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Nuclear export of influenza viral ribonucleoprotein is temperature-dependently inhibited by dissociation of viral matrix protein

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Abstract

The influenza virus copies its genomic RNA in the nuclei of host cells, but the viral particles are formed at the plasma membrane. Thus, the export of new genome from the nucleus into the cytoplasm is essential for viral production. Several viral proteins, such as nucleoprotein (NP) and RNA polymerases, synthesized in the cytoplasm, are imported into the nucleus, and form viral ribonucleoprotein (vRNP) with new genomic RNA. vRNP is then exported into the cytoplasm from the nucleus to produce new viral particles. M1, a viral matrix protein, is suggested to participate in the nuclear export of vRNP. It was found unexpectedly that the production of influenza virus was suppressed in MDCK cells at 41°C, although viral proteins were synthesized and the cytopathic effect was observed in host cells. Indirect immunofluorescent staining with anti-NP or M1 monoclonal antibody showed that NP and M1 remained in the nuclei of infected cells at 41°C, suggesting that a suppression of viral production was caused by inhibition of the nuclear export of these proteins. The cellular machinery for nuclear export depending on CRM1, which mediates the nuclear export of influenza viral RNP, functioned normally at 41°C. Glycerol-density gradient centrifugation demonstrated that vRNP also formed normally at 41°C. However, an examination of the interaction between vRNP and M1 by immunoprecipitation indicated that M1 did not associate with vRNP at 41°C, suggesting that the association is essential for the nuclear export of vRNP. Furthermore, when infected cells incubated at 41°C were cultured at 37°C, the interaction between vRNP and M1 was no longer detected even at 37°C. The results suggest that M1 synthesized at 41°C is unable to interact with vRNP and the dissociation of M1 from vRNP is one of the reasons that the transfer of vRNP into the cytoplasm from the nucleus is prevented at 41°C.

Keywords: Influenza virus; Ribonucleoprotein; Temperature-dependency; Nuclear export; Viral matrix protein

Introduction

We studied the mechanism of membrane fusion, using experimental models of enveloped viral infection and skeletal myoblast differentiation. In the life cycle of an enveloped virus, both viral penetration and budding are associated with membrane fusion (Kielian and Jungerwirth, 1990; Cadd et al., 1997). Also, on the differentiation of skeletal myoblasts, mononucleated myoblasts fuse with each other into multinucleated myotubes (Knudsen and Horwitz, 1977). The study of these fusion mechanisms is important for understanding the biological functions of cell membrane.

To analyze the mechanism of myoblast fusion, we have been using quail myoblasts transformed with a temperature-sensitive mutant of the Rous sarcoma virus (RSV), named QM-RSV (Kim et al., 1992a, b). Cell differentiation in the QM-RSV system is simply regulated by a change of culture temperature. The cells proliferate at 35.5°C, a permissive temperature for RSV, but do not differentiate. The cells begin to differentiate at 41°C, a nonpermissive temperature for RSV, and form myotubes through myoblast fusion. As part of our investigation, the artificial myotubes were made via the fusion of presumptive myoblasts using HVJ (Sendai virus) and compared with spontaneous myotubes formed at 41°C (Saiuchi et al., 1993; Kim et al., 1995; Hirayama et al., 2001).

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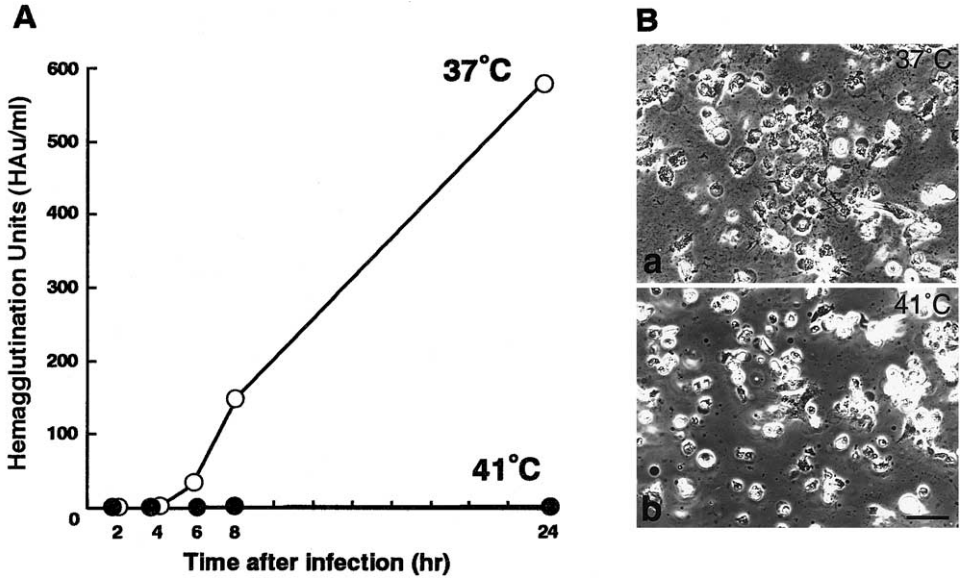

Fig. 1. Production of influenza virus at 37°C or 41°C. (A) MDCK cells were infected with influenza virus (A/Aichi/2/68/H3N2) and incubated at 37°C (○) or 41°C (●). After the infection, culture fluids were harvested when indicated and viral production was assayed in hemagglutination units (HAu/ml). Viral production at 41°C was almost undetectable. (B) Phase-contrast micrographs of infected cells cultured at 37°C (a) or 41°C (b) for 24 h. The cytopathic effect of the infection was observed even at 41°C. Bar, 50 μm.

In the course of characterizing the artificial myotubes from QM-RSV cells, we found unexpectedly that the production of HVJ was markedly suppressed at 41°C, despite that it was normal at 35.5°C. Thus, we examined the temperature-dependency of viral production with other kinds of virus. As a result, the production of the measles virus, influenza virus, herpes simplex virus type-1, and poliovirus was also found to be suppressed at 41°C. However, the inhibitory mechanism of viral production appeared to differ for each virus. For example, in the case of HVJ, the synthesis of viral-specific proteins in host cells was inhibited at 41°C at the level of transcription (Ishida et al., 2002). On the other hand, influenza viral proteins were synthesized even at 41°C. However, our experiments showed that the influenza viral proteins could not be exported from the nucleus into the cytoplasm in host cells at 41°C.

