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H. 知的財産権の出願・登録状況（予定を含む。）

１．特許出願

なし

２．実用新案登録

なし

３．その他

なし

II. 研究成果の刊行に関する一覧表

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III. 研究成果の刊行物・別刷

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Replication-Coupled and Host Factor-Mediated Encapsidation of the Influenza Virus Genome by Viral Nucleoprotein▽

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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/minichromosome maintenance (MCM) complex stimulates a *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 genetic and biochemical evidence suggest that viral nucleoprotein (NP) is involved in successful replication. Here, using cell-free systems, we have shown the precise stimulatory mechanism of 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 findings demonstrate that replication of the virus genome is followed by its encapsidation by NP in collaboration with its chaperone.

The genome of influenza type A viruses consists of eight-segmented and single-stranded RNAs of negative polarity. Transcription from the viral RNA (vRNA) genome is initiated using the oligonucleotide containing the cap-1 structure from cellular pre-mRNAs as a primer, whereas genome replication is primer independent and generates full-length vRNA through cRNA (full-sized complementary copy of vRNA) (reviewed in reference 17). Generally, each viral DNA or RNA genome is not present as a naked form but as a complex with viral basic proteins. The influenza virus genome exists as a ribonucleoprotein (termed vRNP) complex with nucleoprotein (NP), one of the basic viral 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 *Mononegavirales*, 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 that 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 was also shown that NP that is saturated with single-stranded DNA (ssDNA), resulting in the lack of RNA binding activity, stimulates virus genome replication from a model template without primer (18). It is possible that NP stimulates virus genome replication through interaction with the viral polymerase in an RNA binding activity-independent manner. Moreover, the *in vitro* cRNA synthesis using infected cell extracts as an enzyme source depends on a supply of NP free of RNA (27). This finding has been interpreted as indicating that NP prevents the premature termination of RNA synthesis, possibly by binding to nascent RNA chains, that is, encapsidating them. Based on these observations, it could be hypothesized that NP facilitates 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 the 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 involved in 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) is 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 1 (IREF-1) that enabled the viral polymerase to synthesize

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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 a previously established cell-free virus genome replication system and virion-associated vRNP. Exogenously added NP free of RNA stimulated 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 for the stimulation of the 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 small interfering RNA (siRNA)-mediated gene silencing. Based on these observations, we propose an NP- and host factor-dependent mechanism of 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 (ORF) corresponding to the NP gene into pET14b. Rabbit polyclonal antibody against NP was generated by immunization of a 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 manufacturer's 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 glutathione *S*-transferase (GST)-tagged RAF-2p48/UAP56 by PreScission protease (GE Health Care) digestion. Purified proteins were stored in a buffer containing 50 mM HEPES-NaOH (pH 7.9), 300 mM KCl, 20% glycerol, and 1 mM dithiothreitol (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. Cell-free virus genome replication was carried out at 30°C for 90 min in a final volume of 25 μl containing 50 mM HEPES-NaOH (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl, 1.5 mM dithiothreitol, 500 μM each ATP, CTP, and UTP, 25 μM GTP, 5 μCi of [α-^{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 by 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 that 0.3 μM UTP, 250 μM each ATP, CTP, and GTP, and 10 μCi of [α-^{32}P]UTP (3,000 Ci/mmol) were used in a final volume of 200 μl. The coprecipitated RNA products with NP or MCM were separated through 10% PAGE containing 8 M urea.

Gene silencing mediated by siRNA. An siRNA against the RAF-2p48/UAP56 gene corresponding to its open reading frame (5′-AGUACUACGUGAAACU GAAGGACAA-3′) and control double-stranded RNA (dsRNA) targeting none of the cellular mRNAs were designed and synthesized by iGENE Therapeutics Inc. HeLa cells (1×10^5 cells) were transfected with 40 pmol of siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 48 h posttransfection, the cells were infected with influenza A/PR/8/34 at a multiplicity of infection (MOI) of 10 in the absence or presence of 100 μg/ml of cycloheximide (CHX). 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 (Luc) gene in reverse orientation sandwiched between 23-nucleotide (nt)-long 5′-terminal and 26-nucleotide-long 3′-terminal promoter sequences of the influenza virus segment 8, which is placed under the control of the human polymerase I (Pol I) promoter.

Indirect immunofluorescence assay. HeLa cells on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The cells were permeabilized in 0.5% Triton X-100 and incubated in PBS containing 1% bovine serum albumin (BSA). The coverslips were incubated with anti-RAF-2p48/UAP56 rabbit polyclonal antibody (16) for 1 h. After a washing step 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 a 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 h postinfection (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 and 1354, segment 5 m/cRNA (5′-G ATTTCAGTGGCATTCTGGC-3′) complementary to the NP cDNA between nucleotide sequence positions 101 and 120, and 5S rRNA (5′-GGGGTACCTT CGAAAGCCTACAGCACCCGGTA-3′), which were labeled at their 5′ ends with [γ-^{32}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-2p48/UAP56 knockdown cells at 12 h posttransfection for construction of the model viral replicon were subjected to reverse transcription with primers to determine the level of vRNA (5′-TCCATCACGGTTTTGGAATGTTTACTA CAC-3′, which corresponds to the luciferase coding region between nucleotide sequence positions 728 and 757), cRNA (5′-AGTAGAAACAAGGGTGTTTT TTAGTA-3′, which is complementary to the 3′ portion of the segment 8 cRNA), and viral mRNA [oligo(dT)$_{20}$ 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′-TCCATCACGGTTTTGGAAT GTTTACTACAC-3′, which corresponds to the luciferase coding region between nucleotide sequence positions 728 and 757, and 5′-GTGCGCCCCCAGAAGC AATTTC-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 (here, designated exogenous NP) stimulated *de novo* virus genome replication in the absence of MCM and any kind of primer (Fig. 1B, lanes 1 to 5). We confirmed by RNase H digestion analyses with primers corresponding to each segment that RNA products corresponded 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 virus genome replication additively with recombinant NP, suggesting that NP and MCM function through distinct mechanisms (Fig. 1B, lanes 6 to 10 and 16 to 20). The stimulatory activity per molecule of MCM was five times higher than that of NP, as 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) stimulates activity equally as well as recombi-

