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The NPI-1/NPI-3 (Karyopherin α) Binding Site on the Influenza A Virus Nucleoprotein NP Is a Nonconventional Nuclear Localization Signal

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Two cellular proteins, NPI-1 and NPI-3, were previously identified through their interaction with the influenza virus nucleoprotein (NP) by using the yeast two-hybrid system. These proteins were then shown to act as general transport factors (karyopherin α) and nuclear pore-docking proteins to facilitate the transport of the NP and of viral RNA into the nucleus. The yeast two-hybrid assay has now been used to identify the specific domains on the NP that bind to the NPI proteins. Mutational analysis including alanine scanning identified the motifs SxGTKRSYxxM and TKRSxxxM, which are required for binding to NPI-1 and NPI-3, respectively. These sequences were shown to possess nuclear localization signal (NLS) activity following expression of fusion proteins in HeLa cells. These sequences represent a novel nonconventional NLS motif. Another NLS activity not mediated by the NPI binding sites is associated with noncontiguous sequences in the NP.

The influenza A virus has a segmented genome of eight negative-strand RNAs which are packaged into virions as ribonucleoproteins (RNPs). In addition to RNA, RNP contains the viral nucleoprotein (NP) and the three subunits of the RNA-dependent RNA polymerase, PB1, PB2, and PA. When the influenza virus infects cells, it must ultimately deliver its genetic information to the nucleus, where viral RNA transcription and replication take place. In an attempt to learn more about intracellular host-virus interactions which are required for virus replication and which might affect virulence characteristics, we used the yeast two-hybrid system to search for cellular proteins that interact with the viral NP (20, 21). Two NP-interacting proteins (NPI-1 and NPI-3) were identified and shown to facilitate the nuclear import of the viral NP and—in conjunction with the NP—of viral RNA by using digitonin-permeabilized cells. Independent isolation and characterization of NPI-1 and NPI-3 from mammalian cells and of homologs in *Xenopus* and *Drosophila* (3, 4, 10, 15, 18, 27) revealed that these two proteins are general transport factors belonging to the karyopherin α/importin 60 protein family and are involved in the nuclear import of nuclear localization signal (NLS)-containing proteins. In this study we mapped the binding site of NPI-1/karyopherin α1/hSRP-1 (3, 18, 20, 27) and of NPI-3/karyopherin α2/Rch-1 (4, 18, 21) on the influenza A virus NP. Mutational analysis of the NP by the yeast two-hybrid system and expression of NP mutants in mammalian cells helped to define an unusual motif in the N terminus of the viral NP which has NLS activity.

MATERIALS AND METHODS

Yeast, bacterial strain, and plasmids. *Saccharomyces cerevisiae* EGY48 (*MATα trp1 ura3 his3 LEU2::pLEX-Aop6-LEU2*), plasmids pEG202, pSH18-34, and pRFHM1, and the HeLa cell cDNA library constructed in pJG4-5 kindly provided by R. Brent (Harvard Medical School) have been used previously (20). NPI-1 and NPI-3 cDNAs cloned in pJG4-5 were isolated from a HeLa cell cDNA library (20). The *Escherichia coli* strain used for cloning was DH5α [F^- Φ80*dlacZ* ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *supE44* λ^- *thi-1 gyrA96 relA1*] (GIBCO BRL).

Construction of recombinant DNA molecules. NP deletion mutants shown in Fig. 1a were constructed between the *Eco*RI and *Xho*I restriction sites of pEG202 as follows. For mutant A, pBS-NP, which contains the influenza A/PR/8/34 virus NP gene in pBluescript SK+ (Stratagene Cloning Systems), was cut by *Bgl*II (at nucleotide positions 806 and 1061) and religated. The deleted NP was then subcloned into pEG202. For mutant B, pBS-NP was cut by *Bgl*II and *Hin*dIII at nucleotide positions 806 and 1114, respectively, blunted by the Klenow fragment of *E. coli* DNA polymerase I, and religated. The deleted NP was then subcloned into pEG202. For mutants C, E, and F, fragments of the NP with the breakpoints indicated below were subcloned as PCR products from pBS-NP following amplification with the appropriate oligonucleotides. Mutant F was cloned as a *Bgl*II/*Xho*I restriction fragment into the *Bam*HI and *Xho*I restriction sites of pEG202. For mutant D, pBS-NP was digested with *Ppu*MI, blunted with Klenow, and then digested with *Eco*RI. pEG202 was digested with *Xho*I, blunted with Klenow, and then digested with *Eco*RI. The NP fragment was then subcloned into pEG202. For expression in mammalian cells (Fig. 1b), the *Eco*RI to *Xho*I restriction fragments containing the wild-type NP and deletion mutants A, B, and E were subcloned into pcDNAI/Amp (Invitrogen Corp.).

NP deletion mutants used for Fig. 2 were constructed by PCR between the restriction sites indicated below. For construction of the fusion genes used in the yeast two-hybrid assay, the PCR products of mutants G, H, J, K, L, and M were cut by *Bgl*II/*Xho*I and the PCR product of mutant N was cut by *Bam*HI/*Xho*I. These restriction fragments were cloned into the *Bam*HI/*Xho*I sites of pEG202. For constructions used in immunofluorescence assays, the PCR products of mutants G, H, J, K, L, and M were cut by *Bgl*II/*Sal*I and the PCR product of mutant N was cut by *Bam*HI/*Sal*I. These restriction fragments were cloned into the *Bgl*II/*Sal*I sites of PECE (provided by Ronald Harty, Mt. Sinai School of Medicine [7]). The latter plasmid allows expression of the NP fragments as measles virus P fusion proteins. Amino acids 3 to 507 of the P protein were included in fusion proteins.