The influenza virus is a RNA virus with an envelope. Viral RNA is always found in association with viral polypeptides, including the three subunits of RNA-dependent RNA polymerases (PB1, PB2, and PA) and the viral nucleoprotein (NP), to form viral ribonucleoprotein (vRNP) complexes. After the influenza virus has invaded a host cell, vRNP is released into the cytoplasm and enters the nucleus via the cellular nuclear import pathway mediated by importin α/β (Martin and Helenius, 1991b; O'Neill et al., 1995). In the nucleus, vRNP acts as a template for the synthesis of mRNA and progeny genomic RNA. The mRNA for viral proteins is exported into the cytoplasm and translated. Several viral proteins, such as NP and RNA polymerases, are imported into the nucleus and assembled together with newly synthesized genomic RNA into vRNP in the nucleus. The vRNP is then transferred into the cytoplasm via the cellular CRM1-mediated nuclear export pathway to form progeny viral particles with other viral components at the plasma membrane (Elton et al., 2001; Ma et al., 2001; Neumann et al., 2000). Thus, the export of vRNP from the nucleus is one of the most important events in the infectious cycle of the influenza virus (Lamb and Krug, 1996). Current reports suggest that two viral proteins, matrix protein (M1) and nuclear export protein (NEP/NS2) mediate the export of vRNP via formation of a vRNP-M1-NEP/NS2 complex (O'Neill et al., 1998; Neumann et al., 2000), but little is known about its mechanism. The investigation of this process is important for understanding not only the infectious cycle of the influenza virus but also the cellular nuclear transport system.

We investigated the inhibitory mechanism of influenza viral production at 41°C associated with nuclear export of viral proteins. It was clarified that the association of M1 is essential to the export of vRNP from the nucleus to cytoplasm.

Results

Inhibition of viral production at 41°C

To investigate the effects of incubation temperature on the production of influenza virus, MDCK cells were cultured at 37°C or 41°C after infection with A/Aichi/2/68/H3N2. Culture fluids were harvested at various time points after the infection and hemaggulutinating activity (HA) was assayed as viral production. The results were plotted as hemaggultination units (HAu/ml) (Fig. 1A). At 37°C, HA increased from 6-h incuba-

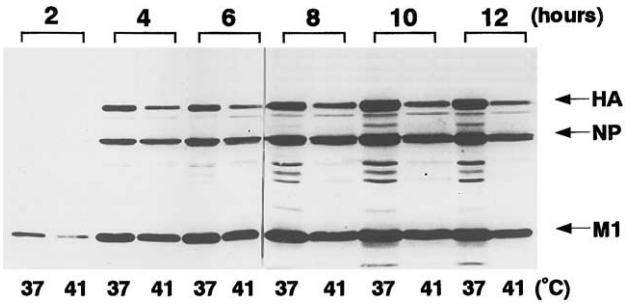

Fig. 2. Synthesis of influenza virus-specific proteins at 37°C or 41°C. Total protein was extracted from infected MDCK cells after culture for each period of time. Viral proteins were detected by immunoblotting using anti-influenza virus serum. Viral proteins were synthesized irrespective of culture temperature.

tion to about 600 HAu/ml within 24 h. After that, viral production reached a plateau. In contrast to this, when the infected cells were incubated at 41°C, the viral production was almost undetectable. The cytopathic effect, however, was seen at 41°C as well as 37°C (Fig. 1B). Similar results were obtained using other influenza viral strains, A/PR/8/34/H1N1 and A/Okuda/57/H2N2. These results imply that viral infection proceeded in host cells, but some steps of viral production were inhibited at 41°C.

Synthesis of viral-specific proteins is not inhibited at 41°C

To examine which steps of viral production were inhibited at 41°C, the synthesis of viral-specific proteins was monitored. Infected cells incubated at 37°C or 41°C were lysed, and viral-specific proteins were detected by immunoblotting using anti-influenza virus serum. As shown in Fig. 2, viral proteins were detected from about 4 h after infection and increased with incubation time at both temperatures, although some nonspecific bands were recognized between NP and M1 bands at 37°C and amounts of viral proteins showed a slight reduction at 41°C.

For a more precise analysis, infected cells were pulse-labeled with [^{35}S]methionine from 5 to 6 h after infection, when the synthesis of viral proteins had definitely started. The proteins were separated by SDS–PAGE and the quantity of radioactive viral proteins at 41°C was compared with that at 37°C using a BAS-2000 system (Fuji Film Co. Ltd.). Viral proteins were basically synthesized at both temperatures, although the amounts showed a slight reduction at 41°C (data not shown).

Inhibition of the export of viral proteins from the nucleus to cytoplasm at 41°C

To further investigate the inhibitory mechanism of viral production at 41°C, the distribution of viral proteins in the infected cells was observed in detail by immunofluorescent staining. Infected cells were fixed at various time points after infection, and first stained with anti-influenza virus serum (data not shown). Viral proteins were detectable in the nucleus but not the cytoplasm of host cells at 2 h after infection both at 37°C and 41°C. On further culture at 37°C, cytoplasmic fluorescence increased gradually from 4 h after infection, showing that viral proteins once imported into the nucleus were transferred into the cytoplasm at 37°C. On the other hand, at 41°C, the nuclear fluorescence was stronger than at 37°C after 4- or 6-h culture, indicating that viral proteins remained in the nucleus. Even after 8 h, viral proteins remained in the nucleus at 41°C. These observations suggest that the nuclear export of viral proteins into the cytoplasm was disturbed at 41°C.

To confirm this possibility, the localizations of NP and M1 were examined by immunofluorescent staining using specific monoclonal antibodies. NP and M1 shuttle between the nucleus and cytoplasm (Martin and Helenius, 1991a, b; Whittaker et al., 1996; Neumann et al., 1997). NP is the major component of vRNP and an indicator of vRNP-localization. Early in the infection, NP synthesized in the cytoplasm immediately enters the nucleus where viral RNA (vRNA) is replicated, and encapsidates the vRNA to form vRNP. Newly synthesized M1 also enters the nucleus to interact with the vRNP. vRNP-M1 complexes are then transported from the nucleus to the cytoplasm.

