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Nuclear Import and Export of Influenza Virus Nucleoprotein

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Influenza virus nucleoprotein (NP) shuttles between the nucleus and the cytoplasm. A nuclear localization signal (NLS) has been identified in NP at amino acids 327 to 345 (J. Davey et al., Cell 40:667–675, 1985). However, some NP mutants that lack this region still localize to the nucleus, suggesting an additional NLS in NP. We therefore investigated the nucleocytoplasmic transport of NP from influenza virus A/WSN/33 (H1N1). NP deletion constructs lacking the 38 N-terminal amino acids, as well as those lacking the 38 N-terminal amino acids and the previously identified NLS, localized to both the cytoplasm and the nucleus. Nuclear localization of a protein containing amino acids 1 to 38 of NP fused to LacZ proved that these 38 amino acids function as an NLS. Within this region, we identified two basic amino acids, Lys7 and Arg8, that are crucial for NP nuclear import. After being imported into the nucleus, the wild-type NP and the NP-LacZ fusion construct containing amino acids 1 to 38 of NP were both transported back to the cytoplasm, where they accumulated. These data indicate that NP has intrinsic structural features that allow nuclear import, nuclear export, and cytoplasmic accumulation in the absence of any other viral proteins. Further, the information required for nuclear import and export is located in the 38 N-terminal amino acids of NP, although other NP nuclear export signals may exist. Treatment of cells with a protein kinase C inhibitor increased the amounts of nuclear NP, whereas treatment of cells with a phosphorylation stimulator increased the amounts of cytoplasmic NP. These findings suggest a role of phosphorylation in nucleocytoplasmic transport of NP.

The genome of influenza viruses is composed of eight negative-strand RNA segments, which are packaged into virions as ribonucleoprotein (RNP) complexes (23). These complexes consist of the viral RNA associated with three polymerase proteins (PA, PB1, and PB2) and the viral nucleoprotein (NP). The viral matrix protein (M1) is also associated with the RNP complex. After binding to cell surface sialic acid-containing receptors, the virus is internalized by receptor-mediated endocytosis. A pH shift in the endosomes results in hemagglutinin-mediated fusion of the viral and endosomal membranes, thereby releasing the viral RNPs (vRNPs) into the cytoplasm (53). The vRNPs then separate from M1, and the RNPs enter the nucleus independently (28, 29). vRNAs are then replicated and transcribed in the nucleus. Newly synthesized PA, PB1, and PB2, as well as NP and M1 proteins, are transported into the nucleus, where new vRNPs are assembled. After transport into the cytoplasm, the RNPs are packaged into budding virus particles.

The transport of proteins, as well as cellular and viral RNPs, from the cytoplasm to the nucleus and vice versa (3, 49, 54) is an active, energy-dependent (41), signal-mediated process (7, 12, 34). Because RNAs are transported as RNPs, rather than as naked RNAs (18, 46), recent work has focused on RNA-binding proteins that are implicated in RNA transport (48). Most of these proteins not only bind RNA but also contain sequences that allow them to be transported from the cytoplasm into the nucleus (32). These nuclear localization signals (NLSs) are enriched in basic amino acids (8). The basic residues can be arranged in a single contiguous stretch of amino acids (19) or in two basic clusters (two to four amino acids each), separated by an amino acid spacer of variable length (50). However, nucleocytoplasmic protein distribution can also be affected by cytoplasmic retention signals in the molecule (47).

Once proteins are transported into the nucleus, protein accumulation is controlled by nuclear retention signals (39, 51) and/or nuclear export signals (NESs) (10). Nuclear retention signals retain proteins in the nucleus, probably by interacting with nuclear proteins. In contrast, proteins that contain NESs are transported back to the cytoplasm in an active, energy-dependent, saturable, and signal-mediated process (9, 24). Recently, NESs have been identified for several RNA-binding and non-RNA-binding proteins (10, 33, 54). The amino acid stretches necessary and sufficient for nuclear export are leucine rich (13, 14). For a small number of proteins, transport across the nuclear membrane is regulated by phosphorylation/dephosphorylation of the transported molecule (35). However, little else is known about the mechanisms underlying the regulation of nucleocytoplasmic transport.

During influenza virus replication, bidirectional transport across the nuclear membrane is required for vRNAs as well as for NP, M1, and the three polymerase proteins PA, PB1, and PB2. M1 has a classic NLS composed of one stretch of basic amino acids (62). Multiple sequence elements are responsible for the nuclear transport of PA, PB1, and PB2 (36, 40, 42). NP, the major structural component of vRNPs, shuttles between the nucleus and the cytoplasm (60, 61). An NLS has been identified in a region spanning amino acids 327 to 345 of NP (6). Although this NLS does not resemble any known NLS, NP interacts with karyopherin α (45), a cellular NLS-binding protein (52). While this work was being prepared for submission, Wang et al. published the characterization of the NP domains that interact with karyopherin α (57). The motif SxGTKRSYxxM [positions 3 to 13 of influenza virus A/PR/8/34

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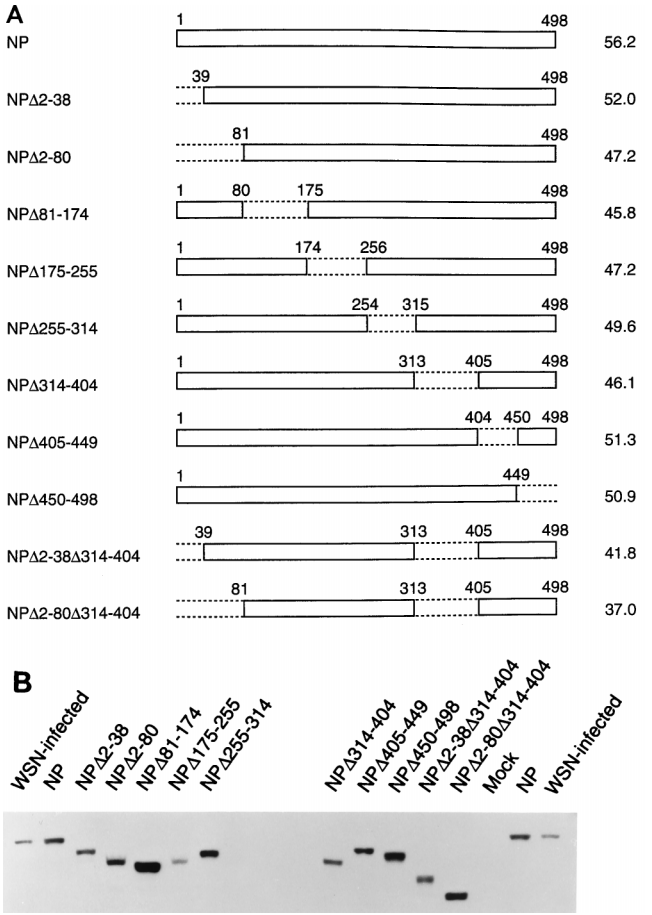


FIG. 1. Expression of NP and NP deletion constructs. (A) Schematic representation of wild-type NP and its deletion constructs. Solid bars represent translated amino acids; dotted bars indicate deleted regions. (B) Western blot analysis of NP and its deletion constructs. Plasmids expressing NP were transfected into COS-1 cells. Western blot analysis was performed 24 h after transfection by using an anti-NP rabbit antiserum (R528, 1:300 dilution) and an ECL kit (Amersham). As a positive control, COS-1 cells were infected with A/WSN/33 and lysed 10 h after infection to determine NP expression.

