

The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins

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Nuclear import and export of viral nucleic acids is crucial for the replication cycle of many viruses, and elucidation of the mechanism of these steps may provide a paradigm for understanding general biological processes. Influenza virus replicates its RNA genome in the nucleus of infected cells. The influenza virus NS2 protein, which had no previously assigned function, was shown to mediate the nuclear export of virion RNAs by acting as an adaptor between viral ribonucleoprotein complexes and the nuclear export machinery of the cell. A functional domain on the NS2 with characteristics of a nuclear export signal was mapped: it interacts with cellular nucleoporins, can functionally replace the effector domain of the human immunodeficiency virus type 1 (HIV-1) Rev protein and mediates rapid nuclear export when cross-linked to a reporter protein. Microinjection of anti-NS2 antibodies into infected cells inhibited nuclear export of viral ribonucleoproteins, suggesting that the Rev-like NS2 mediates this process. Therefore, we have renamed this Rev-like factor the influenza virus nuclear export protein or NEP. We propose a model by which NEP acts as a protein adaptor molecule bridging viral ribonucleoproteins and the nuclear pore complex.

Keywords: HIV/influenza virus NEP(NS2)/nucleocytoplasmic transport/ribonucleoprotein/RNA export

Introduction

Many viruses including influenza, herpes, adeno- and retroviruses replicate their genomes in the nucleus. Therefore, regulated mechanisms must exist by which the nucleic acids of these viruses enter and leave the nucleus to begin and to complete the virus replicative cycle, respectively. The genome of influenza A virus consists of eight segments of negative-sense RNA, which are bound by the viral polymerase at the termini and are coated with the viral nucleoprotein (NP) to form ribonucleoprotein (RNP) complexes. Upon entry, virus is first uncoated and delivers into the cytosol its RNP, which is then transported into the nucleus (O'Neill *et al.*, 1995; Whittaker *et al.*, 1996b). Influenza virus assembly occurs at the plasma membrane at late times after infection; therefore, a switch in the direction of viral RNA transport from the nucleus

to the cytoplasm must occur for the virus replicative cycle to be completed.

The major gateway for nucleocytoplasmic transport of macromolecules is the nuclear pore complex (NPC) (Davis, 1995; Nigg, 1997). We have shown that influenza virus NP is required for transport of viral RNA into the nucleus to initiate virus replication and that the viral NP uses the protein nuclear import pathway, including the four soluble import factors karyopherin α , karyopherin β , Ran and p10 for viral RNA import (O'Neill *et al.*, 1995). The present work addresses the factors involved in the export of viral RNA. Since most RNAs are protein associated, it is believed that protein factors mediate the export of specific RNA classes (reviewed by Izaurralde and Mattaj, 1995). Indeed, direct involvement of several proteins in RNA transport pathways has already been demonstrated. First, the human immunodeficiency virus type 1 (HIV-1) Rev protein mediates the nuclear export of unspliced or partially spliced viral RNAs (Felber *et al.*, 1989; Malim *et al.*, 1989b; Fischer *et al.*, 1994, 1995; Stutz and Rosbash, 1994). Second, cellular mRNAs as RNP particles have been observed in mid-transport through the NPC (Mehlin *et al.*, 1992). Because the hnRNP A1 protein shuttles between the nucleus and the cytoplasm and has mRNA-binding properties, it and other hnRNP proteins are believed to play a direct role as a carrier for the export of cellular mRNAs (Piñol-Roma and Dreyfuss, 1992; Michael *et al.*, 1995). In support of this, the hnRNP A1-like protein hrp36 of *Chironomus tentans* associates with pre-mRNA of the Balbiani ring complex and remains complexed with the RNA during mRNA processing and transport to the cytoplasm (Visa *et al.*, 1996). Third, TFIIIA and L5 proteins of *Xenopus* have been shown to be involved in the transport of 5S rRNA (Guddat *et al.*, 1990).