The mutations and deletions in the first 20 amino acids of the NP shown in Fig. 3 and 4 were also constructed by PCR. For the yeast two-hybrid assay, the PCR products were cut by *Bgl*II/*Xho*I and cloned into the *Bam*HI/*Xho*I sites of pEG202, resulting in pLex A-NP fusion genes. For the immunofluorescence assay, the PCR products were cut by *Bgl*II/*Sal*I and cloned into the *Bgl*II/*Sal*I sites of PECE (7). These constructs allowed the expression of the NP fragments as measles virus P fusion proteins in HeLa cells. All the constructs were confirmed by nucleotide sequence analysis.

Mutants D12 and D12A were generated by PCR from pBS-NP and mutant A, respectively. Mutants D12, D12A, and O were cloned into pEG202 for use in the yeast two-hybrid system and into pcDNAI/Amp for expression in mammalian cells. Mutant O was also cloned as a measles virus P protein fusion in vector PECE.

Interaction of NP mutants and NPI-1 or NPI-3. The interaction between NP mutants and NPI-1 or NPI-3 was measured as described previously (20, 21). Briefly, strain EGY48 was transformed with fusion gene plasmids in addition to the β-galactosidase reporter plasmid pSH18-34 by a high-efficiency lithium ace-

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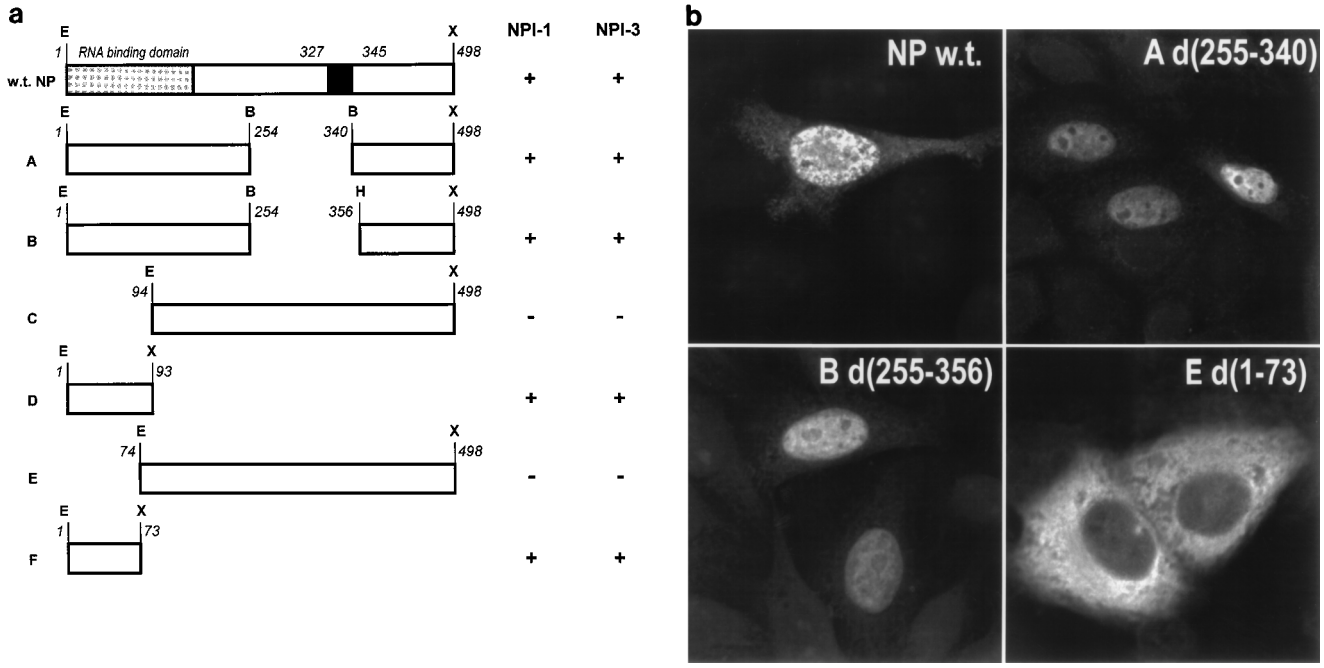


FIG. 1. Amino acids 1 to 73 of the influenza A virus NP contain the binding site for NPI-1 and NPI-3. (a) Interactions of NP deletion mutants and NPI-1 and NPI-3 proteins in the yeast two-hybrid system. Yeast strain EGY48 was cotransformed with pEG202 (containing the different LexA-NP fusion genes), the reporter plasmid pSH18-34, and pHB34 (containing the NPI-1 cDNA in pJG4-5) or pHB81 (containing the NPI-3 cDNA in pJG4-5) (20). The lengths of the NP fragments are indicated by amino acid positions in the wild-type (w.t.) NP. Binding activity was determined by detecting the induction of β-galactosidase. A plus sign indicates that a strong blue color developed within 12 h following streaking on X-Gal-containing plates; a minus sign indicates no reaction. The shaded box indicates RNA binding domains (1a, 14). The black box indicates a putative NLS (5). Restriction enzyme sites used for subcloning of NP fragments are indicated as follows: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; and X, *Xho*I. (b) Localization of NP deletion mutants in HeLa cells. HeLa cells were transfected with pcDNAI/Amp, which contains NP deletion mutants, and analyzed by indirect immunofluorescence assay 24 h posttransfection, using anti-NP polyclonal antibody for construct E d(1–73) or monoclonal antibody for wild-type NP and constructs A and B.