(H1N1) NP] is required for NP binding to the karyopherin α NPI-1, whereas the karyopherin α NPI-3 recognizes the motif TKRxxxxM (positions 6 to 13) for NP binding (57). In addition, fusion constructs containing the 20 N-terminal amino acids of influenza virus A/PR/8/34 NP directed a cytoplasmic protein to the nucleus (57). Wang et al., however, did not study the role of specific amino acids in nuclear localization, nuclear export, or the regulation of nucleocytoplasmic transport in the full-length NP molecule. NP is phosphorylated (1, 2), and its phosphorylation pattern changes during the replication cycle (20); however, the role of phosphorylation in NP transport remains unknown. The goal of this study is to understand the molecular mechanism of nucleocytoplasmic transport of influenza virus NP. To this end, we have investigated the kinetics of NP transport, the structural basis of NP nuclear import and export, and the role of phosphorylation in NP transport.

MATERIALS AND METHODS

Construction of recombinant plasmids. The NP gene was cloned from A/WSN/33 (H1N1) as described previously (17). NP deletion constructs (Fig. 1A) or NP point mutants were generated by PCR (37) or by the method developed by Kunkel (21) and cloned into the *Eco*RI site or the *Eco*RI and *Xho*I sites of the eukaryotic expression vector pCAGGS/MCS (which contains a chicken β-actin promoter) (43). The sequences of the oligonucleotides used for mutagenesis will be provided upon request. Nomenclature of the constructs indicates deleted amino acids; NPΔ81-174, for example, indicates a truncated NP protein containing amino acids 1 to 80 fused to amino acids 175 to 498 (deletion of amino acids 81 to 174).

For NP-LacZ fusion constructs, amino acids 1 to 38 and 39 to 80 (plus the start codon) of NP were generated by PCR. The PCR fragments were cloned into the *Eco*RI and *Bgl*II sites of pCAGGS/MCS, resulting in pCAGGS-NP1-38 and pCAGGS-NP39-80, respectively. The β-galactosidase gene, obtained by digesting pMC1871 (Pharmacia, Piscataway, N.J.) with *Bam*HI, was cloned into the *Bgl*II sites of pCAGGS-NP1-38 and pCAGGS-NP39-80, resulting in pCNP1-38lacZ and pCNP39-80lacZ. To ensure that the constructs did not contain unwanted mutations, NP genes and cloning boundaries were sequenced.

Cells. COS-1 cells (derived from African green monkey kidney) were grown in Dulbecco modified Eagle medium with 10% fetal calf serum. Madin-Darby canine kidney cells (MDCK) and HeLa cells (human cervical carcinoma) were maintained in minimal essential medium supplemented with 5% fetal calf serum. Minimal essential medium with 10% horse serum was used to culture L cells (mouse fibroblasts). Cells were maintained at 37°C and 5% CO_2.

Effects of chemicals on phosphorylation and protein synthesis. H7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine, dihydrochloride; 20 μg/ml; Calbiochem, La Jolla, Calif.] was added to the growth medium 6 h after transfection to inhibit protein kinase C (15). Stimulation of protein kinase C was achieved by adding TPA (phorbol 12-myristate 13-acetate; 1.5 μg/ml; Sigma, St. Louis, Mo.) to the growth medium 6 h after transfection. To stop protein synthesis, cycloheximide (50 μg/ml; Sigma) was added to the growth medium 9 h after transfection (44).

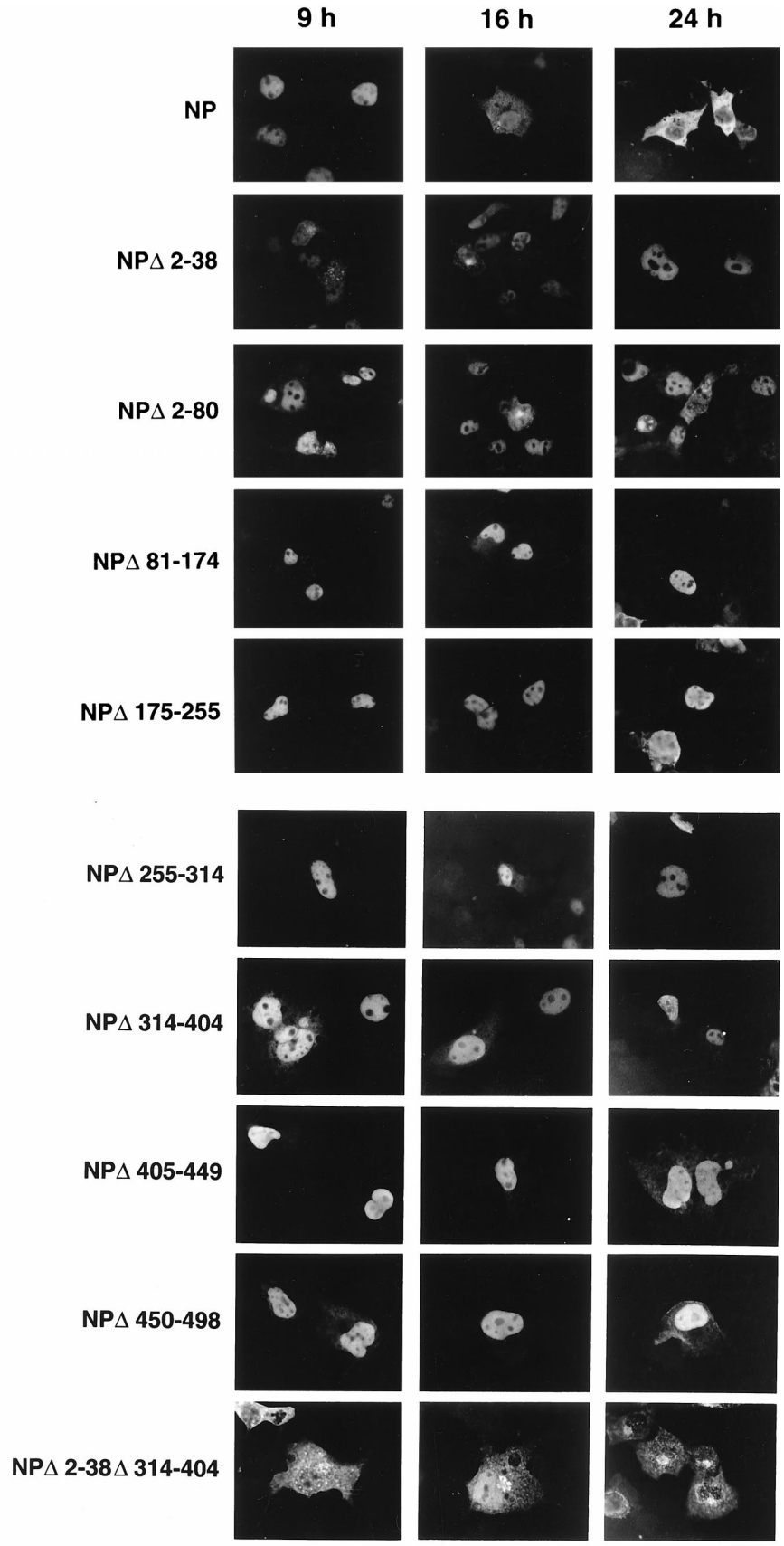
Immunofluorescence. Cells were washed twice with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde (in PBS) for 20 min at room temperature, washed again, and then incubated with ice-cold acetone for 7 min at −20°C. After being blocked with 10% goat serum for 20 min at 37°C, cells were incubated with a rabbit antiserum to NP (R528) at a 1:300 dilution for 30 min at 37°C. Cells were then washed and again blocked with 10% goat serum for 20 min at 37°C. Cells were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G, 1:200 dilution (Boehringer Mannheim, Indianapolis, Ind.) for 30 min at 37°C, washed, and mounted with 10 mM PPD (*p*-phenylenediamine) in glycerol–PBS (9:1), pH 8.5. Samples were observed under a Nikon Biophot microscope. To detect NP-LacZ fusion constructs, cells were incubated with an anti-β-galactosidase antibody (Boehringer Mannheim) at between 1:200 and 1:500 dilution, blocked with goat serum, and incubated with fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G, 1:200 dilution (Boehringer Mannheim).

