

**Title:** Replication-coupled and host factor-mediated encapsidation of the influenza virus genome by viral nucleoprotein

**Running title:** Virus genome encapsidation with NP mediated by host factor

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## ABSTRACT

The influenza virus RNA-dependent RNA polymerase is capable of initiating replication, but mainly catalyzes abortive RNA synthesis in the absence of viral and host regulatory factors. Previously, we reported that IREF-1/MCM stimulates *de novo*-initiated replication reaction by stabilizing an initiated replication complex through scaffolding between the viral polymerase and nascent cRNA to which MCM binds. In addition, several lines of genetical and biochemical evidences suggest that viral nucleoprotein (NP) is involved in successful replication. Here, using cell-free systems, we have shown the precise stimulatory mechanism of the virus genome replication by NP. Stepwise cell-free replication reactions revealed that exogenously added NP free of RNA activates the viral polymerase during promoter escape, while it is incapable of encapsidating the nascent cRNA. However, we found that a previously identified cellular protein, RAF-2p48/NPI-5/UAP56 facilitates replication reaction-coupled encapsidation as an NP molecular chaperone. These demonstrate that replication of the virus genome is followed by its encapsidation by NP in collaboration with its chaperone.

## INTRODUCTION

The genome of influenza type A viruses consists of eight-segmented and single-stranded RNAs of negative polarity. Transcription from the viral RNA genome (vRNA) is initiated using the oligonucleotide containing the cap-1 structure from cellular pre-mRNAs as primer, whereas genome replication is primer-independent and generates full-length vRNA through cRNA (full-sized complementary copy of vRNA) (reviewed in (17)). Generally, each viral DNA or RNA genome is not present as naked form but as a complex with viral basic proteins. The influenza virus genome exists as ribonucleoprotein (termed vRNP) complex with nucleoprotein (NP), one of viral basic proteins, and viral RNA-dependent RNA polymerases consisting of three subunits (PB1, PB2, and PA). NP binds single-stranded RNA without sequence specificity and is required for maintaining the RNA template in an ordered conformation suitable for viral RNA synthesis and packaging into virions (6, 23, 34). In the case of *Mononegaviridae*, nonsegmented and negative-stranded RNA viruses, it is proposed that the nucleocapsid (N) protein forms a trimeric complex with the viral RNA polymerase large (L) protein and phosphoprotein (P) to form a replicase complex to produce the progeny viral genome with concomitant encapsidation of nascent RNA by N protein, and encapsidation is mediated by the chaperone activity of P protein (2, 7, 14, 24). In the case of influenza virus, it is also postulated that NP might regulate the viral polymerase function and encapsidate the virus genome through its interaction with PB1 and/or PB2 (1, 23). Genetic analyses suggest that NP participates in the replication process (15). Recently, it is also shown that NP saturated with ssDNA, resulting in lacking its RNA binding activity, stimulates the virus genome replication from a model template without

primer (18). It is possible that NP stimulates the virus genome replication possibly through the interaction with the viral polymerase in an RNA binding activity-independent manner. Moreover, the *in vitro* cRNA synthesis using infected cell extracts as enzyme source depends on a supply of NP free of RNA (27). This finding has been interpreted as that NP prevents the premature termination of RNA synthesis possibly by binding to nascent RNA chains, that is, encapsidating them. Based on these, it could be hypothesized that NP facilitates the virus genome replication by both RNA binding- and viral polymerase binding-dependent mechanisms. It is proposed that encapsidation is initiated by successive targeting of exogenous NP monomer to a replicating RNA through the interaction between NP and the viral polymerase, which is distinct from replicative enzyme bound to the 5'-end of nascent RNA (1, 8, 11, 22), and then additional NP molecules are subsequently recruited by the NP-NP oligomerization (3, 23). It is also reported that nascent cRNA is degraded by host cellular nucleases unless it is stabilized by newly synthesized viral RNA polymerases and NP (33). However, the precise molecular mechanisms about the virus genome replication and encapsidation by NP are yet unclear.

The cRNA synthesis occurs from incoming vRNA in infected cells, but vRNP complexes isolated from virions by themselves hardly synthesize cRNA (9). Thus, it was reasonable to examine whether a host factor(s) and/or a viral factor(s) are required for the replication process. We reconstituted a cell-free virus genome replication system with virion-associated vRNP and nuclear extracts prepared from uninfected HeLa cells (9). Using biochemical fractionation and complementation assays, we identified influenza virus replication factor (IREF)-1 that enabled the viral polymerase to synthesize full-sized cRNA. Otherwise, the viral RNA polymerase produces mainly

abortive short RNA chains in the absence of IREF-1. IREF-1 was found to be identical with a minichromosome maintenance (MCM) heterohexamer complex. IREF-1/MCM stabilizes replicating polymerase complexes by promoting the interaction between the nascent cRNA and the PA subunit.

Here, we examined the molecular function of NP in influenza virus genome replication using the previously established cell-free virus genome replication system using virion-associated vRNP. Exogenously added NP free of RNA stimulated the virus genome replication with MCM in an additive manner. Further, we found that NP activates the viral polymerase during its transition from initiation to elongation to synthesize the unprimed full-length cRNA, but NP by itself is incapable of encapsidating the nascent cRNA. However, we found that RAF-2p48/NPI-5/UAP56/BAT1, which was identified as a host factor for activation of viral RNA synthesis (16), is required for the encapsidation of nascent cRNA with exogenously added NP free of RNA and the stimulation of elongation process of virus genome replication. We observed that the level of the virus genome replication was decreased in infected cells when the expression of the *RAF-2p48/UAP56* gene was knocked down by siRNA-mediated gene silencing. Collectively, we would propose the NP- and host factor-dependent mechanism of the virus genome encapsidation in concert with its replication.