NP (Fig. 3A) and M1 (Fig. 3B) were already localized in the nuclei within 2-h incubation at both temperatures, indicating that these proteins were transported into the nucleus immediately after synthesis irrespective of the temperature (a, b in Figs. 3A and 3B, respectively). They were then exported from the nucleus into the cytoplasm as vRNP-M1 complexes at 37°C, and as a result, all of the cell was evenly stained after culture for 6 h and 8 h (c, e, g in Figs. 3A and 3B, respectively). However, at 41°C, they were not exported into cytoplasm and remained in the nuclei even at 8 h after infection (d, f, h in Figs. 3A and 3B, respectively). The results demonstrate that the transport of NP and M1 to the cytoplasm was inhibited at 41°C.

In addition, an unique distribution of M1 was observed at 41°C. M1 was localized at the nucleolus (arrows in Figs. 3B: d, f, h), whereas no such distribution was seen at 37°C (arrows in Figs. 3B: c, e, g; also compare the insets in d with c).

The presence of NP and M1 in the nucleus at 41°C was affected by shifting the temperature to 37°C. When infected cells cultured at 41°C were kept at 37°C, cytoplasmic NP began to be detected weakly 1 h after the temperature shift (Fig. 4b) and gradually increased with further incubation at 37°C (Figs. 4c, d). Similar results were obtained in M1 (data not shown).

Effect of temperature on the cellular nuclear export system

The results above suggest that the inhibition of viral production at 41°C was due to a disturbance of the export of some viral-specific proteins from the nucleus to the cyto-

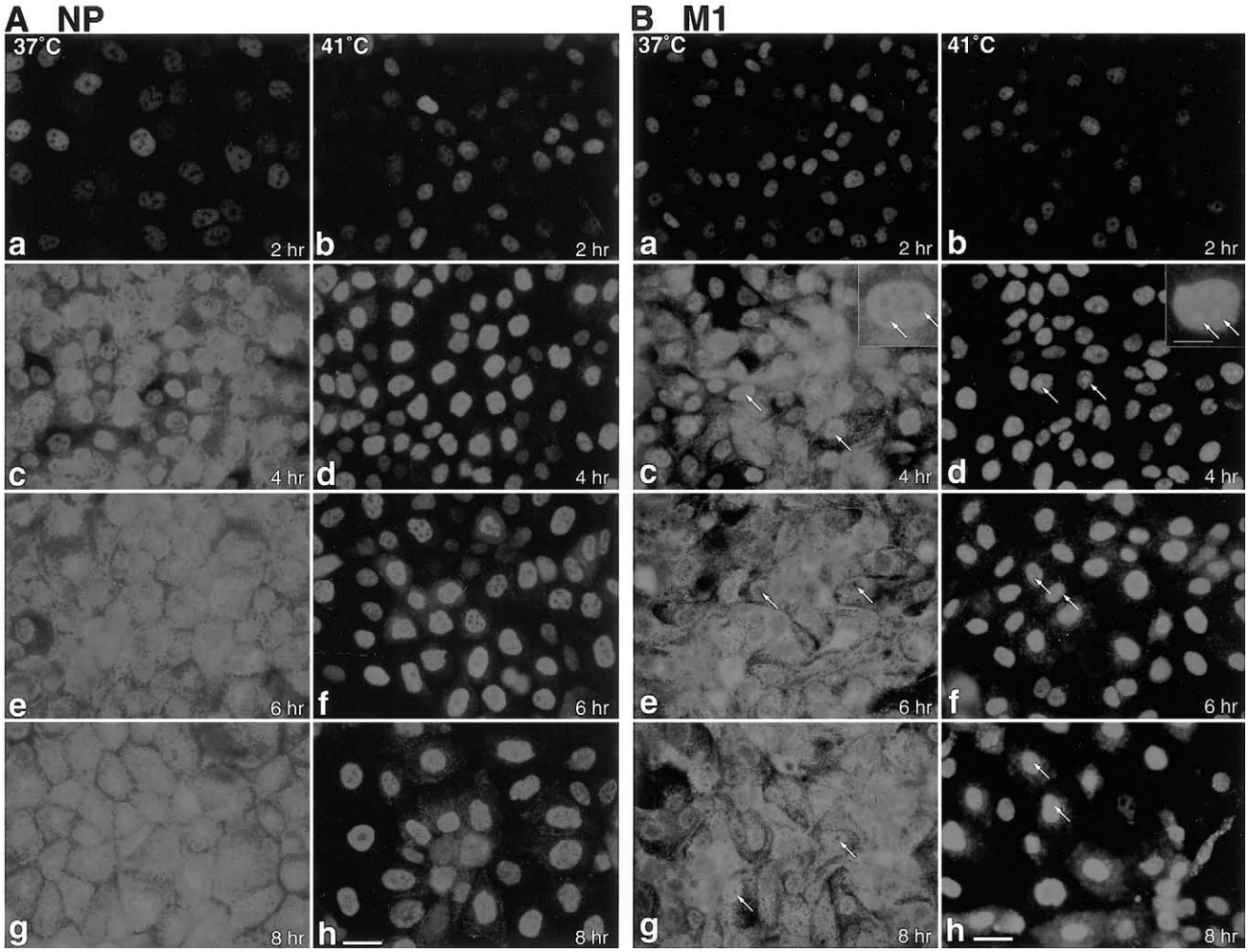

Fig. 3. Intracellular distribution of virus-specific proteins, NP and M1. Infected MDCK cells were incubated at 37°C (a, c, e, g in A and B) or 41°C (b, d, f, h in A and B) for 2 h (a, b), 4 h (c, d), 6 h (e, f), or 8 h (g, h). The cells were indirectly immunofluorescently stained using monoclonal antibody against NP (A) or M1 (B). At 41°C, NP and M1 continued to be detected predominantly in the nucleus even after 8-h incubation (h). M1 was also detected in the nucleolus at 41°C (arrows in B-d, f, h), but not at 37°C (compare insets B-d with B-c). The insets in c or d are high magnification views of a nucleus. Bar, 25 μm, 10 μm in the insets.

plasm. To confirm this, further investigations were performed. Recently, reports have shown that influenza viral RNP is exported from the nucleus by a cellular CRM1-mediated nuclear export pathway (Elton et al., 2001; Ma et al., 2001; Neumann et al., 2000). CRM1 is an essential cellular factor for the nuclear export of proteins containing the leucine-rich nuclear export signal (NES) (Fornerod et al., 1997). Then, it was examined whether the cellular export system is affected at 41°C.