tate protocol (13) and plated on glucose-free synthetic dropout plates lacking histidine, uracil, and tryptophan. In order to assay for β-galactosidase activity, the transformed yeast cells were isolated 2 days posttransformation and streaked onto galactose plates lacking histidine, uracil, and tryptophan and containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (2). The NP mutants (cloned as LexA fusion genes) that induced β-galactosidase only in the presence of galactose in conjunction with NPI-1 or NPI-3 (cloned as fusion genes with a transcriptional activation domain) were considered to retain the ability to interact with NPI-1 or NPI-3.

Localization of expressed proteins by indirect immunofluorescence assay. HeLa cells were grown on glass coverslips to 50 to 85% confluency and transfected with the different constructs (described above) by using Lipofectin according to the manufacturer's recommendations (GIBCO BRL). Twenty-four hours after transfection, the cells were fixed and permeabilized for 15 min in acetone-methanol (1:1) at −20°C. The cells were then treated with polyclonal or monoclonal antibodies. Purified monoclonal antibody PT107 (unpublished data) or polyclonal rabbit anti-X-31 antibody (23) was used for staining of the NP. The epitope on the NP, which is bound by PT107, has not been mapped. Rabbit antibody PV1 against the measles virus P protein was a kind gift of Dalius J. Briedis (McGill University, Montreal, Canada). After staining of the NP or P, the cells were washed and developed with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G labeled with rhodamine. The samples were mounted with Mowiol 40-88 (Aldrich, Inc.) and analyzed by using a 40× or 63× objective and a Leica CLSM confocal imaging system.

RESULTS

The amino terminus of the influenza A virus NP contains a binding site for NPI-1 and NPI-3. In order to study the interaction of the viral NP and cellular NPI-1 and NPI-3 proteins, the yeast two-hybrid system was used (8, 11). The NP and its deletion mutants were expressed in yeast as LexA-NP fusion proteins. The plasmids expressing the NPI-1 or NPI-3 fusion proteins with the transcriptional activation domain had been obtained previously (20, 21). Plasmids containing the NP fusion gene and the NPI-1 or NPI-3 fusion gene were cotransfected into yeast cells together with a reporter plasmid expressing β-galactosidase. Transformants were then transferred to X-Gal plates to allow easy screening for interactions between the NP mutants and either NPI-1 or NPI-3. Earlier results had suggested that a sequence associated with nuclear accumulation of the influenza A virus NP in *Xenopus* oocytes was located between amino acids 327 and 345 (5). Thus, we first made deletions in this region. However, binding activity of the resulting deletion mutants to either NPI-1 or NPI-3 was not affected in the yeast two-fusion protein assay (constructs A and B [Fig. 1a]). When we made deletions of the N-terminal 93 or 73 amino acids in constructs C and E, respectively, interaction with either of the NPI proteins was completely eliminated. Conversely, constructs D and F, which contained only the N-terminal 93 and 73 amino acids, respectively, bound to both NPI proteins.

In order to examine the cellular localization of the mutant NP proteins, several constructs were cloned into an expression plasmid behind a cytomegalovirus (CMV) promoter. Transfection of these plasmids into HeLa cells was monitored 24 h later by indirect immunofluorescence (Fig. 1b). Mutants A and B, which lack the putative nuclear accumulation signal (5), localized to the nucleus, whereas construct E, which had an N-terminal deletion of 73 amino acids, was clearly cytoplasmic. These results suggested a correlation between NPI binding domains and an activity for nuclear localization in mammalian cells.

Amino acids 1 to 20 of the NP contain a binding site for NPI-1 and NPI-3 and NLS activity. The following experiments were done to further define the N-terminal domain containing