Western blot analysis. At 24 h after transfection, cells were washed with PBS, incubated on ice for 1 min, lysed with 100 μl of lysis buffer (26% glycerol, 5.2% sodium dodecyl sulfate, 0.1 M Tris-HCl [pH 6.8]) for 1 min on ice, and spun down at 12,000 rpm for 10 min at 4°C. Aliquots of the supernatant were fractionated on a 10% Tris-glycine gel and electroblotted onto Immobilon-P membranes (Millipore, Bedford, Mass.) in a buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.0375% sodium dodecyl sulfate, and 20% methanol. The membrane was then incubated with a rabbit antiserum to A/Berkeley/1/68 NP (R528), and binding was visualized by using an ECL kit (Amersham, Arlington Heights, Ill.) as instructed by the manufacturer.

RESULTS

Identification of an NLS in the N-terminal region of NP. Davey et al. (6) showed that amino acids 327 to 345 of NP contain an NLS when examined in *Xenopus* oocytes. However, several NP deletion proteins that lack this putative NLS can still enter the nucleus (6), suggesting that NP has an additional NLS. We therefore reexamined NP nuclear localization by generating a series of deletion mutants. cDNAs encoding the wild-type NP of influenza A/WSN/33 virus and our deletion constructs were cloned into a eukaryotic expression vector (pCAGGS) under the control of a chicken β-actin promoter (Fig. 1A). To ensure that the NP constructs were expressed, we transfected COS-1 cells with these plasmids and monitored protein expression 24 h after transfection by Western blot analysis. All of the NP constructs were detected with a rabbit antiserum to NP, and the relative mobilities of the NP deletion mutants on polyacrylamide gels corresponded to the extent of deletion in each mutant (Fig. 1B).

To identify an NP NLS, we transfected the plasmids into COS-1 cells and performed indirect immunofluorescence assays at 9 h after transfection, the earliest time point at which NP expression was detected. Wild-type NP exclusively local-


9 h
16 h
24 h
NP
NPΔ 2-38
NPΔ 2-80
NPΔ 81-174
NPΔ 175-255
NPΔ 255-314
NPΔ 314-404
NPΔ 405-449
NPΔ 450-498
NPΔ 2-38Δ 314-404

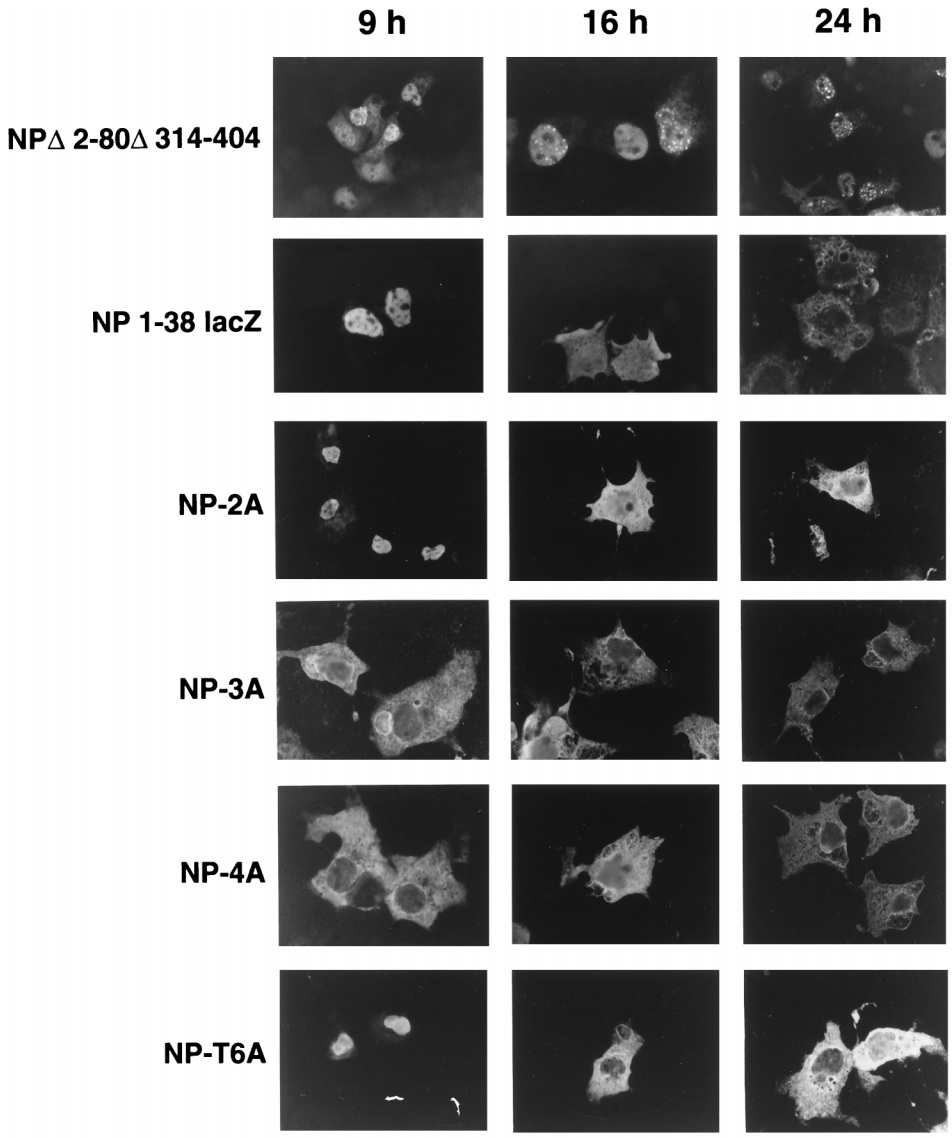


FIG. 2. Kinetics of cellular localization of NP and its derivative proteins. COS-1 cells were transfected, and immunofluorescence assays were performed 9, 16, and 24 h after transfection. NP localization was assessed with an anti-NP rabbit antiserum (R528, 1:300 dilution). NP-LacZ fusion protein was detected by using an anti-β-galactosidase monoclonal antibody (1:500 dilution; Boehringer Mannheim).

ized to the nucleus (Fig. 2; Table 1). Intracellular protein localization varied among the NP deletion mutants. NPΔ81-174, NPΔ175-255, NPΔ255-314, NPΔ405-449, and NPΔ450-498 predominantly localized to the nucleus. NPΔ314-404, which lacks the previously identified NLS, was found in the nuclei of most of the cells; however, in some cells, nuclear and cytoplasmic NPΔ314-404 was detected. The fact that NPΔ314-404 did not predominantly localize to the cytoplasm suggested the presence of an additional NLS in NP. Interestingly, NPΔ2-80 was also found both in the nucleus and in the cytoplasm. The N-terminal 80 amino acids may therefore contain an NLS, deletion of which may have been partially compensated for by the NLS spanning amino acids 327 to 345. To further define the region responsible for nuclear localization, we tested NPΔ2-38 in indirect immunofluorescence assays. NPΔ2-38 localized to both the nucleus and the cytoplasm. These results suggested that NP contains two NLSs, one at amino acids 327 to 345 and the other at amino acids 1 to 38. To test this possibility, NP deletion constructs lacking both potential NLSs (NPΔ2-38Δ314-404 and NPΔ2-80Δ314-404) were examined. Although both of these deletion mutants were still found in the nucleus and the cytoplasm (Fig. 2), more NP localized in the cytoplasm for these mutants than for others. In addition, NPΔ2-38, NPΔ2-80, NPΔ2-38Δ314-404, and NPΔ2-80Δ314-404 exhibited vesicular aggregation, unlike NPΔ314-404, which was evenly distributed in the cytoplasm. Taken together, these results suggest the existence of an NLS in the 38 N-terminal amino acids of NP.