First, it was examined whether CRM1 is associated with the nuclear export of influenza viral RNP in our experimental system. For this, Leptomycine B (LMB), which inhibits CRM1 specifically (Kudo et al., 1998; Nishi et al., 1994), was used. MDCK cells were cultured for 3 h at 37°C after the infection to enable the accumulation of viral proteins in the nucleus. Then, the cells were treated with LMB for another 3 h at 37°C. NP remained predominantly in the nucleus in the cells treated with LMB even at 37°C, indicating that CRM1 is associated with the nuclear export of influenza viral RNP (data not shown).

Thus, it was investigated whether the function of CRM1 is influenced at 41°C. MDCK cells were cultured at 41°C or 37°C for 6 h, and GFP (green fluorescent protein)-NES was injected into the nuclei of the cells (Fig. 5 a). The cells were incubated at 41°C or 37°C for another 30 min for nuclear export of general NES-containing proteins and the localization of GFP-NES was observed. GFP-NES injected into the nucleus (Fig. 5a) was transported into the cytoplasm at 41°C (Fig. 5g) as well as at 37°C (Fig. 5d). These results indicate that the cellular machinery for nuclear export via CRM1 is not impaired at 41°C.

vRNP forms even at 41°C

The formation of vRNP is required for the nuclear export of viral proteins (Hamaguchi et al., 1985). Then, experi-

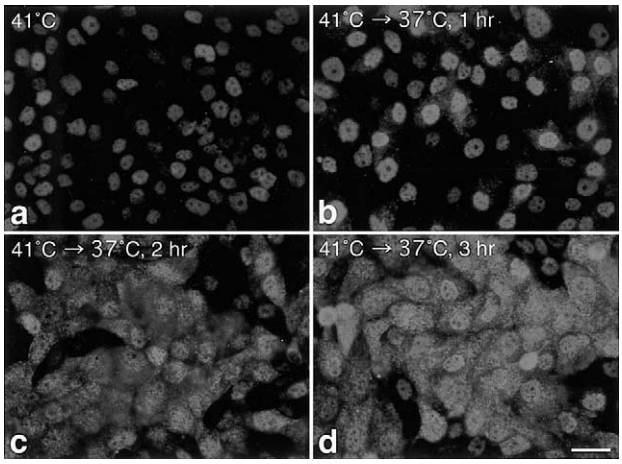

Fig. 4. Intracellular localization of NP after shifting down the temperature to 37°C from 41°C. Infected MDCK cells were incubated at 41°C for 4 h (a), and then further cultured at 37°C for 1 h (b), 2 h (c), or 3 h (d). The localization of NP was examined by immunofluorescent staining as in the case of Fig. 3A. NP was gradually exported from the nucleus into the cytoplasm with incubation time after the shift to 37°C. Bar, 25 μm .

ments were conducted to determine whether vRNP formed normally in the nucleus at 41°C. The formation of vRNP can be detected from the separation pattern of density-gradient centrifugation (Bui et al., 2000). Infected cells incubated at 37°C or 41°C for 6 h were lysed and separated into 15 fractions by glycerol-density gradient centrifugation. The position of vRNP was visualized by immunoblotting using anti-NP antibody. As shown in Fig. 6A, vRNP was sedimented in the expected position in the glycerol gradient, peaking in fraction-11 and -12 at both temperatures. The measurement of the intensity of the bands showed that the peaks of vRNP-fractions were similar in infected cells incubated at both 37°C and 41°C (Fig. 6B). The data suggest that the formation of vRNP proceeds as normal even at 41°C.

Effects of incubation temperature on the formation of vRNP-M1 complexes

Some reports have shown that the export of vRNP into the cytoplasm is blocked in the absence of M1 (Bui et al., 2000; Martin and Helenius, 1991a Whittaker et al., 1996), suggesting that the process requires M1. The interaction between vRNP and M1 at 41°C was examined by immunoprecipitation assay. To investigate the association of M1 with vRNP, the extracts of infected cells were immunoprecipitated with anti-NP monoclonal antibody, and the precipitates were analyzed by immunoblotting using anti-influenza virus serum (Fig. 7). In the infected cells cultured at 37°C, M1 was coprecipitated with NP as expected (arrowhead in Fig. 7, Lane 2), showing that M1 associates with vRNP. In contrast, M1 was not coprecipitated with NP in the cells cultured at 41°C (Fig. 7, Lane 3). This indicates that vRNP-M1 complexes were not formed at 41°C.

To understand why M1 did not associate with vRNP at 41°C, attempts were made to further characterize the M1 synthesized at 41°C. Infected cells were incubated at 41°C or 37°C for 4 h, and cultured for another 2 h at 37°C with cycloheximide, an inhibitor of protein translation. As the synthesis of new viral proteins at 37°C was inhibited by cycloheximide, viral components synthesized at 41°C could be chased in this assay. In control cells incubated at 37°C for the first 4 h, M1 was coprecipitated with NP (arrowheads in Fig. 8A, Lanes 2 and 3, respectively). This shows that cycloheximide did not affect the formation of complexes between M1 and vRNP. On the other hand, M1 was not coprecipitated with NP in the cells first cultured at 41°C (Fig. 8A, Lanes 4 and 5). The results suggest that M1 synthesized at 41°C could not associate with vRNP even at 37°C.

The localization of vRNP in these cells was also observed (Fig. 8B). vRNP was distributed both in the cytoplasm and in the nucleus (Fig. 8B-a) in control cells, in which M1 associates with vRNP (Fig. 8A, Lane 3) even in the presence of cycloheximide. By contrast, the export of vRNP into the cytoplasm was inhibited (Fig. 8B-b) in the cells first cultured at 41°C in which M1 did not associate with vRNP (Fig. 8A, Lane 5). The results suggest that the association of M1 with vRNP is necessary for the migration of vRNP into the cytoplasm from the nucleus.