## MATERIALS AND METHODS

**Biological materials.** vRNP was prepared from purified influenza A/Puerto Rico/8/34 virus as previously described (28). For the expression of His-tagged NP (His-NP), we cloned the open reading frame corresponding to the *NP* gene into pET14b. Rabbit polyclonal antibody against NP was generated by immunization of 2 month-old female rabbit with His-NP protein. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

**Preparation of recombinant proteins.** His-tagged recombinant proteins were prepared and purified according to the manufacturers' protocol. In addition, to remove the bacterial RNA possibly bound to NP, we treated recombinant proteins with RNase A before purification, and washed them with a buffer containing 1 M NaCl. Recombinant RAF-2p48/UAP56 was prepared from GST-tagged RAF-2p48/UAP56 by PreScission protease (GE healthcare) digestion. Purified proteins were stored in a buffer containing 50 mM HEPES-NaOH (pH 7.9), 300 mM KCl, 20% glycerol, and 1 mM DTT at -80°C until use. Recombinant MCM complex was prepared as previously described (9). These purified recombinant proteins were separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue in Fig. 1A.

**Cell-free virus genome replication system.** A cell-free virus genome replication was carried out at 30°C for 90 min in a final volume of 25  $\mu$ l containing 50 mM HEPES-NaOH (pH 7.9), 5 mM  $MgCl_2$ , 50 mM KCl, 1.5 mM dithiothreitol, 500  $\mu$ M each ATP, CTP, and UTP, 25  $\mu$ M GTP, 5  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]GTP (3,000 Ci/mmol), 8 U of RNase inhibitor, and vRNP (10 ng of NP equivalents) in the presence or absence of purified proteins. RNA products were purified, subjected to 4% PAGE in the presence

of 8 M urea, and visualized by autoradiography. For limited elongation assays, RNA synthesis was performed with vRNP (150 ng of NP equivalents) in the absence of UTP, and RNA products were separated through 15% PAGE containing 8 M urea. To address the encapsidation of nascent cRNA with NP, RNA synthesis was carried out by following the standard protocol described above except the concentration of nucleotides, that is, 0.3  $\mu$ M UTP and 250  $\mu$ M each ATP, CTP, and GTP, 10  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP (3,000 Ci/mmol) in a final volume of 200  $\mu$ l. The co-precipitated RNA products with NP or MCM were separated through 10% PAGE containing 8 M urea.

**Gene silencing mediated by siRNA.** An siRNA against *RAF-2p48/UAP56* gene corresponding to its open reading frame (5'-AGUACUACGUGAAACUGAAGGACAA-3') and control dsRNA targeting none of cellular mRNAs, were designed and synthesized by iGENE Therapeutics Inc.. HeLa cells ( $1 \times 10^5$  cells) were transfected with 40 pmol of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. At 48 h post transfection, the cells were infected with influenza A/PR/8/34 at moi of 10 in the absence or presence of 100  $\mu$ g/ml of cycloheximide. The *RAF-2p48/UAP56* knockdown cells were also transfected with viral protein expression plasmids encoding PB1, PB2, PA, and NP, and pHH21-vNS-Luc reporter plasmid to reconstitute a model viral replicon (19, 30). This reporter plasmid carries the *luciferase* gene in reverse orientation sandwiched between 23 nucleotide-long 5'- and 26 nucleotide-long 3'-terminal promoter sequences of the influenza virus segment 8, which is placed under the control of the human Pol I promoter.

**Indirect immunofluorescence assay.** HeLa cells on coverslips were fixed with 4% paraformaldehyde in PBS. The cells were permeabilized in 0.5% Triton

X-100, and incubated in PBS containing 1% BSA. The coverslips were incubated with anti-p48/UAP56 rabbit polyclonal antibody (16) for 1 h. After wash with 0.1% Tween-20 in PBS, coverslips were incubated with Alexa Fluor 568-conjugated anti-rabbit IgG (Invitrogen) for 1 h. Images were acquired under the same exposure time by fluorescence microscope system (Axiovision, Carl Zeiss).

**Primer extension assay.** Total RNAs isolated from control and RAF-2p48/UAP56 knockdown cells at 0, 3, 6, and 9 hpi were subjected to reverse transcription at 42°C for 1 h with primers specific for segment 5 vRNA (5'-GGGAATACAGAGGGGAGAA-3') corresponding to the *NP* cDNA between nucleotide sequence positions 1336 to 1354, segment 5 m/cRNA (5'-GATTTTCAGTGGCATTCTGGC-3') complementary to the *NP* cDNA between nucleotide sequence positions 101 to 120, and 5S rRNA (5'-GGGGTACCTTCGAAAGCCTACAGCACCCGGTA-3'), which were labeled at their 5'-end with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase (TOYOBO). The products purified with phenol/chloroform extraction and ethanol precipitation were separated through 6% polyacrylamide gel containing 7 M urea, and visualized by autoradiography.

**Real-time quantitative PCR.** Total RNAs isolated from control and RAF-p48/UAP56 knockdown cells at 12 h post transfection for construction of the model viral replicon were subjected to reverse transcription with primers to determine the level of vRNA (5'-TCCATCACGGTTTTGGAATGTTTACTACAC-3', which corresponds to the *luciferase* coding region between nucleotide sequence positions 728 and 757), cRNA (5'-AGTAGAAACAAGGGTGTTTTTTAGTA-3', which is complementary to the 3' portion of the segment 8 cRNA), and viral mRNA (oligo(dT)<sub>20</sub>



for poly(A) tail) synthesized from the reconstituted model viral replicon. The synthesized single-stranded cDNAs were subjected to real-time quantitative PCR analysis (Thermal Cycler Dice Real Time System TP800; TaKaRa) with two specific primers, 5'-TCCATCACGGTTTTGGAATGTTTACTACAC-3', which corresponds to the *luciferase* coding region between nucleotide sequence positions 728 and 757, and 5'-GTGCGCCCCCAGAAGCAATTTC-3' complementary to the *luciferase* coding region between nucleotide sequence positions 931 and 952. The amount of *NP* mRNA transcribed from the expression plasmid, which is transcribed by cellular RNA polymerase II, was detected as an internal control.