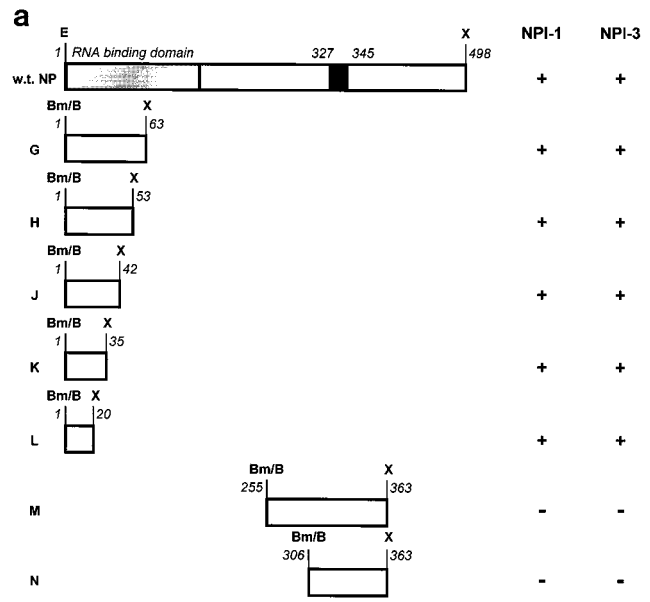
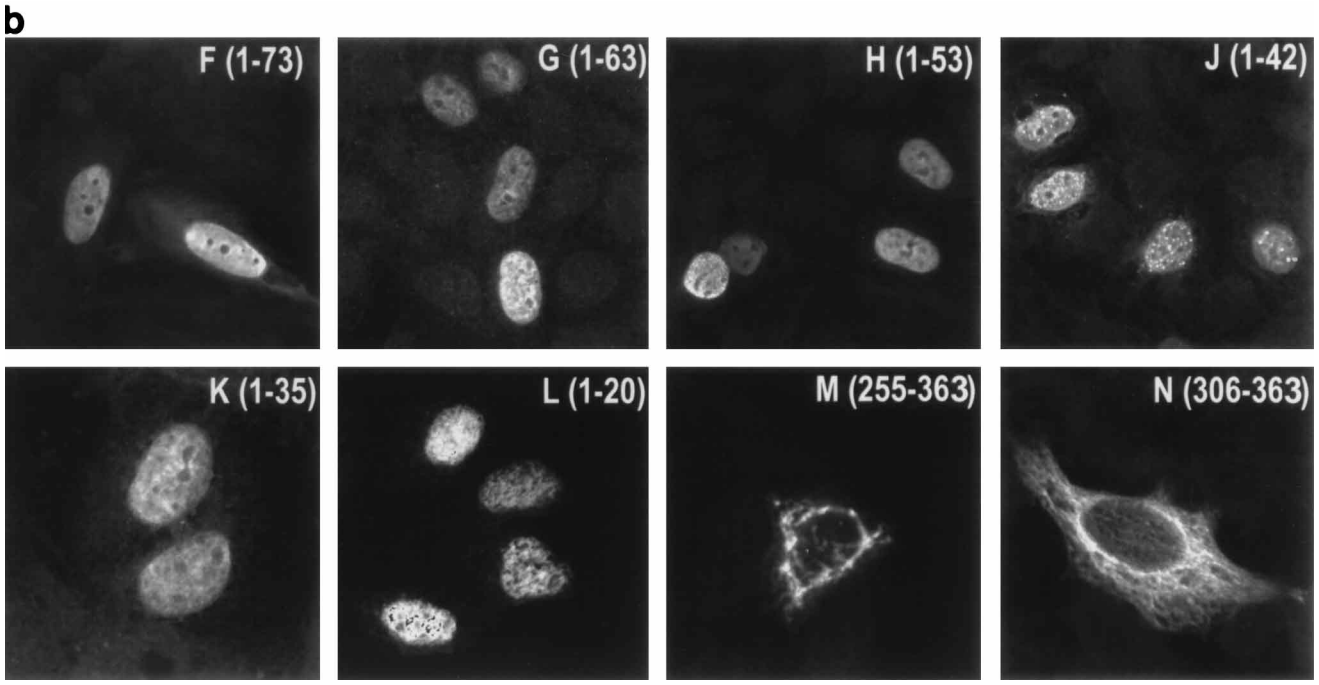


FIG. 2. Amino acids 1 to 20 of the influenza A virus NP possess NLS activity. (a) Interactions of NP deletion mutants and NPI-1 and NPI-3 in the yeast two-hybrid system. The NP deletion mutants were cloned into pEG202 and assayed as described in the legend to Fig. 1. Restriction enzyme sites used for subcloning of NP fragments are as follows: B, *Bgl*II; E, *Eco*RI; X, *Xho*I; Bm, *Bam*HI. (b) Localization of fusion proteins containing NP fragments and measles virus P protein in HeLa cells. The fusion genes were cloned into PECE (provided by Ronald Harty), transfected into HeLa cells, and expressed as NP-P fusion proteins. The localization of the fusion proteins was analyzed by indirect immunofluorescence assay 24 h posttransfection, using anti-measles virus P protein antibody PV1 (kindly provided by Dalius J. Briedis). Constructs F to L (Fig. 1a and 2a) contain NP-specific amino acids as indicated. Constructs M and N contain NP-specific amino acids 255 to 363 and 306 to 363, which include the sequences previously thought to possess NLS activity (5). Constructs K, M, and N were photographed with the 63× objective. The remaining photographs were taken with the 40× objective.