NP is transported back from the nucleus to the cytoplasm. Whittaker et al. studied nucleocytoplasmic transport of RNP complexes in virus-infected cells (61). Cell-cell fusion assays revealed that once NP is imported into the nucleus, it is transported to the nucleus of another cell, where it accumulates (61). These findings suggest that NP is a shuttling protein. To determine which domains of NP are required for export, we analyzed the intracellular localization of wild-type NP and our

TABLE 1. Cellular localization of NP and its derivative proteins

Protein	Protein localization after transfection[a]		
	9 h	16 h	24 h
NP	N	N+C	C
NPΔ2-38	N, N+C_{Gr}	**N**, N+C	**N**, N+C
NPΔ2-80	N, N+C_{Gr}	**N**, N+C_{Gr}	**N**, N+C_{Gr}
NPΔ81-174	N	N	N
NPΔ175-255	N	N	N
NPΔ255-314	N	N	N
NPΔ314-404	**N**, N+C	**N**, N+C	**N**, N+C
NPΔ405-449	N	N	**N**, N+C
NPΔ450-498	N	N	**N**, N+C
NPΔ2-38Δ314-404	**N+C_{Gr}**, N	**N+C_{Gr}**, N	**C_{Gr}**, N+C_{Gr}
NPΔ2-80Δ314-404	**N+C_{Gr}**, N	**N**, N+C_{Gr}	**N**, N+C_{Gr}
NP1-38lacZ	N	N+C, N	C
NP39-80lacZ	C	C	C
NP-2A	N	N, N+C, C	C, N+C, N
NP-3A	C	C	C
NP-4A	C	C	C
NP-T6A	N	C	C

[a] Determined by indirect immunofluorescence assays 9, 16, and 24 h after transfection of COS-1 cells. N, nuclear staining only; C, cytoplasmic staining only; C_{Gr}, granular cytoplasmic staining; N+C, cells with both nuclear and cytoplasmic staining. Bold letters indicate the predominant condition; **N+C_{Gr}**, N, for example, indicates that NP localized to both the nucleus and the cytoplasm of most cells, but some cells showed exclusive nuclear NP localization. NP deletion constructs are shown in Fig. 1. For mutants NP-2A, NP-3A, NP-4A, and NP-T6A, see Fig. 6.

deletion mutants from 9 h to 24 h after transfection by immunofluorescence assays (Fig. 2; Table 1). Wild-type NP had localized to the nucleus 9 h after transfection but was found in both the nucleus and the cytoplasm 16 h after transfection. By 24 h after transfection, NP was localized exclusively to the cytoplasm, demonstrating NP export from the nucleus to the cytoplasm in the absence of any other viral proteins. The NP remained cytoplasmic, even 36 and 48 h after transfection (data not shown). Therefore, in our system, either cytoplasmic NP did not reenter the nucleus or the reimported NP was rapidly exported back to the cytoplasm. In contrast to wild-type NP, the NP deletion mutants were not transported back to the cytoplasm. Only NPΔ2-38Δ314-404 localized predominantly to the cytoplasm 24 h after transfection, compared to nuclear and cytoplasmic NP 9 and 16 h after transfection. These results demonstrate that NP, after being imported into and exported from the nucleus, has an intrinsic ability to accumulate in the cytoplasm without any other viral proteins. NP deletion analysis did not reveal an export signal, suggesting that multiple signals and/or formation of conformational domains are required for NP export, nuclear retention signal release, or cytoplasmic retention.

Discrepancies between the findings by Whittaker et al. (NP shuttling in HeLa and L cells) (61) and by us (NP export but not reimport in COS-1 cells) may originate from differences in the cell types. We therefore analyzed NP transport in COS-1, MDCK, HeLa, and L cells from 9 to 48 h after transfection. Although essentially the same localization pattern was observed in COS-1, MDCK, and HeLa cells, NP export was delayed in MDCK and HeLa cells (data not shown); NP remained in the nucleus until 16 h after transfection but localized to the cytoplasm between 24 and 48 h after transfection. As with COS-1 cells, NP remained in the cytoplasm of MDCK and HeLa cells. In contrast, NP was found in the cytoplasm of only a small number of L cells 36 and 48 h after transfection, suggesting either that the export process in this cell line is incomplete or that the localization equilibrium for NP favors the nucleus.

To prove that the cytoplasmic NP localization 24 h after transfection is the result of exported rather than nonimported NP, we performed time course experiments in the presence of cycloheximide, an inhibitor of protein synthesis. When COS-1 cells were transfected with wild-type NP and incubated for 9 h, we found detectable amounts of protein in the nucleus (Fig. 2). To inhibit protein synthesis, cycloheximide was added to the cultures and the cells were further incubated. We determined protein localization 16, 24, and 32 h after transfection (i.e., 7, 15, and 23 h after cycloheximide addition). We found NP in the cytoplasm and in the nucleus 16 and 24 h after transfection (Fig. 3). By 32 h after transfection, NP was localized to the cytoplasm for most of the cells. Because protein synthesis was inhibited 9 h after transfection, when NP was localized to the nucleus, the cytoplasmic NP at 32 h after transfection cannot be the result of nonimported NP. Instead, NP detected in the cytoplasm at 32 h after transfection must have been exported from the nucleus. These results demonstrate that once NP is imported into the nucleus, it is exported back to the cytoplasm, where it accumulates.

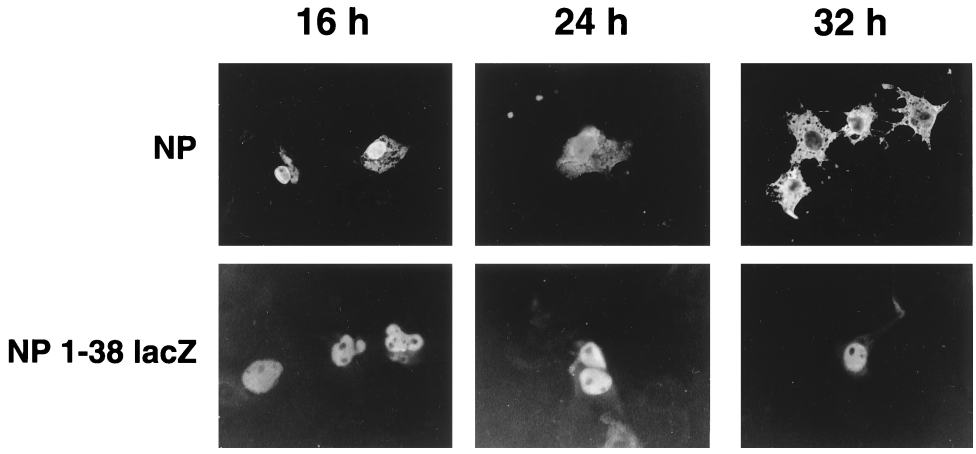


FIG. 3. Effects of cycloheximide on the cellular localization of NP and NP-LacZ fusion constructs. COS-1 cells were transfected and treated with cycloheximide (50 μg/ml) 9 h after transfection. NP localization was determined at 16, 24, and 32 h after transfection (i.e., 7, 15, and 23 h after the addition of cycloheximide). NP protein was detected with an anti-NP rabbit-antiserum (R528), and NP-LacZ fusion protein was detected with an anti-β-galactosidase monoclonal antibody.