## RESULTS AND DISCUSSION

**Stimulation of *de novo* cRNA synthesis by NP.** Exogenously added recombinant NP free of RNA (hereafter designated exogenous NP) stimulated the *de novo* virus genome replication in the absence of MCM and any kind of primers (Fig. 1B, lanes 1-5). We confirmed by RNase H digestion analyses with primers corresponding to each segment that RNA products correspond to those synthesized from each segment (data not shown). Then, we examined whether exogenous NP and MCM coordinately stimulate the virus genome replication reaction. MCM stimulated the virus genome replication additively with recombinant NP, suggesting that NP and MCM function through distinct mechanisms (Fig. 1B, lanes 6-10 and 16-20). The stimulatory activity per molecule of MCM was 5 times higher than that of NP judged by the slopes of the lines in Fig. 1C (Fig. 1D). We observed that authentic NP free of RNA purified from virions by CsCl glycerol density gradient centrifugation (5, 34) also possesses the stimulatory activity as well as recombinant NP (data not shown). We used, as the enzyme source, the vRNP containing authentic NP that is bound to the template RNA. Thus, it is quite likely that RNA-free NP but not template-bound NP is required for the *de novo* virus genome replication. The RNA synthesis level varied among segments as previously described (9). For instance, the segments 1, 2, and 3 were hardly replicated compared with other segments. The reason for this variation in cRNA synthesis is unknown presently.

**NP facilitates the promoter escape of the viral RNA polymerase.** Previously, we demonstrated that MCM does not enhance the frequency of the replication initiation, but rather makes a nonproductive viral polymerase override the

step for abortive synthesis. To examine whether NP is involved in the initiation reaction of the virus genome synthesis, we carried out a limited elongation assay, in which UTP is omitted from the reaction mixture and the RNA polymerase pauses at the first adenine residue on the template. The expected lengths of limited elongation products are 12 nt for segments 1, 3, and 7; 13 nt for segments 5 and 8; 14 nt for segment 6; 18 nt for segment 4; and 19 nt for segment 2. Since we detected the comparable amount of each RNA product in the absence or presence of exogenous NP (Fig. 1E), it is concluded that NP does not stimulate the initiation reaction as MCM does not (9). Thus, NP may be required for a step(s) post the initiation and the early elongation steps, in which short cRNAs are synthesized.

To examine whether NP stimulates the transition of the viral polymerase from initiation to elongation, that is, the promoter escape of the viral polymerase, unprimed limited elongation assays were first performed in the absence of UTP, and elongation reactions were restarted by the addition of UTP (Fig. 1F). MCM (0.5 pmol) or exogenous NP (3 pmol) was also added either before or after the limited elongation. The full-length cRNA was synthesized by restarting the limited elongation reaction performed in the presence of MCM (lane 2) or exogenous NP (lane 4) during the limited elongation reaction. Thus, it is quite likely that, to avoid the abortive RNA synthesis by the viral polymerase, MCM and NP are required for the viral polymerase prior to its movement along 12 to 19 nt-long vRNA template from the 3'-terminus of vRNA, where hairpin loop and double-stranded promoter region are located. Since the initiation reaction was not stimulated by NP (Fig. 1E) and the viral polymerase could not transit from initiation to elongation in the absence of NP (Fig. 1F), it is possible that NP stimulates elongation complexes during the promoter escape of the viral polymerase as

does MCM (9). The cell-free virus genome replication reaction was also carried out (Fig. 1G) in the presence of MCM (lane 2; 0.5 pmol) or NP (lane 3; 3 pmol) with a low concentration of UTP to slow down the reaction and synthesize a ladder of nascent cRNA chains in order to examine the length of elongated nascent cRNA chains. We found that comparable amounts of cRNA with shorter length (~100 nt) are synthesized in the presence of either MCM or NP. In contrast, the amount of longer one (>100 nt) stimulated by MCM was more than that by NP (Fig. 1G, compare lane 2 with lane 3). Therefore, it is quite likely that MCM promotes the elongation process more effectively than NP, possibly due to the weak interaction of exogenously added NP with long nascent cRNA as described later. Taken together, it is strongly suggested that NP stimulates the promoter escape of the viral polymerase as does MCM. Previous reports showed that the target of MCM is PA (9), whereas that of NP is PB1 and PB2 (1). Therefore, it is possible that the replication stimulation mechanism by NP and MCM are distinct each other.