		NPI-1	NPI-3
w.t.	MASQGTKRSYEQMETDGERQ	+	+
A3	MA**A**QGTKRSYEQMETDGERQ	-	+
A4	MAS**A**GTKRSYEQMETDGERQ	+	+
A5	MASQ**A**TKRSYEQMETDGERQ	-	+
A6	MASQG**A**KRSYEQMETDGERQ	-	-
A7	MASQGT**A**RSYEQMETDGERQ	-	-
A8	MASQGTK**A**SYEQMETDGERQ	-	-
A9	MASQGTKR**A**YEQMETDGERQ	-	-
A10	MASQGTKRS**A**EQMETDGERQ	-	+
A11	MASQGTKRSY**A**QMETDGERQ	+	+
A12	MASQGTKRSYE**A**METDGERQ	+	+
A13	MASQGTKRSYEQ**A**ETDGERQ	-	-
A14	MASQGTKRSYEQM**A**TDGERQ	+	+
A15	MASQGTKRSYEQME**A**DGERQ	+	+
A16	MASQGTKRSYEQMET**A**GERQ	+	+
A17	MASQGTKRSYEQMETD**A**ERQ	+	+
A18	MASQGTKRSYEQMETDG**A**RQ	+	+
A19	MASQGTKRSYEQMETDGE**A**Q	+	+
A20	MASQGTKRSYEQMETDGER**A**	+	+
D5	M----TKRSYEQMETDGERQ	-	+
D5A7	M----T**A**RSYEQMETDGERQ	-	-
D5A8	M----TK**A**SYEQMETDGERQ	-	-
D5D9	M----TKR-YEQMETDGERQ	-	-
D5D10	M----TKRS-EQMETDGERQ	-	-
D5A19	M----TKRSYEQMETDGE**A**Q	-	+
NPI-1 Motif	**SxGTKRSYxxM**		
NPI-3 Motif	**TKRSxxxM**		

FIG. 3. Fine mapping and alanine scanning of the NPI-1/NPI-3 interacting site on the NP. Mutations and deletions of the first 20 amino acids of the NP were cloned into pEG202 and assayed as described in the legend to Fig. 1 for binding to NPI-1 or to NPI-3. Alanine mutations are in boldface and underlined. Hyphens indicate deletions. Minimal NPI-1 and NPI-3 binding motifs are indicated at the bottom. w.t., wild type.

the NPI-1/NPI-3 binding site. Carboxy-terminal deletions were made in the first 73 amino acids, and fusions were constructed in vector pEG202. Fusions containing as few as 20 amino acids of the NP N terminus bind to both NPI-1 and NPI-3 when the yeast two-hybrid system is used (Fig. 2a). In order to test if these sequences function as NLS motifs, these N-terminal sequences were expressed as fusion proteins by using a reporter protein usually found in the cytoplasm. Use of a reporter was necessary because the small N-terminal NP fragments could not be visualized with the available NP-specific antisera. Fusion constructs were made with the measles virus P protein, which localizes to the cytoplasm (12). Fusion constructs containing the 73, 63, 53, 42, 35, and 20 N-terminal amino acids of the NP relocate the reporter protein into the nucleus (constructs F, G, H, J, K, and L, respectively [Fig. 2b]). Control constructs which express NP-specific amino acids 255 to 363 and 306 to 363 in the context of the measles virus P protein are found only in the cytoplasm, confirming the absence of a nuclear accumulation signal in these sequences.

Thus, the NPI-1/NPI-3 binding site of the NP also functions as an NLS motif. Comparison of the N-terminal 20 amino acids of the NP and the sequences of classical NLSs reveals no clear sequence identity. The NLS of the simian virus 40 large T antigen contains a stretch of 5 basic amino acids (PKKKRKV), and the bipartite NLS of nucleoplasmin has two basic clusters of amino acids separated by 10 nonbasic amino acids (KRXXXXXXXXXXKKKK) (6). Since the first 20 amino acids of the NP contain 3 basic amino acids separated by 10 nonbasic amino acids (Fig. 3), we decided to further analyze the sequences responsible for NPI binding and NLS activity.

Fine mapping of the NPI-1/NPI-3 binding site. In order to identify the specific amino acids involved in binding to NPI-1 and NPI-3, we performed a mutational analysis, including alanine scanning mutagenesis, followed by assaying of the mutants in the yeast two-hybrid system. Figure 3 lists 24 mutants which were analyzed. The results suggest slight differences in the binding site recognized by NPI-1 and NPI-3. First, it appears that the five amino acids at the N terminus (or at least amino acids two to five) are not required for binding to NPI-3. This conclusion is based on the binding characteristics of mutants A3, A4, A5, D5, and D5A19. Second, mutant A10 is recognized by NPI-3 and not by NPI-1, again suggesting a difference in the binding pattern. These data reveal minimal binding motifs of SxGTKRSYxxM and TKRSxxxM for NPI-1 and NPI-3, respectively.

In addition, the number of amino acids between the serine and methionine is important for NPI-3 binding to the NP. Mutant D5 is recognized by NPI-3, but D5D10 is not. Since mutation of Y10 to alanine does not disrupt the NPI-3 binding site (mutant A10), but deletion of this residue does ablate binding, we conclude that a three-amino-acid spacer between the serine and methionine is necessary for recognition of this NLS by NPI-3.

Several mutants were also cloned as fusion genes (with the measles virus P gene) into the CMV-driven expression plasmids. They were then transfected into HeLa cells and analyzed for cellular localization by indirect immunofluorescence (Fig. 4). Mutants A7, A8, D5A7, D5A8, and D5D10, which have lost the ability to bind either NPI-1 or NPI-3, localize to the cytoplasm. However, mutants D5A19 and D5, which retain NPI-3 binding activity, are clearly nuclear in this assay. These results suggest a good correlation between NPI binding and NLS activity. It also appears that the binding site for NPI-1 and NPI-3 is contained within the first 13 amino acids at the N terminus. Finally, the data suggest a redundancy of two slightly different NLS signals mediated by binding either to NPI-1 or to NPI-3.