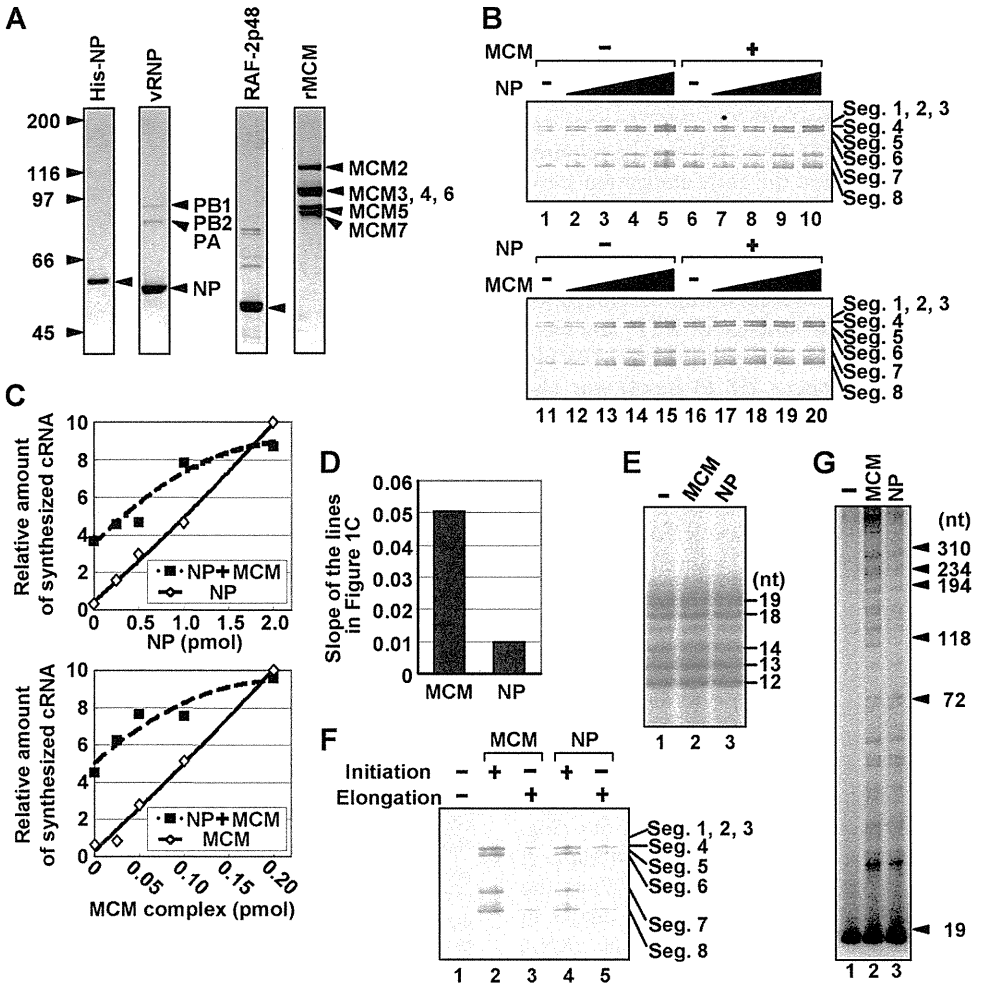

FIG. 1. NP and MCM additively stimulate virus genome replication. (A) Purified recombinant proteins and vRNP. Purified His-NP, vRNP, RAF-2p48/UAP56, and MCM complexes were separated by 7.5% SDS-PAGE and visualized by staining with Coomassie brilliant blue. (B) Stimulatory activity of NP and MCM in cell-free virus genome replication. RNA synthesis was carried out in the absence (lanes 1 to 5) or presence (lanes 6 to 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 experiments shown in the lower panel, we performed the RNA synthesis assay in the absence (lanes 11 to 15) or presence (lanes 16 to 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 were 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 panel C were determined. (E) Limited elongation assays. Unprimed limited elongation assays were carried out in the absence (lane 1) or presence (lane 2; 0.5 pmol) of MCM 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 μM UTP, 250 μM each ATP, CTP, and GTP, and 10 μCi of [α-^{32}P]UTP (3,000 Ci/mmol). The purified products were separated through 4 to 15% linear gradient PAGE containing 8 M urea and visualized by autoradiography.

nant 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 *de novo* virus genome replication. The RNA synthesis level varied among segments, as previously described (9). For instance, segments 1, 2, and 3 were hardly replicated compared with replication of other segments. The reason for this variation in cRNA synthesis is presently unknown.

NP facilitates the promoter escape of the viral RNA polymerase. Previously, we demonstrated that MCM does not enhance the frequency of 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 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 elon-