#### **The encapsidation of newly synthesized virus genome by NP.**

Previously, we proposed that MCM stimulates the virus genome replication by acting as a scaffold between nascent cRNA chains and the viral polymerase during the promoter escape of the polymerase (9). Since NP has both RNA and viral polymerase binding activities, it should be speculated that NP also functions as a scaffold between newly synthesized RNA and the viral polymerase as does MCM. To address this, we tried to pull down the replicated cRNA chains associated with His-tagged either MCM or NP using Ni-NTA resin (Fig. 2A). The cell-free virus genome replication reaction was carried out in the presence of equal molar amount of MCM (lanes 1 and 3) or NP (lanes 2 and 4) with a low concentration of UTP in order to examine the length of co-purified

RNA as described in Fig. 1E. As shown in input lanes, MCM stimulated the elongation process more effectively than NP as shown in Fig. 1E (Fig. 2A, compare lane 1 with lane 2). Further, longer nascent cRNA chains were preferentially co-purified with MCM (lane 3), suggesting that MCM stabilizes the elongation complex and thereby makes the viral polymerase to escape the promoter successfully. It also seems likely that MCM has a role in the elongation process, but its precise mechanism is still unknown. In contrast, rather shorter cRNA chains were associated with exogenous NP (lane 4). After or along with the virus genome replication, the newly synthesized virus genome has to be encapsidated by exogenous NP to form RNP complexes as templates for the next phase of the virus genome replication and to protect from degradation by cellular nucleases (33). It is hypothesized that the encapsidation proceeds by targeting exogenous NP to the nascent RNA through the interaction between NP and the viral polymerase bound to the 5'-end of the nascent RNA to allow NP to interact with the viral RNA preferentially to other cellular RNA species (1, 8, 11, 22), and then subsequently NP is recruited through NP-NP oligomerization (3, 23). In our cell-free system, we found that exogenous NP interacts with shorter cRNA (Fig. 2A, lane 4) without the addition of soluble viral polymerases, which could bind to the 5'-end of the nascent RNA and be a target of NP. It might be explained that the primary targeting of NP to the nascent RNA easily occurs since there is no RNA target other than the nascent RNA in our system. However, it is worthwhile to note that encapsidation of longer nascent cRNA by NP was not achieved, when NP was simply added to the system (lane 4). This raises a question how newly synthesized virus genome is encapsidated with NP free of RNA.

NP recognizes the phosphodiester backbone of ssRNA in a specific

sequence-independent manner. We used, as the enzyme source, the vRNP containing authentic NP, which is bound to the template RNA. Thus, it is reasonably hypothesized that newly synthesized cRNA chains remain associated with the template RNP possibly by partial hybridization of the nascent cRNA with template vRNA and/or the interaction of nascent cRNA with template-bound authentic NP instead of exogenous NP. To address this, we immunopurified the template-bound authentic NP of vRNP in the presence of exogenous His-NP using anti-NP antibody (Fig. 2B). The length of RNA products associated with authentic NP or both authentic NP and exogenous His-NP (lane 3) was clearly distinct from that interacting with only exogenous His-NP (lane 4). From these results, it is assumed that the nascent cRNA product is hardly encapsidated with exogenous NP since the nascent cRNA tends to interact with template-bound NP more than exogenous NP and might partially hybridize with the template.

**The encapsidation with NP mediated by RAF-2p48/ UAP56.** As shown in Fig. 2, it is assumed that some factor(s) may be missing in the encapsidation of nascent cRNA products with exogenous NP. Previously, RAF-2p48/UAP56/BAT1 (hereafter, designated RAF-2p48/UAP56) belonging to the DExD-box family of ATP-dependent RNA helicase (13), also reported as NPI-5 (20), was identified as a host factor that binds to NP and stimulates influenza virus RNA synthesis from exogenously added model vRNA templates (16) and is involved in splicing of cellular pre-mRNAs and messenger RNP maturation of cellular and viral transcripts (4, 25, 29). RAF-2p48/UAP56 binds to NP free of RNA but not NP-RNA complex, and facilitates NP-RNA complex formation as a molecular chaperone for NP. Therefore, it was proposed that RAF-2p48/UAP56 is involved in the arrangement of NP on the template. However, its

precise roles, including the requirement for encapsidation process, have been yet uncovered. Thus, we tried to examine whether RAF-2p48/UAP56 facilitates the encapsidation of newly synthesized RNA with exogenous NP (Fig. 3). We found that long nascent cRNA was encapsidated with exogenous NP by the addition of RAF-2p48/UAP56 (compare lane 4 (in the presence of RAF-2p48/UAP56) with lane 3 (in the absence of RAF-2p48/UAP56)). The ATP-dependent RNA unwinding activity of RAF-2p48/UAP56 was not required for the encapsidation of nascent chains since the encapsidation occurred in the presence of ATP $\gamma$ S, which is a nonhydrolyzable analog of ATP (data not shown). Therefore, we propose a model that RAF-2p48/UAP56 facilitates the formation of RNP complexes by co-replicationally transferring exogenous NP to the nascent cRNA chain but not remodeling secondary structures of template and newly synthesized cRNA by its potential RNA helicase activity (see Fig. 5). Furthermore, RAF-2p48/UAP56 stimulated the elongation activity of the viral polymerase possibly due to facilitating the encapsidation of nascent cRNA (Fig. 3, lane 2). It is speculated that the co-replicative encapsidation of nascent cRNA by NP may prevent the premature termination of RNA synthesis by avoiding a secondary structure of nascent RNA, which is hypothesized as one of causative factors in the termination process of other RNA polymerases (10, 27). Therefore, it is possible that the encapsidation of the nascent RNA with exogenous NP mediated by RAF-2p48/UAP56 increases the processivity of the viral polymerase through avoiding inappropriate secondary structures of nascent cRNA.