We further examined whether vRNP-M1 complexes once formed at 37°C were dissolved at 41°C. Infected cells were incubated at 37°C for 4 h to form vRNP-M1 complexes and incubated for another 2 h at 41°C with cycloheximide. M1 was detected in immunoprecipitates with NP (arrowheads in Fig. 8A, Lanes 6 and 7), suggesting that the

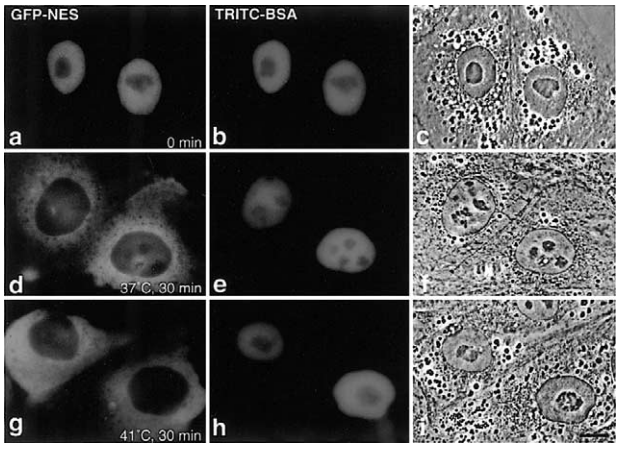

Fig. 5. Effect of temperature on nuclear export pathway depending on CRM1. MDCK cells were incubated at 41°C or 37°C for 6 h and then green fluorescence protein containing nuclear export signal (GFP-NES) was microinjected into the nucleus (a, d, g). TRITC-BSA was coinjected as an injection marker (b, e, h). Injected cells were fixed immediately after injection (a-c), or after incubation for another 30 min at 37°C (d–f) or 41°C (g–i). c, f, and i are phase-contrast images of a–b, d–e, and g-h, respectively. Injected GFP-NES was exported into the cytoplasm even at 41°C (g). Bar, 10 μm.

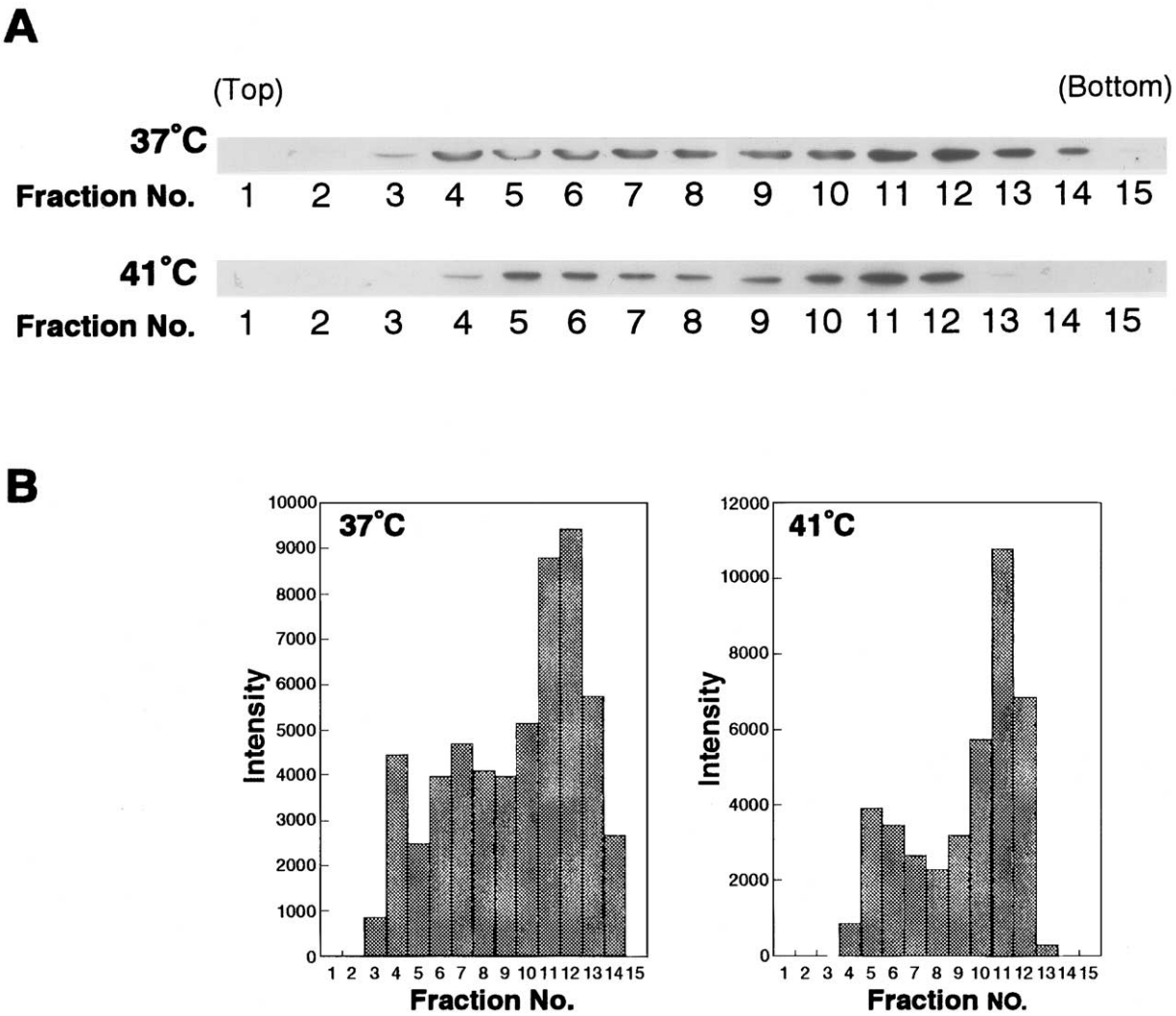

Fig. 6. Segmentation of vRNP by glycerol-density gradient centrifugation. Infected MDCK cells were incubated at 37°C or 41°C for 6 h, and lysed as described in Materials and methods. The extracts were layered onto a step-glycerol gradient, and separated into 15 fractions. vRNP was detected by immunoblotting with anti-NP antibody (A). The intensity of the bands of NP was measured by NIH image (B). The peaks of vRNP were at fraction-11 and -12 at both temperatures, suggesting that the formation of vRNP was not impaired at 41°C.

complex between vRNP and M1 formed at 37°C was maintained even at 41°C. Over this period of time, the export of vRNP into the cytoplasm was not inhibited (Fig. 8B-c).