Presence of an NLS whose activity is not mediated by binding to NPI-1 or NPI-3. In order to confirm the localization of the NPI-1/NPI-3 binding site in the full-length NP molecule, the first 12 amino acids were deleted (construct D12 [Fig. 5a]). As expected, this mutant did not bind NPI-1 or NPI-3 in the yeast two-hybrid system. However, this construct—when inserted into the CMV-driven expression plasmid and transfected into HeLa cells—unexpectedly localized mostly to the nucleus (Fig. 5b). This phenomenon was further investigated by examining a construct that lacked amino acids 1 to 12 and 255 to 340. Although this mutant lacked the NPI binding site and the putative NLS previously described (5), it again was found predominantly in the nucleus. We then made an attempt to further define the domain which is associated with this NLS activity. Construct O contained the D5A7 mutation at the N terminus and was truncated at amino acid position 254 (so that it also lacked an NPI binding site). This construct localized in the cytoplasm when expressed as a fusion protein (containing the measles virus P protein) (Fig. 5b). We then discovered that

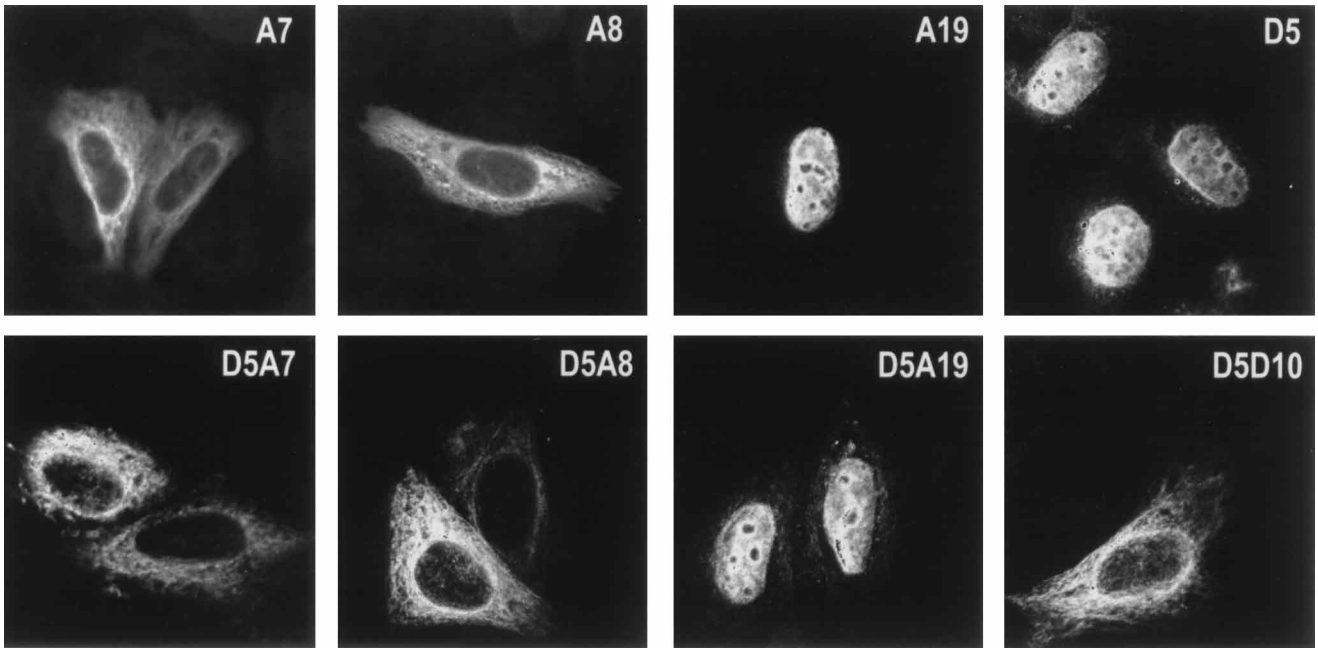


FIG. 4. Mutations and deletions in the first 20 amino acids of the influenza A virus NP fragment affect NLS activity. The mutations and deletions in the first 20 amino acids of the NP were cloned into the fusion gene vector PECE, transfected into HeLa cells, and expressed as fusion proteins (containing the measles virus P protein). The localization of the fusion proteins was analyzed by indirect immunofluorescence assay 24 h posttransfection, using anti-measles virus P protein antibody PV1.

constructs O and E (neither of which alone carries an NLS) allowed localization to the nucleus when cotransfected. This result suggests that the NLS activity associated with the D12 mutant is mediated by noncontiguous regions in the NP molecule and that NP deletions O and E can interact in *trans*, thereby reconstituting a conformational NLS.

DISCUSSION

Nuclear protein import is accomplished by two sequential events: docking at and translocation through the nuclear membrane (9, 10, 16–19, 22). Through the use of the yeast two-hybrid system, with the NP as "bait," we identified two proteins, NPI-1 and NPI-3 (docking proteins), which were shown to be involved in the nuclear import of the influenza virus NP and of viral RNA (20, 21). The present study was done in order to identify the NPI binding site on the NP and to study its biological activity. We assayed the interaction of the NP with NPI-1 and NPI-3 in the yeast two-hybrid system by using NP deletion mutants and found the NPI binding site localized to the amino terminus (Fig. 1). Furthermore, the amino terminus of the NP showed NLS activity, since it could confer nuclear localization on a protein (the measles virus P protein) which normally localizes to the cytoplasm (Fig. 2).