**Involvement of RAF-2p48/UAP56 in influenza virus genome replication in infected cells.** Finally, we tried to examine whether RAF-2p48/UAP56 functions in the influenza virus genome replication in cultured cells using siRNA-mediated gene

silencing. After 48 h post transfection of siRNA corresponding to the *RAF-2p48/UAP56* ORF, the expression level of *RAF-2p48/UAP56* in knockdown cells decreased to approximately 30% of the cells transfected with non-targeting siRNA used as a negative control (Fig. 4A and 4B). We carried out quantitative primer extension assays with appropriate primers specific for each vRNA and m/cRNA of segment 5 (Fig. 4C and 4D). We confirmed that the product corresponding to cRNA was not found from a fraction bound with oligo(dT) cellulose (data not shown). This result showed that the accumulation of vRNA and cRNA was reduced and delayed in *RAF-2p48/UAP56* knockdown cells compared with control cells (Fig. 4C, lanes 1-7 and 14-20, and Fig. 4D). The same results were obtained for other segments (data not shown). It is proposed that nascent cRNA is degraded unless it is encapsidated with viral RNA polymerase and NP (33). In addition, the results in Fig. 3 and the previous report (16) demonstrated that *RAF-2p48/UAP56* stimulates the viral polymerase activity. Thus, *RAF-2p48/UAP56* might be involved in the virus genome replication and encapsidation in infected cells. We also found that the level of *NP* mRNA in *RAF-2p48/UAP56* knockdown cells decreased to 15% in control cells at 3 hpi (Fig. 4C, lanes 15 and 18, and Fig. 4E). In contrast, the comparable amounts of *NP* mRNA were found in both control and *RAF-2p48/UAP56* knockdown cells at 6 and 9 hpi (Fig. 4C, lanes 16, 17, 19, and 20) since the amount of vRNA template enough for the viral mRNA synthesis might be accumulated at 6 and 9 hpi but the replication activity was reduced and delayed in *RAF-2p48/UAP56* knockdown cells. To confirm the effect of *RAF-2p48/UAP56* on viral transcription, we utilized cycloheximide (CHX), a potent protein synthesis inhibitor (Fig. 4C, lanes 8-13 and 21-26, and Fig. 4E). A previous report showed that CHX suppresses viral protein synthesis and thereby leads to



degradation of replicated viral RNA but not mRNA since newly vRNP formation was repressed (33). Therefore, we could examine the amount of viral mRNA synthesized from incoming vRNP independent of the level of vRNA accumulation in the presence of CHX (Fig. 4C, lanes 8-13, and Fig. 4E). The level of NP mRNA in RAF-2p48/UAP56 knockdown cells was reduced to 70% in control cells in the presence of CHX at 3 hpi (Fig. 4C, lanes 21 and 24, and Fig. 4E). Therefore, it is likely that the reduction of viral mRNA synthesis in RAF-2p48/UAP56 knockdown cells is mainly due to the decrease of vRNP accumulation in the absence of CHX, although RAF-2p48/UAP56 has a stimulatory role in the viral transcription possibly by arrangement of NP on template and/or the nuclear export-competent messenger RNP formation (25). To rule out the possibility that the reduction of vRNA and cRNA synthesis was caused by the reduction of viral protein synthesis, we carried out a viral model replicon assay (19, 30), in which active vRNP complexes are reconstituted with PB1, PB2, PA, and NP, and the model vRNA encoding *luciferase* gene as described in Materials and Methods (Fig. 4F). With this system, we could examine the viral polymerase activity independent of the expression level of viral proteins since viral proteins were expressed from plasmids under the control of cellular RNA polymerase II promoter in this assay. Fig. 4F showed that the vRNA, cRNA, and viral mRNA synthesis were decreased in RAF-2p48/UAP56 knockdown cells compared with that in control cells even in the presence of comparable amounts of NP in both cells. We found that NP synthesized in RAF-2p48/UAP56 knockdown cells migrates differently from that in control cells (Fig. 4F). Previous reports showed that NP is modified by phosphorylation (23) and its N-terminal region is digested by caspase (35), but the involvement of RAF-2p48/UAP56 in these is not known at present.

It is well known that NP is one of proteins responsible for the virus genome replication (15, 18, 27, 33). Recently, it is reported that ubiquitination of NP regulates the virus genome replication (12). It is proposed that the soluble viral polymerase might act as a replicative enzyme *in trans*, but transcription occurs from template-bound viral polymerase *in cis* (8). In this study and recent reports (9, 31-33), the *de novo* cRNA synthesis is found from template-bound viral polymerase, thus it could be explained that the soluble viral polymerase might have the stimulatory activity but is not completely essential for the synthesis of nascent cRNA. The viral nuclear export protein (NEP/NS2) is also involved in the accumulation level of vRNA and cRNA (26). Further, it is reported that small non-coding RNAs derived from the influenza virus genome might regulate the viral transcription and replication through its interaction with viral polymerase complexes (21). Further to understand the mechanism of the influenza viral genome replication, precise analyses of a functional replicative enzyme including viral and cellular factors are required.

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## FIGURE LEGENDS

FIG. 1. NP and MCM additively stimulate the virus genome replication. (A) Purified recombinant proteins and vRNP. Purified His-NP, vRNP, RAF-2p48/UAP56, and MCM complexes were separated by 7.5% SDS-PAGE and visualized by staining with Coomassie brilliant blue. (B) Stimulatory activity of NP and MCM in a cell-free virus genome replication. RNA synthesis was carried out in the absence (lanes 1-5) or presence (lanes 6-10) of recombinant MCM complex (0.05 pmol of MCM complex) with 0 (lanes 1 and 6), 0.25 (lanes 2 and 7), 0.5 (lanes 3 and 8), 1.0 (lanes 4 and 9), and 2.0 pmol (lanes 5 and 10) of recombinant NP (upper panel). For the lower panel, we performed the RNA synthesis assay in the absence (lanes 11-15) or presence (lanes 16-20) of recombinant NP (0.50 pmol) with 0 (lanes 11 and 16), 0.025 (lanes 12 and 17), 0.05 (lanes 13 and 18), 0.10 (lanes 14 and 19), and 0.20 pmol (lanes 15 and 20) of MCM complex (lower panel). (C) Quantitative summary of panel A. The amounts of newly synthesized cRNA corresponding to segment 7 are determined by the ImageJ software. (D) Stimulatory activity per molecule of MCM and NP. The slopes of the lines in the presence of NP or MCM in Fig. 1C were determined, respectively. (E) Limited elongation assays. Unprimed limited elongation assays were carried out in the absence (lane 1) or presence of MCM (lane 2; 0.5 pmol) or NP (lane 3; 3.0 pmol). (F) NP functions during transition from initiation to elongation reaction. Unprimed limited elongation reactions were performed without (lanes 1, 3, and 5) or with (lanes 2 and 4) either MCM (lane 2; 0.5 pmol) or NP (lane 4; 3.0 pmol). After incubation for 1 h, elongation reactions were restarted by the addition of UTP. For lanes 3 and 5, MCM (0.5 pmol) and NP (3.0 pmol) were added at the restart of elongation reaction,