It was desirable, however, that these results be confirmed without cycloheximide, because the drug may influence indirectly the results. To chase the viral proteins, [^{35}S]methionine was used. Infected cells were incubated at 41°C or 37°C for 3 h and pulse-labeled with [^{35}S] methionine for another 1 h. The temperature was then shifted down to 37°C or up to 41°C for another 2 h in normal medium and an immunoprecipitation assay was performed similar to that in Figs. 7 and 8. In the shift-down assay (41°C, 4 h → 37°C, 2 h), labeled M1, which was synthesized at 41°C, was not coprecipitated with vRNP (date not shown) consistent with the above experiments with cycloheximide (Fig. 8A, Lanes 4 and 5). However, in the shift-up assay (37°C, 4 h → 41°C, 2 h), it was found unexpectedly that the vRNP-M1 complexes once formed at 37°C dissociated gradually at 41°C (Fig. 9, Lanes 5 and 6). This was in contradiction to the above results with cycloheximide (Fig. 8A, Lanes 6 and 7). Since the results obtained without the drug more reasonably reflected the normal state of cells, it is considered that the complexes between vRNP and M1 were dissociated at 41°C, and that cycloheximide inhibited the dissociation at 41°C (see Discussion).

Discussion

Viral production has been found to be suppressed at 41°C in many kinds of virus (Ishida et al., 2002). Production of the influenza virus was also inhibited at 41°C. In this study, we showed that the suppression of influenza viral production was due to a disturbance of the nuclear export of

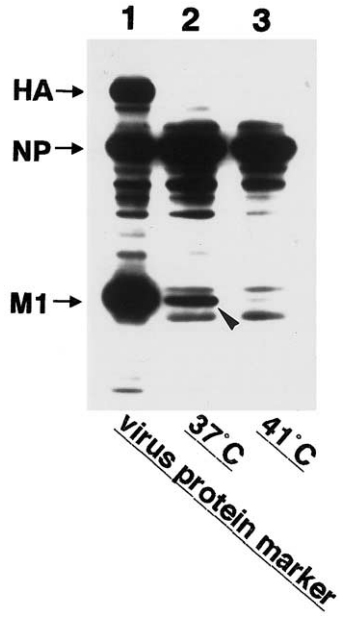

Fig. 7. Interaction of M1 with vRNP in infected cells. Infected cells were incubated at 37°C or 41°C for 4 h and total protein was extracted as described in Materials and methods. Cell lysates were immunoprecipitated with anti-NP antibody and immunoblotted using anti-influenza virus serum. Lane 1; markers of viral proteins, Lane 2; precipitates from infected cells incubated at 37°C, Lane 3; precipitates from infected cells incubated at 41°C. M1 was not coimmunoprecipitated with vRNP at 41°C.

viral proteins. There are no reports that the nuclear export of influenza viral proteins is temperature-sensitive, although some studies report that viral production is influenced at high temperature (Scholtissek and Rott, 1969; Kendal et al., 1977; Oxford et al., 1980; Chu et al., 1982; Giesendorf et al., 1984). Further investigation significantly demonstrated that M1 did not associate with vRNP at 41°C and this dissociation caused the viral proteins to remain in the nucleus at 41°C. M1 is reported to be required for nuclear export of vRNP in the influenza virus. When a M1-defective virus is used or the synthesis of M1 is inhibited, the export of vRNP from the nucleus fails to occur (Martin and Helenius, 1991a; Bui et al., 2000). Microinjection of antibodies against M1 blocks the process of vRNP-export (Martin and Helenius, 1991a). On the other hand, electron microscopic observation and in vitro binding assay suggest that M1 interacts with vRNP, especially with viral RNA (Martin and Helenius, 1991a; Ye et al., 1999). Thus, it is conceivable that the association of M1 is necessary for nuclear export of vRNP (Martin and Helenius, 1991a; Bui et al., 2000). However, no direct proof had been obtained. Our results directly indicate that the binding of M1 with vRNP is closely related to their migration from the nucleus. This is the first report showing that the formation of a complex of M1 and vRNP is essential for their nuclear export into the cytoplasm. NEP/NS2, a small component protein of the influenza virus, is also reported to mediate the export of vRNP from the nucleus by acting as an adaptor protein between CRM1, a nuclear export machinery, and the M1-vRNP complex (O'Neill et al., 1998; Neumann et al., 2000). Further investigating the function of NEP/NS2 at 41°C would provide a more accurate model of the nuclear export of influenza viral proteins. An investigation is in progress.

M1 was uniquely distributed in the nucleolus at 41°C. Concerning this, there is a report that M1 is localized in the nucleolus when it alone is expressed in CV-1 cells (Bucher