In contrast, earlier studies (5) had suggested that nuclear accumulation of the NP was dependent not on the N terminus but rather on amino acids 327 to 345 of the protein. The proposed nucleophilic signal (AAFEDLRVLS) was found to be important for the nuclear accumulation of the NP as well as of NP-globin fusion proteins in *Xenopus* oocytes. However, we found that expression of NP mutants A and B, lacking amino acids 255 to 356 and 255 to 340, respectively, showed nuclear localization (Fig. 1b). Furthermore, two fusion proteins containing amino acids 255 to 363 and 306 to 363 (both encompassing the putative NLS) of the viral NP were cytoplasmic in HeLa cells (Fig. 2b). These findings suggest that the earlier data concerning the nucleophilic signal of the NP (located between amino acids 327 and 345) were most likely an artifact of the *Xenopus* system and could not be verified in mammalian cells (at least not in HeLa cells).

A more refined analysis of the NPI binding site suggested that the N-terminal 13 residues of the NP contain the necessary amino acids involved in binding of NPI-1 and NPI-3. Alanine scanning identified the motif TKRSxxxM as sufficient for NPI-3 binding as well as for NLS activity. Mutants which have lost the ability to bind NPI-1 (but retain NPI-3 binding activity) are able to direct a fusion protein to the nucleus and thus appear to retain NLS activity by virtue of their interaction with NPI-3 (mutants D5 and D5A19 [Fig. 4]). In a fractionated nuclear import assay either NPI-1 or NPI-3 was able to direct free NP and NP in the form of an RNP complex to the nucleus (21). Thus, there is a redundant mechanism in terms of the NLS located at the N terminus. Binding of the NP to either NPI-1 or to NPI-3 may facilitate docking to the nuclear membrane and subsequent translocation through the membrane.

Nothing is known about the differential expression of the docking proteins in various cell types, and thus it is not possible to identify the NPI(s) involved in natural influenza virus infections. It should be noted, however, that there appear to be additional members of the NPI-1–NPI-3 protein family. There are more than 50 human expressed sequence tags in the database at the National Center for Biotechnology Information. Sequence alignments of some of these expressed sequence tags with the region spanning nucleotides 800 to 1200 of NPI-1 demonstrate that there are at least four different human NPI-1 protein family members (including NPI-1 and NPI-3). The sequence of a partial clone homologous to NPI-1 and NPI-3 was published by Adams et al. (1). It is thus possible that there is a high degree of redundancy (as well as specificity) for the nuclear import of NLS-containing proteins and that the nuclear localization of proteins may be mediated by the presence of different NPI proteins in the cell.

The NLS motifs SxGTKRSYxxM and TKRSxxxM, which were defined by the interaction with NPI-1 and NPI-3, respec-