respectively. (G) MCM stimulates the elongation process more effectively than NP. RNA synthesis was carried out in the absence (lane 1) or presence of either MCM (lane 2; 0.5 pmol) or NP (lane 3; 3.0 pmol) with 0.3  $\mu$ M UTP and 250  $\mu$ M each ATP, CTP, and GTP, 10  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP (3,000 Ci/mmol). The purified products were separated through 4-15% linear gradient PAGE containing 8 M urea and visualized by autoradiography.

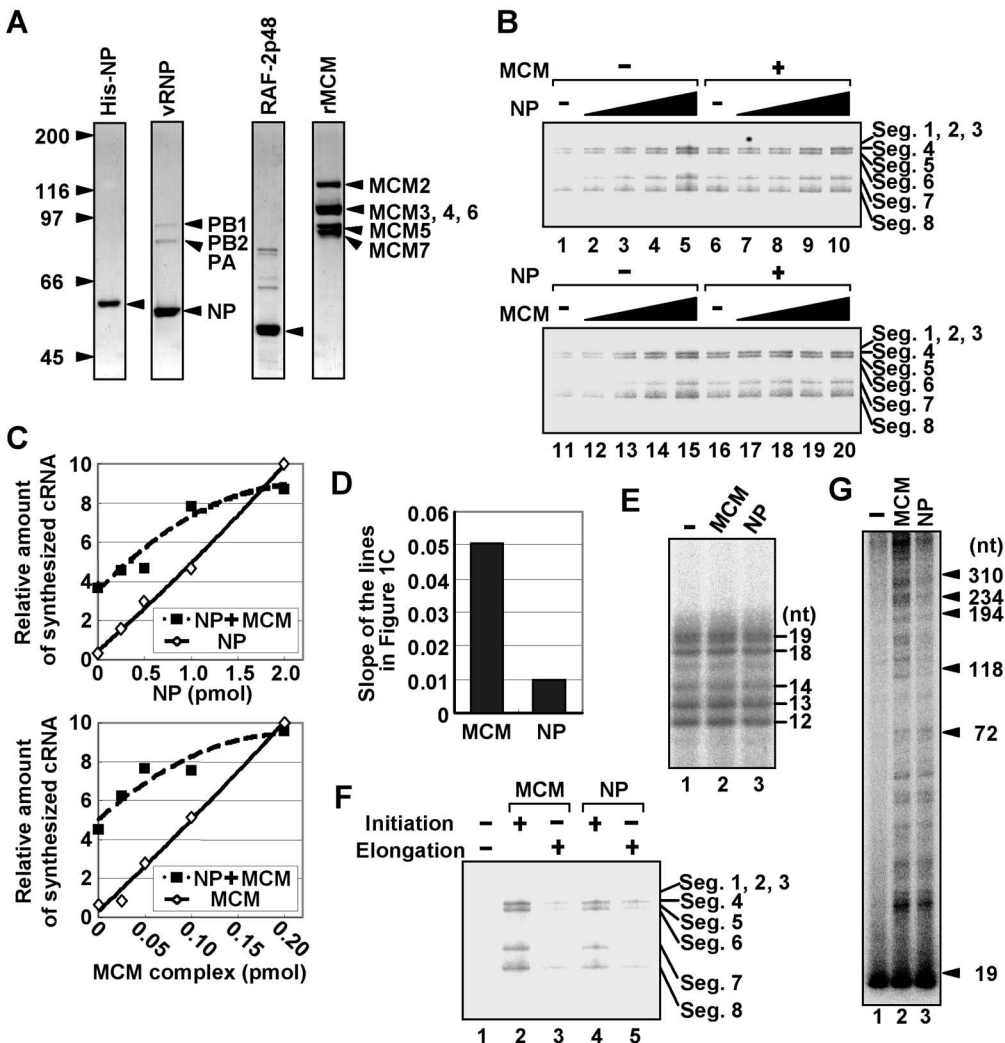
FIG. 2. Encapsidation of nascent cRNA with NP. (A) *De novo* RNA synthesis was carried out in the presence of His-MCM (lanes 1 and 3; 20 pmol) or His-NP (lanes 2 and 4; 20 pmol) with 0.3  $\mu$ M UTP and 250  $\mu$ M each ATP, CTP, and GTP, 10  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP (3,000 Ci/mmol) in a final volume of 200  $\mu$ l. The products were purified with His-MCM (lane 3) or His-NP (lane 4) by using Ni-NTA resin. Lanes 1 and 2 represent the 20% of input amounts. The band intensity of short (\*) and long (\*\*) nascent cRNA products (indicated positions by asterisks) was quantitatively measured with ImageJ software, and the relative intensity of newly synthesized cRNA co-precipitated with MCM or NP against input fraction is indicated. (B) *De novo* RNA synthesis was carried out with the authentic vRNP in the presence of His-NP as described in Fig. 2A. The newly synthesized RNA products were co-immunoprecipitated without (lane 2) or with (lane 3) anti-NP antibody. Lane 1 shows the 20% of input amount. The product purified by Ni-NTA resin is also represented in lane 4. The band intensity of short (\*) and long (\*\*) nascent cRNA products was quantitatively measured with ImageJ software, and the relative intensity of newly synthesized cRNA precipitated by using anti-NP antibody or Ni-NTA resin against input fraction is indicated.