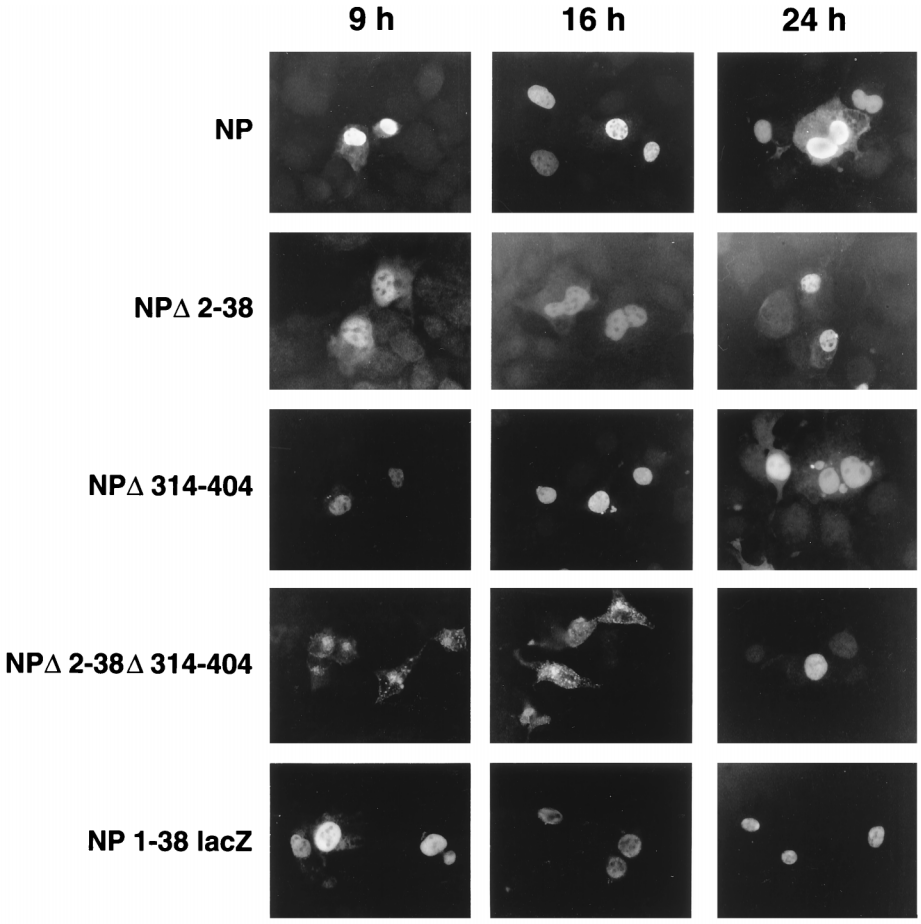


FIG. 4. Effects of H7 on the cellular localization of NP and its derivative proteins. COS-1 cells were transfected and treated with H7 (20 μg/ml) 6 h after transfection. Immunofluorescence assays were performed 9, 16, and 24 h after transfection by using an anti-NP rabbit antiserum (R528) to detect NP and an anti-β-galactosidase monoclonal antibody to detect the NP-LacZ fusion constructs.

Inhibition of phosphorylation enhances nuclear localization of NP. Influenza virus NP is phosphorylated (1, 20), but the role of phosphorylation in NP nucleocytoplasmic transport is unknown. To address this issue, we studied NP transport in the presence of H7, a protein kinase C inhibitor. COS-1 cells were transfected, and 6 h later, H7 was added. Wild-type NP localized to the nucleus 9 h after transfection, as seen in untreated cells (Fig. 4; Table 2). However, unlike the case for untreated cells, NP was still localized to the nucleus 16 h after transfection and remained predominantly nuclear even 24 h after transfection.

Deletion constructs that lack either the putative NLS at amino acids 2 to 38 or that at amino acids 314 to 404 localized predominantly to the nucleus (compare Fig. 2 and 4 and Tables 1 and 2). Interestingly, the construct that lacks both putative signals (NPΔ2-38Δ314-404) showed nuclear and vesicular cytoplasmic localization in both untreated and H7-treated cells. In addition, this mutant predominantly localized to the cytoplasm in untreated cells 24 h after transfection, whereas in H7-treated cells, it accumulated in the nucleus. For reasons that remain unknown, the construct that contains the larger N-terminal deletion (NPΔ2-80Δ314-404) resulted in increased nuclear localization under all conditions tested, compared to that of NPΔ2-38Δ314-404. These data indicate that inhibition of phosphorylation results in increased amounts of NP in the

TABLE 2. Effects of H7 on the cellular localization of NP and its derivative proteins

Protein	Protein localization after transfection[a]		
	9 h	16 h	24 h
NP	N	N	**N**+C
NPΔ2-38	N	N	N
NPΔ2-80	N	N	N
NPΔ81-174	N	N	N
NPΔ175-255	N	N	N
NPΔ255-314	N	N	N
NPΔ314-404	N	N	N
NPΔ405-449	N	N	N
NPΔ450-498	N	N	N
NPΔ2-38Δ314-404	C_{Gr}	**C_{Gr}**, N	N
NPΔ2-80Δ314-404	N	N	**N**, N+C_{Gr}
NP1-38lacZ	N	N	N
NP39-80lacZ	C	C	C
NP-2A	N	N	N
NP-3A	C	C	C
NP-4A	C	C	C
NP-T6A	N	C+N, N	C, N+C

[a] Determined by indirect immunofluorescence assays 9, 16, and 24 h after transfection of COS-1 cells (i.e., 3, 10, and 18 h after the addition of H7). N, nuclear staining only; C, cytoplasmic staining only; C_{Gr}, granular cytoplasmic staining; N+C, cells with both nuclear and cytoplasmic staining. Bold letters indicate the predominant condition.