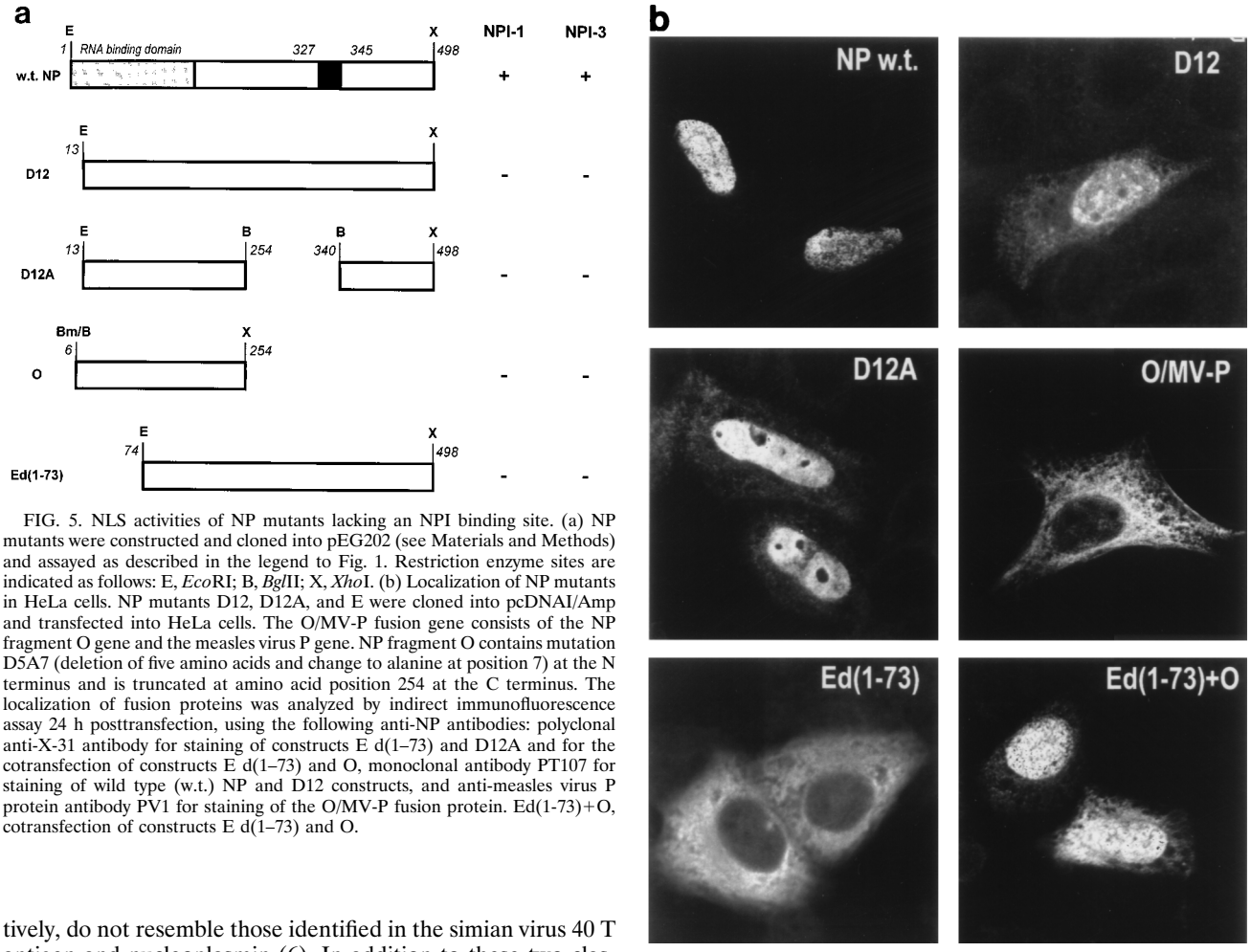


FIG. 5. NLS activities of NP mutants lacking an NPI binding site. (a) NP mutants were constructed and cloned into pEG202 (see Materials and Methods) and assayed as described in the legend to Fig. 1. Restriction enzyme sites are indicated as follows: E, *Eco*RI; B, *Bgl*II; X, *Xho*I. (b) Localization of NP mutants in HeLa cells. NP mutants D12, D12A, and E were cloned into pcDNAI/Amp and transfected into HeLa cells. The O/MV-P fusion gene consists of the NP fragment O gene and the measles virus P gene. NP fragment O contains mutation D5A7 (deletion of five amino acids and change to alanine at position 7) at the N terminus and is truncated at amino acid position 254 at the C terminus. The localization of fusion proteins was analyzed by indirect immunofluorescence assay 24 h posttransfection, using the following anti-NP antibodies: polyclonal anti-X-31 antibody for staining of constructs E d(1–73) and D12A and for the cotransfection of constructs E d(1–73) and O, monoclonal antibody PT107 for staining of wild type (w.t.) NP and D12 constructs, and anti-measles virus P protein antibody PV1 for staining of the O/MV-P fusion protein. Ed(1-73)+O, cotransfection of constructs E d(1–73) and O.

tively, do not resemble those identified in the simian virus 40 T antigen and nucleoplasmin (6). In addition to these two classical NLS sequences, nonconventional NLS motifs have been described (24–26). None of these appear to be similar or identical to those identified in the influenza virus NP.

RNA binding domains of the NP have been mapped to amino acids 1 to 77, 79 to 188, and 91 to 188 (1a, 14). However, at this time RNA binding domains have not been fine mapped on the NP, so we do not know whether the NPI binding sites overlap the RNA binding domain. Import of the viral RNA most likely involves a piggyback mechanism by which the RNA is bound to viral NP that gets transported into the nucleus (21).

Unexpectedly, we also identified an NLS activity which does not appear to be mediated by binding to either NPI-1 or NPI-3 (constructs D12 and D12A [Fig. 5b]). This NLS activity is most likely the result of a discontinuous amino acid sequence on the NP, since cotransfection of two fragments (constructs O and E [Fig. 5b]) results in nuclear localization measured by immunofluorescence. The signals have not been further investigated, and the mechanism by which this nuclear transport is effected is not known. Since each fragment alone has a cytoplasmic localization, the two fragments may interact directly or indirectly and thereby reconstitute a biologically active NLS. Indeed, data from the two-hybrid assay indicate that full-length NP can interact with itself (data not shown). In any case, this NLS activity does not appear to involve binding to NPI-1 or NPI-3 (as measured in the yeast two-hybrid system). This signal may be weaker than that associated with the N terminus, since the constructs containing the bipartite NLS show partial cytoplasmic localization (mutant D12 [Fig. 5b]). However, we cannot be certain about the relative strengths of these NLS activities, since we lack a quantitative in vivo assay. This bipartite NLS may also represent an NLS which is assembled from adjacent NPs in RNP complexes rather than a signal assembled from domains normally contained within a single NP molecule. Finally, based on the analysis of the amino acid sequence of NP, the above-described NLS activity does not appear to be mediated by classical NLS sequences.

In summary, the present data characterize the NPI binding site on the influenza A virus NP. The N-terminal 13 amino acids contain a motif which is associated with binding to NPI-1 and NPI-3. In addition, this sequence possesses an NLS activity, as can be demonstrated by the nuclear localization of fusion proteins which contain this short motif. The N-terminal 13 amino acids of influenza A virus NPs are highly conserved. Of the 56 avian and mammalian NP sequences available in the data bank, 54 are identical to the sequence of the A/PR/8/34 virus NP. The specific NPI binding motifs identified in the influenza A virus NP appear not to be present in influenza B and C virus NPs. It will be important to examine whether the NPs of these viruses also bind to NPI-1 and NPI-3 and whether these binding sites have NLS activities.

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