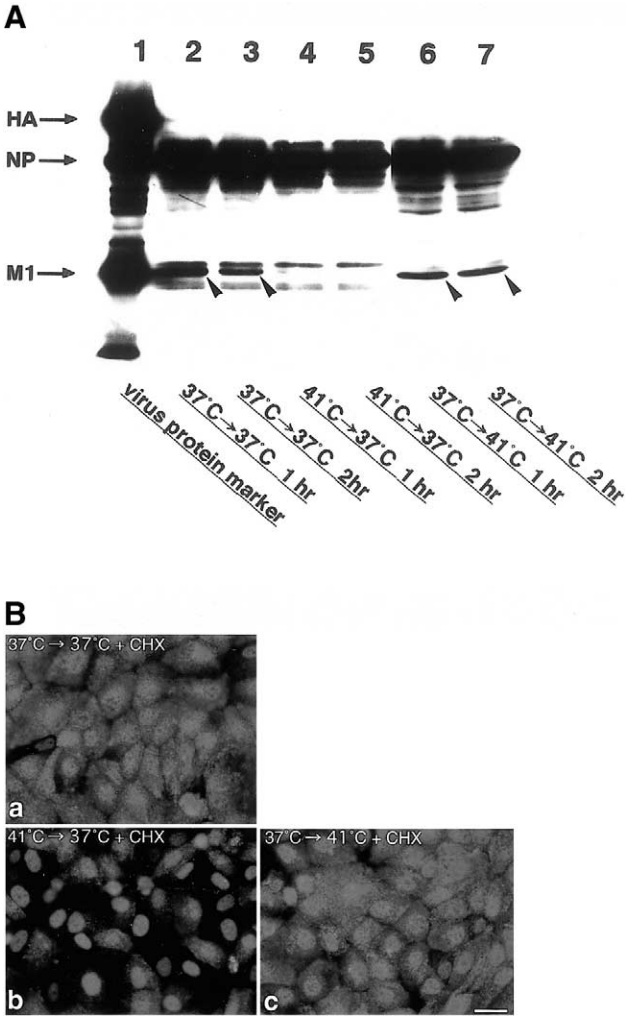

Fig. 8. Interaction of M1 synthesized at 41°C or 37°C with vRNP. (A) Infected cells were incubated at 37°C or 41°C for 4 h, and then further cultured at 37°C or 41°C for 2 h with 50 μM cycloheximide (CHX). The interaction between vRNP and M1 was examined by immunoprecipitation using anti-NP antibody similar to in Fig. 7. Lane 1; markers of viral proteins, Lanes 2 and 3; precipitates from infected cells incubated at 37°C for 4 h and incubated another 1 h (Lane 2) or 2 h (Lane 3) at 37°C with cycloheximide, Lanes 4 and 5; precipitates from infected cells incubated at 41°C for 4 h and another 1 h (Lane 4) or 2 h (Lane 5) at 37°C with cycloheximide, Lanes 6 and 7; precipitates from infected cells incubated at 37°C for 4 h and another 1 h (Lane 6) or 2 h (Lane 7) at 41°C with cycloheximide. (B) Fluorescent images of NP in the cells shown in Lane 3, 5, or 7 in (A). Namely, infected cells were incubated at 37°C (a, c) or 41°C (b) for 4 h and incubated for another 2 h at 37°C (a, b) or 41°C (c) with cycloheximide. The binding of M1 with vRNP is associated with the migration of vRNP from the nucleus to cytoplasm. Bar, 25 μm.

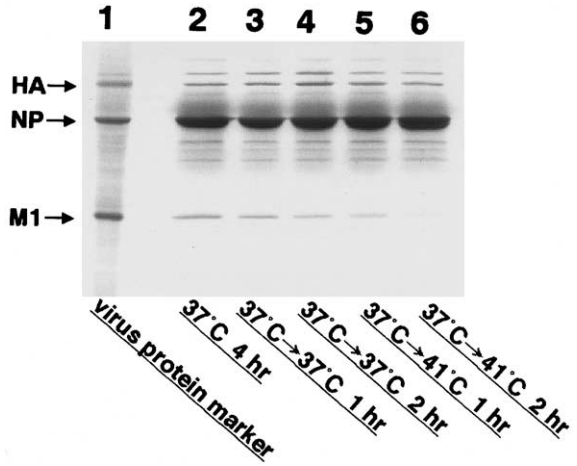

Fig. 9. Effect of incubation temperature on the association of M1 with vRNP. Infected cells were incubated at 37°C for 3 h and labeled with [^{35}S]methionine for 1 h. Labeled cells were then incubated for another 2 h at 37°C or 41°C in normal medium. Cells were lysed and immunoprecipitated using anti-NP antibody similar to in Fig. 7 or 8. The association between vRNP and M1 was detected from the radioactivity of M1. Lane 1; markers of viral proteins, Lane 2; precipitates from infected cells incubated at 37°C for 3 h and labeled for 1 h, Lanes 3 and 4; precipitates from infected cells incubated for another 1 h (Lane 3) or 2 h (Lane 4) at 37°C after labeling, Lanes 5 and 6; precipitates from infected cells shifted up to 41°C for 1 h (Lane 5) or 2 h (Lane 6) after labeling. M1 was dissociated from vRNP by shift up to 41°C (Lanes 5 and 6).

et al., 1989). Consistent with this report and our observation, M1 does not associate with vRNP. M1 may tend to be localized in the nucleolus when it exists alone, but is transferred into the cytoplasm when it forms a complex with vRNP in the nucleus.

The shift-down assay with cycloheximide and [^{35}S]methionine suggested that M1 synthesized at 41°C could not associate with vRNP even at 37°C. However, the results were reversed in the shift-up assay using cycloheximide and [^{35}S]methionine. In the presence of cycloheximide, the M1-vRNP complex that formed at 37°C appeared even at 41°C. However, M1 labeled at 37°C was separate from vRNP at 41°C, suggesting that the complex was dissociated at 41°C. As described in the "Results", the behavior of labeled proteins reflects the natural state in the cell. From the results, it is considered that cycloheximide is associated with the inhibition of the dissociation of the M1-vRNP complex at 41°C. Namely, certain proteins synthesized only at 41°C, such as heat shock proteins, obstruct the association of M1 with vRNP. As such proteins are not synthesized in the presence of cycloheximide, the M1-vRNP complex does not dissociate even at 41°C. Cellular phosphorylation events are reported to affect the nuclear export of vRNP (Bui et al., 2000, 2002; Pleschka et al., 2001). In our experimental system, however, M1 synthesized at 41°C did not bind with vRNP when the cells were then cultured at 37°C, suggesting that the change to vRNP or M1 at 41°C is irreversible. This implies another mechanism to prevent the nuclear export of vRNP at 41°C, other than phosphorylation. If phosphorylation is simply involved, the association between M1 synthesized at 41°C and vRNP will be recovered by shifting down to 37°C, at which temperature the cellular phosphorylation system is normal. We assume that the obstructive proteins induce irreversible, perhaps conformational, changes in vRNP or M1 at 41°C. Identifying such obstructive factors will provide important information not only on the mechanism of nuclear export of viral proteins, but also on how to inhibit influenza viral infection.

Materials and methods

Cells and virus

MDCK cells, derived from canine kidney, were grown in minimum essential medium (MEM) supplemented with 10% calf serum (CS) as described previously (Kim et al., 1990). When indicated, the cells were treated with 10 ng/ml of Leptomycine B (a gift from Dr. Minoru Yoshida, Tokyo University, Tokyo, Japan) or 50 μM cycloheximide (Sigma Chemical Co., CA).

Influenza A virus (A/Aichi/2/68/H3N2) (kindly provided by Dr. Yosinobu Okuno, Osaka Prefectural Institute of Public Health, Osaka, Japan) was propagated in chick embryonated eggs as described previously (Yamaoka et al., 1995). The harvested infectious chorioallantoic fluid was stored at −80°C until use.