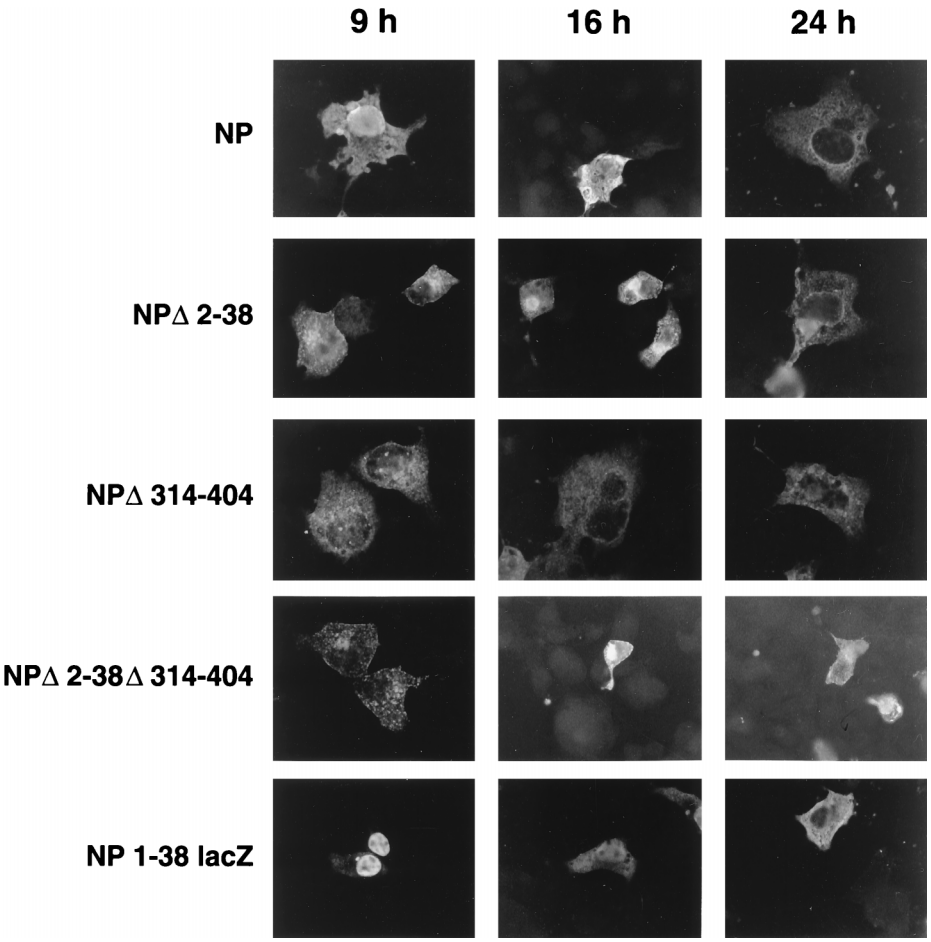


FIG. 5. TPA inhibits nuclear localization of NP and its derivative proteins. COS-1 cells were transfected and treated with TPA (1.5 μg/ml) 6 h after transfection. The localization of cellular NP or NP-LacZ fusion protein was monitored 9, 16, and 24 h after transfection with an anti-NP rabbit antiserum (R528) or an anti-β-galactosidase monoclonal antibody.

nucleus, which suggests that phosphorylation of NP and/or cellular proteins is crucial for NP transport.

Stimulation of phosphorylation affects NP nucleocytoplasmic transport. If phosphorylation of NP and/or cellular proteins is important for NP nucleocytoplasmic transport, stimulation of phosphorylation may also affect transport. We therefore analyzed the effects of TPA, which stimulates protein phosphorylation, on NP transport. In contrast to untreated cells, NP did not localize exclusively to the nucleus even 9 h after transfection (Fig. 5; Table 3). This result could mean that TPA prevents the nuclear import of NP or that if NP is imported to the nucleus, it is exported back to the cytoplasm very rapidly. Overall, more NP localized to the cytoplasm in the presence of TPA than in its absence (compare Fig. 2 and 5 and Tables 1 and 3). Constructs that lack the putative transport signal(s) differed in cellular localization: NPΔ2-38, NPΔ314-404, and NPΔ2-38Δ314-404 localized exclusively to the cytoplasm, while constructs NPΔ2-80 and NPΔ2-80Δ314-404 localized to both the nucleus and the cytoplasm (compare Tables 1 and 3). These findings further demonstrate that NP transport is regulated by phosphorylation.

Amino acids 1 to 38 of NP contain a signal(s) required for nuclear import and export. To investigate whether the 38 N-terminal amino acids of NP act as an active import signal, we constructed fusion proteins with β-galactosidase, a cytoplasmic protein. Amino acids 1 to 38 or 39 to 80 (plus the start codon) of NP were fused to the β-galactosidase reading frame without any additional linker amino acids. Whereas NP39-80lacZ localized to the cytoplasm (Table 1), NP1-38lacZ localized to the nucleus 9 h after transfection (Fig. 2; Table 1). These data prove that the N-terminal 38 amino acids of NP contain an active NLS and are consistent with the finding by Wang et al. that fusion of amino acids 1 to 20 of influenza virus A/PR/8/34 to a cytoplasmic protein results in nuclear import of the fusion construct (57).

To determine if the 38 N-terminal amino acids of NP contain signals required not only for nuclear import but also for export, we examined the intracellular localization of NP1-38lacZ 16 and 24 h after transfection. NP1-38lacZ localized to both the nucleus and the cytoplasm 16 h after transfection but was found only in the cytoplasm 24 h after transfection (Fig. 2; Table 1). NP39-80lacZ predominantly localized to the cytoplasm at all three time points (data not shown). Interestingly, for NP1-38lacZ, incubation with cycloheximide resulted in its nuclear accumulation even 24 h after transfection, in contrast to wild-type NP (Fig. 3).

To assess whether transport of NP-lacZ fusion proteins is regulated by phosphorylation, the intracellular localization of these proteins was determined following incubation with H7 or TPA. For NP1-38lacZ, inhibition of phosphorylation resulted in increased amounts of nuclear protein, whereas stimulation of phosphorylation resulted in increased amounts of cytoplas-

TABLE 3. Effects of TPA on the cellular localization of NP and its derivative proteins

Protein	Protein localization after transfection[a]		
	9 h	16 h	24 h
NP	N+C	N+**C**	C
NPΔ2-38	C_{Gr}	C_{Gr}	C_{Gr}
NPΔ2-80	N+C_{Gr}	N+C_{Gr}	N
NPΔ81-174	N+C	N+C_{Gr}	**N**, C_{Gr}
NPΔ175-255	N	N	N
NPΔ255-314	C	C	**C**, C+N
NPΔ314-404	C	C	C
NPΔ405-449	N	N, N+C	N+C
NPΔ450-498	N+C	N, N+C	N, N+C
NPΔ2-38Δ314-404	C_{Gr}	C_{Gr}	C_{Gr}
NPΔ2-80Δ314-404	N+C_{Gr}	**N**, N+C_{Gr}	N, C_{Gr}
NP1-38lacZ	**N**, N+C	**N+C**, C	C
NP39-80lacZ	C	C	C
NP-2A	N, N+C	N, N+C, C	C+N, C
NP-3A	C	C	C
NP-4A	C	C	C
NP-T6A	N, N+C, C	C	C

[a] Determined by indirect immunofluorescence assays 9, 16, and 24 h after transfection of COS-1 cells (i.e., 3, 10, and 18 h after the addition of TPA). N, nuclear staining only; C, cytoplasmic staining only; C_{Gr}, granular cytoplasmic staining; N+C, cells with both nuclear and cytoplasmic staining. Bold letters indicate the predominant condition.

mic protein, similar to the wild-type NP level (compare Fig. 4 and 5 and Tables 2 and 3). NP39-80lacZ localized predominantly to the cytoplasm under these conditions (data not shown). These data indicate that the 38 N-terminal amino acids of NP contain the information required for nuclear import, nuclear export, and transport regulation. However, there may be differences between the mechanism for wild-type NP transport and that for NP1-38lacZ, as exemplified by the nuclear accumulation of the NP1-38lacZ in the presence of cycloheximide.

