific genes was quantitated using the SYBR Select master mix (Thermo Fisher Scientific) according to the manufacturer's instructions. The primer sequences for IL-6 were 5′-GACCCAACCACAAATGCCA-3′ (forward) and 5′-GTCATGTCCTGCAGCCACTG-3′ (reverse); those for IL-8 were 5′-CTGGCCGTGGCTCTCTTG-3′ (forward) and 5′-CCTTGGCAAAACTGCACCTT-3′ (reverse); those for β-actin were 5′-GCACCCAGCACAATGAAGA-3′ (forward) and 5′-CGATCCACACGGAGTAC TTG-3′ (reverse); those for MCP-1 were 5′-CTCTGCCGCCCTTCTGTG-3′ (forward) and 5′-TGCATCTGGCTGAGCG AG-3′ (reverse); those for HPRT1 were 5′-CGAGATGTGATGAAGGAGATGG-3′ (forward) and 5′-TGATGTAATCC AGCAGGTCAGC-3′ (reverse); while the primers for HHV-6A-encoded IE1 were 5′-AATCAATCTTCTGGGTGGGAA GAAAATCCAGCAATGTAATAATTGATGGGTGCAATCG-3′ (forward) and 5′-ACTATTCTCAAGAAGTGGCTCCGGAG AACATTCTCATCACAGACATTCTTTCTTATATCG-3′ (reverse); those for U14 were 5′-TTCGACACCGAAGAAGCCA-3′ (forward) and 5′-GTCCGGTCGATTATGAAAGGAG-3′ (reverse). The amount of mRNA expression was normalized to the amount of β-actin mRNA expression. The relative abundance of each gene was calculated using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method (65, 66).

Immunoblotting and immunofluorescence. Immunoblotting and immunofluorescence were performed as described previously (16, 67–69). The band intensities of p65 in the nuclear and cytoplasmic fractions were evaluated using ImageJ software and normalized to those of Lamin A/C and α-tubulin, respectively. For immunofluorescence, the cells were fixed with methanol/acetone and stained with the indicated antibodies. Nuclear DNA was stained with Hoechst 33342, and specific signals were detected using a confocal laser-scanning microscope (Olympus Fluo-View FV1000; Olympus or LSM800 microscope; Zeiss).

Affinity precipitation. Affinity precipitation was performed as previously described (16, 60, 70). At the indicated time point, either infected or transfected cells were lysed in buffer (50 mM Tris [pH 8.0], 0.1% NP-40, 150 mM NaCl) for 1 h at 4°C and then centrifuged at high speed. The supernatants of the cell lysates were collected and incubated with Strep-Tactin beads (transfected cells) or protein G-Sepharose coupled with anti-U14 antibody (infected cells). These beads were rinsed with the same buffer to remove unbound protein, and sample buffer containing 1,4-dithiothreitol (DTT) was added to the beads. After boiling for 5 min, the samples were used for immunoblotting.

Separation of cellular fractions. Separation of cellular fractions was performed as previously described (71). Briefly, cells were collected at the indicated times, rinsed with PBS, and lysed with hypotonic lysis buffer (10 mM HEPES [pH 7.5], 10 mM KCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 10 mM NaF, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, protease inhibitor mixture). The lysate was then incubated for 30 min prior to centrifugation at 500 $\times$ *g* for 5 min at 4°C. The supernatant was then transferred into fresh tubes as cytoplasmic fractions. The nuclear pellets were rinsed twice in hypotonic lysis buffer containing increased amounts of Nonidet P-40 (0.1%) and lysed with buffer containing 50 mM HEPES (pH 7.9), 250 mM KCl, 1% Nonidet P-40, 5% glycerol, 0.1 mM EDTA, 1 mM DTT, 10 mM NaF, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and protease inhibitor mixture. The samples were frozen and thawed three times and incubated on ice for 30 min. Insoluble material was pelleted at 14,000 rpm for 10 min at 4°C. The supernatant was then used as nuclear extracts. The lack of tubulin (cytoplasmic marker) and Lamin A/C (nuclear marker) were used as controls for purity of the nuclear and cytoplasmic compartments, respectively.

Calculation of virus genome copy numbers. A total of 1 $\times$ 10^6 cells (JJhan) were infected with U1102 in triplicate (1 $\times$ 10^5 genome copies/ml). At 24 h postinfection, infected cells were divided into three aliquots. One aliquot was treated with 5 μM SC75741, another was treated with 10 μM QNZ, and the remaining aliquot was kept untreated. At 72 h after infection, the infected cells were freeze-thawed, DNA was extracted from the supernatant of the cells treated under each condition using the DNeasy blood and tissue kit (Qiagen), and the genome copy number per milliliter of infected cell was quantitated by qPCR using SYBR Select master mix (Thermo Fisher Scientific). The sequence of primer used for this purpose was 5′-CGCTAGGTTGAGAATGATCGA-3′ (forward) and 5′-CAAAGCCAAATTATCCAGAGCG-3′ (reverse) as described previously (72).

Statistical analysis. For the comparison of two groups, statistical analysis was performed using the unpaired Student *t* test. Tukey's test was used for multiple comparisons. A *P* value of >0.05 was considered not significant (n.s.).

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