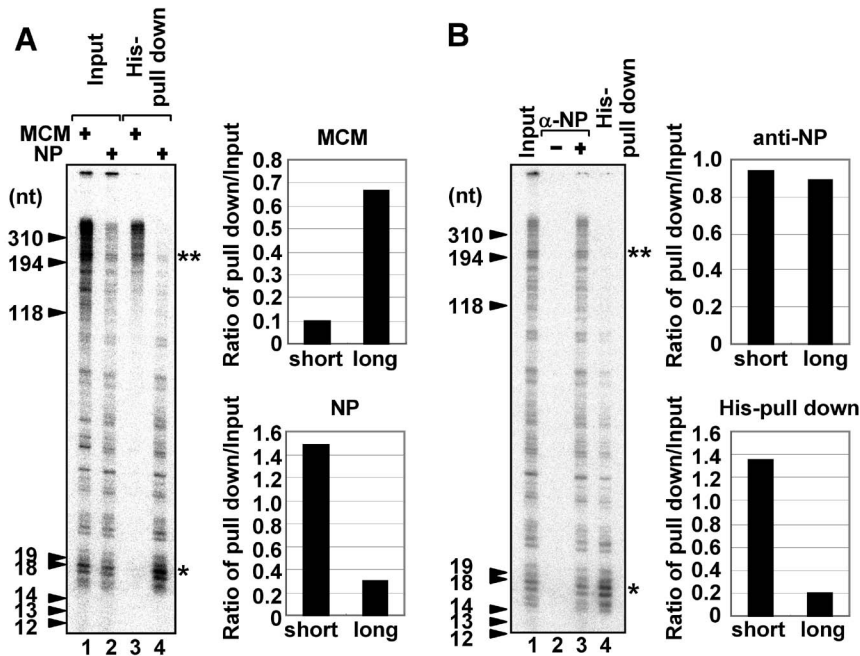
FIG. 3. The stimulatory activity of RAF-2p48/UAP56 in encapsidation of nascent cRNA. (A) RNA synthesis was performed in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of recombinant RAF-2p48/UAP56 with His-NP as described in Fig. 2. The products were purified with His-NP by using Ni-NTA resin (lanes 3 and 4). The 20% of input amounts are shown in lanes 1 and 2. The band intensity of short (\*) and long (\*\*) nascent cRNA products was quantitatively measured with ImageJ software, and the relative intensity of cRNA co-precipitated with NP in the absence or presence of RAF-2p48 against input fraction is indicated. (B) The band intensities of the regions corresponding to less than 30 nt-, 30~70 nt-, 70~120 nt-, and more than 120 nt-long RNA in each lane in panel A were quantitatively measured with ImageJ software, and the results were indicated as a percentage of the total intensity of each lane.

FIG. 4. Involvement of RAF-2p48/UAP56 in influenza virus genome replication in infected cells. (A) At 48 h post transfection, cells transfected with either control or siRNA against *RAF-2p48/UAP56* ORF were subjected to indirect immunofluorescence assay with anti-RAF-2p48/UAP56 antibody. Nuclear DNA stained with 4', 6-diamidino-2-phenylindole (DAPI) was also represented. Images were acquired under the same exposure time by a fluorescence microscope system (Axiovision, Carl Zeiss). (B) Expression level of RAF-2p48/UAP56. The lysates prepared from control and RAF-2p48/UAP56 knockdown cells ( $5 \times 10^3$ ,  $1 \times 10^4$ , and  $2 \times 10^4$  cells) were separated by SDS-PAGE, and then visualized by western blotting assays with anti-NP and  $\beta$ -actin antibodies. (C, D, and E) The level of viral RNAs in infected RAF-2p48/UAP56 knockdown cells. Control and RAF-2p48/UAP56

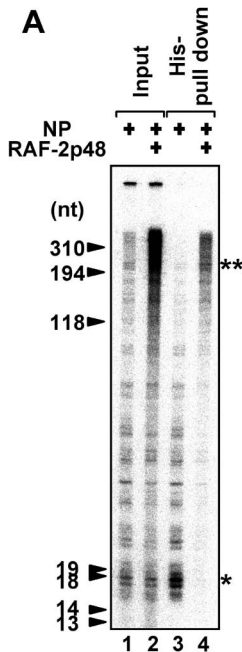
knockdown cells were infected with influenza virus in the absence (lanes 1-7 and 14-20) or presence of cycloheximide (lanes 7-13 and 21-26) for 0, 3, 6, and 9 h. Primer extension assays were carried out with primers specific for segment 5 vRNA or m/cRNA as described in Materials and Methods. As a loading control, 5S rRNA was also detected (lanes 27-39). The band intensity was quantitatively measured by ImageJ software, and the results of three independent experiments are summarized in panel D and indicated as a ratio of mRNA amount in RAF-2p48/UAP56 knockdown cells to that in control cells with or without CHX in panel E. (F) The level of viral RNAs synthesized from a reconstituted model replicon in RAF-2p48/UAP56 knockdown cells. Control and RAF-2p48/UAP56 knockdown cells were transfected with plasmids expressing PB1, PB2, PA, and NP, and model vRNA encoding *luciferase* gene as described in Materials and Methods. At 12 h post transfection, total RNAs were purified, and then subjected to reverse transcription followed by quantitative real-time PCR with primer sets specific for vRNA, cRNA, and *luciferase* mRNA. The expression level of NP protein in control and RAF-2p48/UAP56 knockdown cells was also detected by western blotting assay with anti-NP antibody.