Basic amino acids in the N-terminal region of NP are crucial for NP nuclear import. Within the 38 N-terminal amino acids of NP, there are no classic nuclear import or nuclear export signals. However, WSN-NP contains a cluster of basic amino acids at positions 4 to 8, where three (underlined) of the five amino acids are positively charged (KGTKR). In addition, the consensus motif for protein kinase C ([S/T]X[K/R]) is found at positions 6 to 8. The serine residue at position 3 of the NP of A/Victoria/3/75 is phosphorylated (2, 20), although WSN-NP contains a threonine residue at this position. To determine the significance of the putative NLS and/or phosphorylation sites for NP nuclear import, Thr3 and the basic amino acids at positions 4, 7, and 8 were replaced by alanine, resulting in the mutant NP-4A (Fig. 6). NP-4A localized exclusively to the cytoplasm 9, 16, and 24 h after transfection in untreated cells (Fig. 2) and in cells that had been exposed to TPA and H7 (data not shown; compare Tables 1, 2, and 3). These results indicate that the putative NLS and/or phosphorylation sites spanning amino acids 4 to 8 of NP have an important role in NP nuclear import.

WSN-NP	MAT**K**G**T**K**R**
NP-2A	--AA----
NP-3A	---A--AA
NP-4A	--AA--AA
NP-T6A	-----A--

FIG. 6. Amino acid residues 1 to 8 of WSN wild-type NP and its mutants. Basic amino acids are shown in bold letters. The protein kinase C consensus sequence ([S/T]X[K/R]) is underlined. Dashes indicate the same amino acids as those shown in the wild-type NP.

To better define the amino acids that are crucial for NP nuclear import, we determined the nucleocytoplasmic transport of additional NP point mutants (NP-2A, NP-3A, NP-T6A) (Fig. 6). In NP-3A, the basic amino acids at positions 4, 7, and 8 were replaced by alanine, thereby destroying the putative NLS and the putative protein kinase C site. However, the threonine at position 3 was unchanged. NP-3A, like NP-4A, localized exclusively to the cytoplasm under all conditions tested (Fig. 2; Tables 1 to 3). From these data, we conclude that the basic amino acids at positions 4, 7, and 8 are crucial for NP nuclear import. The basic amino acid at position 4 of WSN-NP is not conserved among the NPs of influenza A viruses, which suggests that it may be dispensable for NP nuclear import. To confirm that neither the threonine at position 3 nor the lysine at position 4 is crucial for NP nuclear import, both amino acids were replaced by alanine (NP-2A). NP-2A showed an overall transport pattern similar to that of wild-type NP under all conditions tested (Fig. 2; Tables 1 to 3). These data indicate that Thr3 and Lys4 are dispensable for NP nuclear import but that the basic amino acids at positions 7 and/or 8 are crucial for this transport process.

The cytoplasmic NP localization observed for NP-3A and NP-4A may be the result of inactivation of either the NLS at positions 7 and 8 or the putative phosphorylation site (positions 6 to 8). To differentiate between these possibilities, the nucleocytoplasmic transport of NP-T6A (Fig. 6) was investigated. Replacement of Thr6 by alanine destroyed the putative protein kinase C site; the two basic amino acids at positions 7 and 8 were unchanged. NP-T6A localized to the nucleus 9 h after transfection but exclusively to the cytoplasm 16 and 24 h after transfection (Fig. 2; Table 1). Compared to its effect on wild-type NP, H7 had a minimal effect on NP-T6A; the mutant localized to both the cytoplasm and the nucleus 24 h after transfection even in the presence of H7 (Table 2). Exclusive cytoplasmic NP-T6A was observed following incubation with TPA (Table 3). Taken together, these results show that the putative phosphorylation site spanning amino acids 6 to 8 is not essential for NP nucleocytoplasmic transport and that the basic amino acids Lys7 and/or Arg8 are crucial for NP nuclear import.

DISCUSSION

In this report, we have demonstrated that the 38 N-terminal amino acids of NP contain the information required for nuclear import and export and that these transport processes are regulated by phosphorylation. By characterizing NP deletion constructs and NP-globin fusion proteins in a *Xenopus* oocyte system, Davey et al. had identified an NLS at amino acids 327 to 345 (6). Here, we defined an additional NLS in the 38 N-terminal amino acids of NP. Our finding is thereby consistent with the data recently published by Wang et al., who reported an NLS in the 20 N-terminal amino acids of NP (57). Neither amino acids 327 to 345 nor amino acids 1 to 20 resemble any of the known NLSs. However, three basic amino acids (underlined) are found at positions 4, 7, and 8 of influenza A/WSN/33 virus NP (KGTKR). Site-directed mutagenesis of these residues resulted in exclusive cytoplasmic NP localization, indicating the importance of these basic amino acids for NP import (Fig. 2). Interestingly, all of the NPs of influenza

A viruses, with the exception of A/WSN/33, have glutamine at position 4, suggesting that this residue is not crucial for NP nuclear localization. We proved this hypothesis by creating NP-2A, an NP mutant with alanine at position 4, which was still imported into the nucleus. We therefore conclude that the conserved basic residues at positions 7 and/or 8 are crucial for NP nuclear import.

The minimal binding motif of karyopherin α NPI-1 and NPI-3 includes amino acids 6 to 9 and residue 13 (57), as determined in the yeast two-hybrid system. We, however, found that NP-T6A was still imported into the nucleus. The replacement of Thr6 by alanine may therefore have weakened the NP-karyopherin α interaction, resulting in negative results in the yeast two-hybrid system. It is, however, possible for the interaction between NP and karyopherin α to still be strong enough to direct NP-T6A in the nucleus. These findings suggest that individual amino acids within the karyopherin α-binding motif may contribute to different extents to karyopherin α-binding and hence to NP nuclear import.

Mutagenesis of positions 3 and 4 (NP-2A) did not significantly affect NP nucleocytoplasmic transport. NP-karyopherin α binding studies revealed that the replacement of Ser3 by alanine (for influenza A/PR/8/34 virus) destroyed the NP binding of karyopherin α NPI-1 but not that of NPI-3 (57). Recently, several different cell lines were shown to contain different sets of karyopherin α-proteins, and each of these karyopherin α molecules was able to direct NLS-containing proteins into the nucleus (38). Therefore, nuclear import of NP-2A is presumably executed by karyopherin α NPI-3, and the slightly different transport kinetics compared to wild-type NP likely result from the loss of the karyopherin α NPI-1 binding site. Based on our findings and those of others (57), we present a model for NP nucleocytoplasmic transport (Fig. 7). The basic amino acids 7 and/or 8 play a crucial role in karyopherin α binding and NP nuclear import, while amino acids 3, 4, and 6 contribute to these processes to a lesser extent (Fig. 7, bottom).

Trace amounts of NPΔ2-38Δ314-404, which lacks both putative NLSs, were still imported into the nucleus, suggesting that NP may contain additional NLSs (Fig. 7, center). Wang et al. made a similar observation (57). Consistent with this notion, Weber et al. found that amino acids 198 to 216 of A/PR/8/34 NP serve as an NLS when fused with MxA protein (57a). A number of proteins contain multiple NLSs (56, 58), which can act independently (e.g., the NLSs for the hepatitis delta antigen, the B-*myb* gene product, and the herpes simplex virus type 1 regulatory protein ICP27) (5, 30, 55) or cooperate to target proteins to the nucleus (63). The NP-NLSs seem to function independently, as determined by our NP-LacZ fusion studies, by NP-MxA fusion studies, and by NP-globin fusion studies (6), in which deletion of one of the signals was, at least partially, compensated for by one of the other NLSs. Previous studies (6, 57) were inconclusive in determining the relative strengths of the different NP NLSs. We demonstrated that the N-terminal NLS is dominant over other NP-NLSs by inactivating the former (e.g., NP-3A [Fig. 2]). Alternatively, the NLS at positions 327 to 345 may not be an authentic signal; deletion of a portion of NP may have altered its three-dimensional structure and unmasked a potential NLS (at amino acids 327 to 345) that is normally buried in the intact molecule.