Protein export requires a specific nuclear export signal (NES), which functions independently of surrounding peptide sequences and can confer the ability to be transported out of the nucleus on another protein to which it is fused. Recently, several NES-containing proteins have been identified, including the lentivirus proteins Rev and Rex (Fischer *et al.*, 1995; Palmieri and Malim, 1996), the cAMP-dependent protein kinase inhibitor (PKI) (Wen *et al.*, 1995), hnRNP A1 (Michael *et al.*, 1995), amphibian TFIIIA (Fridell *et al.*, 1996b) and yeast Gle1 (Murphy and Wenthe, 1996). In the case of the HIV-1 Rev protein, the function of its NES correlated with the ability to bind to the FG-repeat elements of nucleoporins (Bogerd *et al.*, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995). Human (Rab/hRip1) and yeast (yRip1) nucleoporins were identified using the yeast two-hybrid system as Rev-binding proteins, which interact with the Rev NES/effector domain. Mutations which disrupt the Rev NES also abolish the ability of Rev to interact with Rab/hRip1 and yRIP1.

Nuclear export signals within the proteins Gle1 and PKI also interact with Rab, further strengthening the connection between NESs and nucleoporin function in nucleocytoplasmic transport (Fridell *et al.*, 1996a; Murphy and Wenthe, 1996).

We hypothesized that influenza virus promotes the export of its own RNA by producing a factor(s) which interact(s) with nucleoporins in much the same way that Rev does. By screening influenza virus-encoded proteins for the ability to bind nucleoporins we hoped to identify the protein(s) which mediate viral RNA export. In the yeast two-hybrid system, only the viral NS2 protein was able to interact with different nucleoporins. Moreover, the NS2 or a small NS2 amino-terminal peptide could functionally replace the Rev effector/NES domain as a Rev-NS2 fusion protein. In addition, an amino-terminal peptide of NS2 promoted the nuclear export of a heterologous protein to which it was cross-linked. These experiments suggest that NS2 is a Rev-like, NES-containing protein. Significantly, antibodies directed against the NS2 protein, when injected directly into the nucleus, inhibited the cytoplasmic accumulation of viral NP and RNP at late times after infection. These experiments suggest a role for the NS2 protein in transport of newly synthesized viral RNA from the nucleus to the cytoplasm. Since NS2 (non-structural protein 2) is misnamed, i.e. it has been demonstrated to be a structural protein (Lamb *et al.*, 1978; Richardson and Akkina, 1991; Yasuda *et al.*, 1993), we renamed this protein in accordance with the function we have identified—the viral nuclear export protein or NEP.

Results

Influenza virus NEP protein interacts with cellular nucleoporins

Because the export function of the HIV-1 Rev protein correlated with its ability to interact with FG-repeat-containing cellular proteins called nucleoporins (Bogerd *et al.*, 1995; Fischer *et al.*, 1995; Stutz *et al.*, 1995, 1996), we examined whether any influenza virus protein interacted in the yeast two-hybrid system with nucleoporins. In this way, we hoped to identify influenza viral proteins which function as nuclear export factors. Only viral proteins which are known to enter the nucleus were examined (Figure 1). Five nucleoporins with distinct characteristics were chosen for this study: Rab/hRip1 and yRip1, which contain XXFG repeats, were cloned originally in yeast two-hybrid screens with the Rev protein as bait (Bogerd *et al.*, 1995; Fritz *et al.*, 1995; Stutz *et al.*, 1995); yNup100 and yNup116 are members of the GLFG-repeat family of nucleoporins, and bind Rev weakly; yNup1 is a member of the FXFG-repeat family and does not bind to Rev (Stutz *et al.*, 1995, 1996).