Viral infection

Viral infection was carried out as described previously (Yamaoka et al., 1995). Briefly, 1×10^6 MDCK cells were seeded into a 35-mm diameter dish and incubated at 37°C for 48 h in 10% CS-MEM. The cells were washed once with PBS (phosphate-buffered saline; 8 g/l NaCl, 0.2 g/l KCl, 2.9 g/l $Na_2HPO_4{\cdot}12H_2O$, 0.2 g/l KH_2PO_4) containing 2 mM $CaCl_2$ (PBS(+)) and 0.2 ml of infectious chorioallantoic fluid diluted with PBS(+) was inoculated onto MDCK monolayers at a multiplicity of infection (m.o.i.) of 2×10^3 (2×10^3 virions per cell) at 4°C for 60 min with occasional tilting. Infected cells were washed twice with PBS and cultured in 1 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% CS. To measure the viral production in host cells, 0.05 ml of culture fluid was harvested at various time points after the infection and hemagglutinating activity (HA) was measured using 0.5% (v/v) chick red blood cells in PBS as described previously (Salk, 1944).

Antibodies

Anti-influenza virus polyclonal antibodies were prepared by immunizing rabbits with purified influenza virus from infectious chorioallantoic fluid. Monoclonal antibodies against NP or M1 of the influenza virus were prepared by a method described previously (Hyodo and Kim, 1994).

Briefly, viral proteins extracted from purified virus with 0.5% sodium dodecyl sulfate (SDS) were injected into 4-week-old female Balb/c mice. Spleen cells were fused with myeloma cells (X63-Ag8-6.5.3.) by polyethylene glycol-4000 (Nacalai Tesque, Kyoto, Japan). Hybridoma cells producing antibodies to NP or M1 were cloned by limited dilution. Monoclonal antibodies were purified from the culture supernatant of hybridoma cells.

SDS–PAGE and immunoblotting

All procedures were performed as described previously (Laemmli, 1970; Saiuchi et al., 1993). Briefly, SDS-extracts of infected cells were separated in 11% or 13% polyacrylamide gels, and transferred onto nitrocellulose membranes. The blotted membranes were incubated in Tris-buffered saline (2.42 g/l Tris-(methyl hydroxy)-aminomethane, 29.24 g/l NaCl, pH adjusted to 7.5 with HCl) containing 4% skim milk to block the nonspecific absorption of antibodies. The membranes were reacted with anti-influenza virus serum and viral proteins on the membranes were visualized with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories, CA) and 4-chloro-1-naphthol.

Pulse-labeling

Infected MDCK cells were labeled with 50 μCi/ml [^{35}S]methionine (American Radiolabeled Chemicals, Inc., MO) in DMEM deficient in methionine (GIBCO BRL, NY) from 5 to 6 h after infection. Total protein was extracted from infected cells as described previously (Saiuchi et al., 1993) and separated on 13% SDS-polyacrylamide gel. The gel was dried and exposed to a Fuji Imaging Plate Type BAS-III (Fuji Film Co. Ltd., Tokyo, Japan) for 6 h. Radioactive viral proteins were analyzed with a Bio-Imaging Analyzer BAS-2000II system (Fuji Film Co.).

Indirect immunofluorescent staining

Indirect immunofluorescent staining was performed as described previously (Kim et al., 1995). The cells grown on glass coverslips were fixed in a mixture of acetone and methanol (1:1), subjected to antibody-treatment with anti-influenza virus serum, anti-NP monoclonal antibody, or anti-M1 monoclonal antibody, and counterstained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or mouse immunoglobulin goat antibodies (Cappel/ICN Pharmaceuticals, Inc., OH).

Microinjection assay

MDCK cells were seeded on glass coverslips and cultured for 48 h at 37°C. The cells were further incubated for 6 h at 41°C or 37°C as a control. Green fluorescence protein (GFP) fused with nuclear export signal of protein kinase inhibitor (GFP-NES) (a gift from Dr. Yoshihiro Yoneda, Osaka University, Osaka, Japan) (Tachibana et al., 1996) was microinjected through a glass capillary into the nuclei of cells with TRITC-BSA (Sigma Chemical Co.), which enables one to monitor the actual site of injection. Injected cells were then incubated for 30 min at 41°C or 37°C. After fixation for 10 min with 3.7% formaldehyde in PBS at room temperature, the localization of injected proteins was observed by fluorescent microscopy (Carl Zeiss, Oberkochen, Germany).

Glycerol-density gradient centrifugation

Glycerol-density gradient centrifugation was performed according to the method of Bui et al. (2000). The cells incubated at 37°C or 41°C for 6 h after viral infection were scraped and lysed into PBS containing 0.1% Triton X-100. The cell lysate was treated with DNase I (50 μg/ml) for 30 min at 37°C, followed by the addition of NaCl to a final concentration of 1 M. The mixture was centrifuged, and the supernatants were loaded onto a step glycerol-gradient (1 ml of 70%, 0.75 ml of 50%, 0.375 ml of 40%, and 1.8 ml of 33% [wt/vol] glycerol). The gradient was centrifuged at 45000 rpm for 4 h at 4°C in a SW50.1 rotor (Beckman, CA). Fractions were collected from the bottom and concentrated. Each fraction was analyzed by SDS–PAGE and immunoblotting using anti-NP monoclonal antibody. The intensity of the bands of NP was measured by NIH Image, an image processing and analysis program for the Macintosh computer provided free by the National Institutes of Health (MD, USA).

Immunoprecipitation assay

Infected cells incubated for 4 h at 37°C or 41°C were harvested in TNE buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 150 mM NaCl, 1 mM EDTA, and 25 μg/ml aprotinin), and lysed by 20–30 strokes through a 27 gauge needle until the nuclear membrane was almost disrupted for monitoring by microscopy. After centrifugation at 12000 rpm for 15 min at 4°C in a TMA-4 rotor (TOMY SEIKO, Co. Ltd., Tokyo, Japan), the cell lysate was treated with anti-NP monoclonal antibody (9 μg/ml) and incubated for 4 h at 4°C. Then 2.5% [v/v] protein A-Sepharose CL-4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was added to the antibody-cell extract mixture and incubated for 2 h at 4°C with gentle shaking. Immunoprecipitates bound to the Sepharose beads were pelleted by centrifugation at 2000 rpm in a TMA-4 rotor and washed 6 times with TNE buffer. Immunoprecipitates were then suspended in SDS sample buffer (Hyodo and Kim, 1994) containing 0.125 M DTT and boiled for 3 min. The samples were analyzed by 11% SDS–PAGE followed by immunoblotting using anti-influenza virus serum.

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