In this report, we showed that NP has an intrinsic ability to be exported from the nucleus to the cytoplasm (Fig. 7, top). How does NP accumulate first in the nucleus and later in the cytoplasm? A mechanism that triggers NP export may exist. Such a switch mechanism may simply depend on the protein concentration. Formation of multimers, for example, which

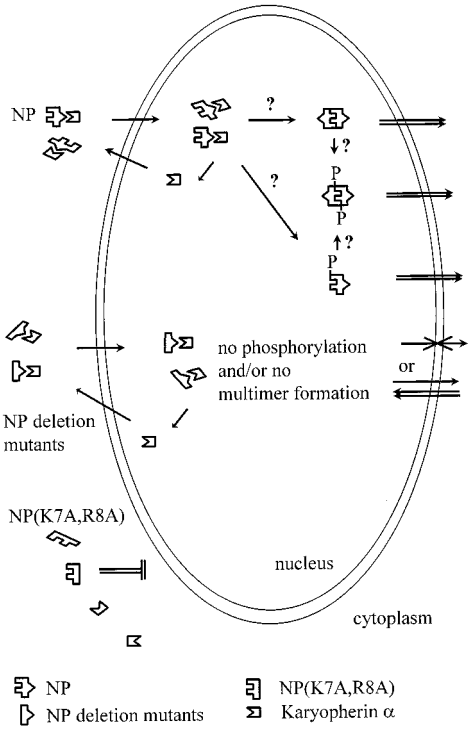


FIG. 7. Schematic diagram of NP nucleocytoplasmic transport. (Top) NP protein is imported into the nucleus via its interaction with the NLS-binding protein karyopherin α. NP nuclear export may be triggered by multimer formation, phosphorylation, or both. (Center) NP deletion constructs are imported into the nucleus. Multimer formation, phosphorylation, or both may be abolished as a direct result of the deletion by itself and/or the altered protein structure due to the deletion. The result would be a block in NP nuclear export. Alternatively, NP deletion constructs may be exported and rapidly reimported into the nucleus. (Bottom) NP constructs that lack a functional NLS do not interact with karyopherin α and are not imported into the nucleus.

also depends on protein concentration, may be a prerequisite for NP export (Fig. 7, top). Protein concentration-dependent triggering of NP export could also explain the differences in NP localization observed by us (cytoplasmic NP accumulation) and by others (nuclear NP accumulation) (57, 61). Transient transfection, as used in our study, usually results in high levels of protein expression, whereas protein levels in constitutively expressing cell lines, as used by others (26, 61), are often much lower. Thus, the concentration required to trigger NP nuclear export in the latter cell lines may not be reached.

Another switch mechanism to regulate nucleocytoplasmic transport of NP may be phosphorylation (Fig. 7, top). The phosphorylation patterns of NP differ between early and late infection (20). It is therefore possible that bidirectional NP or RNP transport is regulated by phosphorylation. Although this notion is supported by our results, we cannot conclude whether NP nuclear transport is regulated by phosphorylation/dephosphorylation of cellular proteins and/or by NP itself. For a number of proteins, nucleocytoplasmic transport is regulated by phosphorylation of sites near or in an NLS (35). Moreover, the effects of phosphorylation vary depending on the protein (31, 35). Inactivation of phosphorylation sites enhances nuclear protein localization for some proteins, but reduces it for others (25, 27). The 38 N-terminal amino acids of A/WSN/33 NP contain both a protein kinase C (residues 6 to 8) and a casein kinase II (residues 15 to 18) motif. Analysis by mutagenesis demonstrated that the putative protein kinase C site (po-

sitions 6 to 8) is not crucial for NP import. We are currently investigating a role for the casein kinase II motif in NP transport.

A third mechanism for triggering NP import and/or export would be a combination of protein concentration (NP oligomerization) and phosphorylation (Fig. 7, top). Recently, Kurihara et al. showed that nuclear localization signal peptides activate a protein kinase (22). Therefore, accumulation of NP may be required to activate a kinase. The activated kinase might then phosphorylate NP and/or cellular factors, thereby affecting transport kinetics.

Protein export from the nucleus is an active, energy-dependent, and signal-mediated process. Interestingly, after it had been imported into the nucleus, the NP-LacZ fusion construct containing amino acids 1 to 38 of NP accumulated in the cytoplasm, suggesting that the information for nuclear import as well as for export and cytoplasmic accumulation is located within this amino acid stretch. However, unlike wild-type NP, NP1-38lacZ did not accumulate in the cytoplasm following incubation with cycloheximide, suggesting that two separate export pathways or mechanisms for cytoplasmic accumulation exist. Sequences previously identified as important for nuclear export are relatively short, leucine-rich amino acid stretches (59), yet our analysis of the N-terminal 38 amino acids of NP did not reveal a leucine-rich region.

Notably, some of our NP deletion constructs (e.g., NPΔ81-174, NPΔ175-255, and NPΔ255-314) that contain the N-terminal 38 amino acids were imported into the nucleus and accumulated there. These findings suggest that an NES may be necessary but not sufficient for cytoplasmic NP accumulation. The nuclear NP accumulation may therefore be the result of a deleted NES, a deleted phosphorylation site, and/or prevented multimer formation (Fig. 7, center). Conformational changes that affect multimer formation and/or phosphorylation might also account for the differences observed between NPΔ2-38Δ314-404 and NPΔ2-80Δ314-404. In addition, other signals, such as nuclear retention (51) or cytoplasmic retention (47) signals, may play a role in the nucleocytoplasmic distribution of proteins. The nuclear accumulation of some of the NP deletion constructs would, in this case, be the result of a deleted cytoplasmic retention signal: NP deletion constructs may be exported but rapidly reimported to the nucleus (Fig. 7, center). Cell fusion experiments, similar to those done by Whittaker et al. (61), are now in progress to determine whether the NP deletion constructs are export incompetent or are exported and than rapidly reimported into the nucleus.

In our system, wild-type NP accumulated in the cytoplasm. Others, using a mouse fibroblast C127 cell line that expresses the three influenza virus polymerase proteins and NP (26, 61), have found that NP localizes to the nucleus and that after these cells are fused with HeLa cells, NP is transported to the HeLa nuclei (61). Influenza virus infection of both HeLa cells and L cells results in abortive infections (11, 16), and for both cell lines, NP accumulates in the nucleus (our data and reference 57). The import and/or export kinetics of proteins may therefore be cell type dependent and may affect influenza virus replication.

Data presented by Whittaker et al. (60, 61) and Bui et al. (4) suggest that influenza virus RNP transport is regulated by M1 protein. Most of the data addressing this issue, however, were obtained from virus-infected cells. Therefore, little is known about the role of NP in RNP transport without the contribution by other viral proteins, including M1. Because bidirectional transport across the nuclear membrane was shown for NP protein alone (reference 61 and this study), it may be that NP is involved in RNP transport. M1 and NP may contribute independently or cooperatively to RNP transport via their transport signals and phosphorylation status. On the other hand, the contribution of one of these proteins may be indirect, for example, if the protein-protein interaction resulted in the masking or unmasking of transport and/or phosphorylation sites. Further studies of NP intracellular transport would both promote our knowledge of the role of this protein in influenza virus infection and improve our understanding of protein transport in general.

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