FIG. 5. A proposed model. NP facilitates the promoter escaping of the viral polymerase through the interaction between NP and the viral polymerase in the RNA binding activity-independent manner. During elongation step, RAF-2p48/UAP56 stimulates the co-replicative encapsidation of newly synthesized cRNA with exogenous NP, thereby increasing the processivity of the viral polymerase.



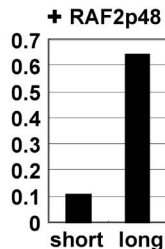
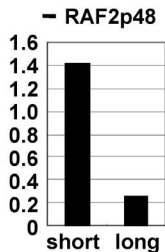


**A**

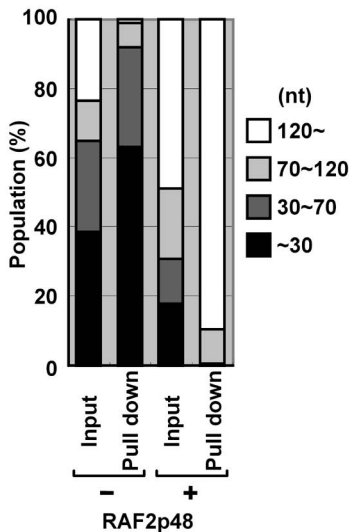


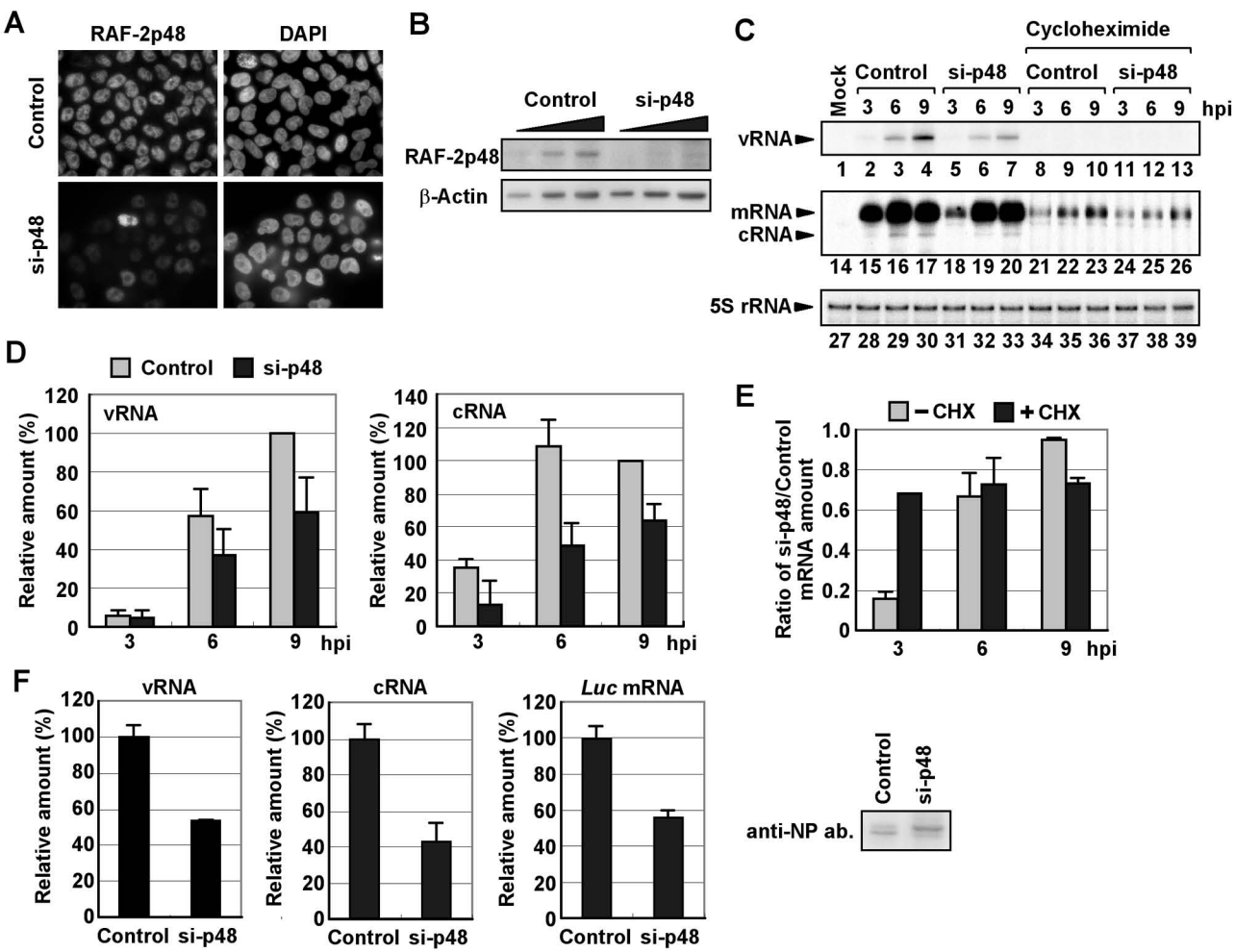
Ratio of pull down/Input

Ratio of pull down/Input



**B**





# Kawaguchi A. et al., Figure 5