The viral matrix protein (M1) failed to interact with any of the nucleoporins tested (Figure 1). The M1 protein was the prime candidate to provide an export signal for influenza virus RNPs since M1 was shown previously to be required for nuclear export of influenza virus RNPs at late times after infection (Martin and Helenius, 1991; Whittaker *et al.*, 1996a). In addition to full-length M1, we found that a fragment of M1 (i.e. amino acids 89–252), which was shown previously to interact with the NEP (Ward *et al.*, 1995), also failed to interact with

	Cellular Nucleoporin (FG-repeat element type)				
	Rab/hRIP1 (XXFG)	yRip1 (XXFG)	yNup1 (FXFG)	yNup100 (GLFG)	yNup116 (GLFG)
M1	-	-	-	-	-
M1⁸⁹⁻²⁵²	-	-	-	-	-
NP	-	-	-	-	-
PB1	-	-	-	-	-
PB2	-	-	-	-	-
PA	-	-	-	-	-
NEP	+++	+++	-	+	+
NS1	+	-	-	-	-
Rev	+++	+++	-	+	+

Fig. 1. Yeast two-hybrid interactions of influenza virus proteins and nucleoporins. The bait constructs express LexA fused to influenza virus NEP (amino acids 1–121), NP (amino acids 2–498), M1 (amino acids 1–252), an M1 fragment (amino acids 89–252), PA (amino acids 1–716), PB1 (amino acids 1–757), PB2 (amino acids 1–759) and NS1 (amino acids 2–230). For comparison, a LexA fusion to Rev (amino acids 1–116) is included. The prey constructs express the acidic B42 domain fused to yRip1 (amino acids 148–275), yNup100 (amino acids 278–534), yNup116 (amino acids 458–701) and yNup1 (amino acids 438–737). The prey construct expressing Rab/hRip1 (amino acids 1–562) was expressed from pVP16, which contains the VP16 activation domain. The predominant class of FG-repeats within each nucleoporin is indicated. The number of plus signs denotes the relative strength of blue color on X-Gal indicator plates, which was displayed by yeast strains bearing the reporter plasmid pSH18-34 and the indicated two-hybrid plasmids.

nucleoporins in the yeast two-hybrid system. Thus, our data suggest that M1 may not be the viral component which directly interacts with the cellular machinery involved in the nuclear export of proteins.

Since virion RNA is packaged as a complex with NP and the heterotrimeric viral polymerase, each of these proteins was also a candidate to provide the signal for transport of viral RNPs out of the nucleus. Viral RNAs and their cRNA copies exist in the infected cell as RNP complexes, with multiple copies of the viral influenza virus NP along the length of the RNA. Neither the influenza virus NP, nor the components of the viral polymerase (PB1, PB2 or PA) were able to interact with any of the nucleoporins which were tested, again suggesting that these viral factors do not interact directly with the nuclear export machinery.

The NEP of influenza virus was able to interact strongly with Rab/hRIP1 and yRip1, less strongly with yNup100 and yNup116, and not with yNup1. Interestingly, the particular nucleoporins which strongly interact with the NEP are identical to those which interact with Rev, suggesting that, like Rev, NEP is a virus-encoded nuclear export factor. In fact, the strength of the interaction of NEP with each nucleoporin tested was similar to that of Rev with these same nucleoporins (Figure 1). Since it is the Rev effector domain which interacts with the FG-repeat region of particular nucleoporins, this experimental result also suggested that NEP contains a similar functional domain.

The NS1 protein interacts only weakly with Rab/hRip1, and did not bind to the other nucleoporins, which bound NEP and Rev. Therefore, for the purpose of this study, the NS1 protein was not considered further.

NEP can functionally replace the Rev effector domain

Since the NEP was able to bind efficiently to nucleoporins, we examined whether the NEP could substitute for the

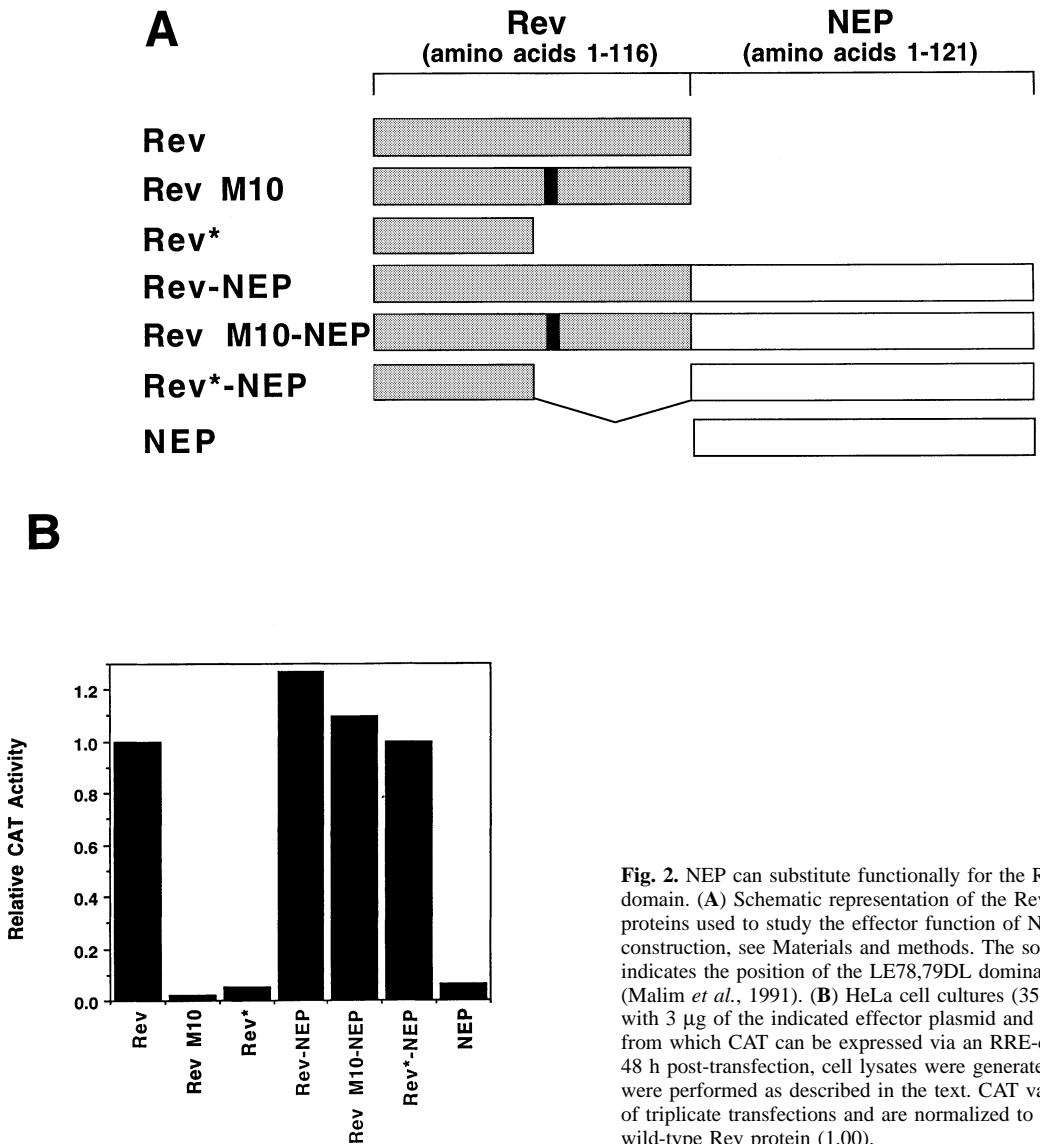


Fig. 2. NEP can substitute functionally for the Rev effector/NES domain. **(A)** Schematic representation of the Rev and NEP fusion proteins used to study the effector function of NEP. For methods of construction, see Materials and methods. The solid bar in Rev M10 indicates the position of the LE78,79DL dominant-negative mutation (Malim *et al.*, 1991). **(B)** HeLa cell cultures (35 mm) were transfected with 3 µg of the indicated effector plasmid and 2 µg of pDM128, from which CAT can be expressed via an RRE-containing RNA. At 48 h post-transfection, cell lysates were generated and CAT assays were performed as described in the text. CAT values are the average of triplicate transfections and are normalized to levels induced by wild-type Rev protein (1.00).

Rev effector domain. NEP was subcloned as a translational fusion gene to the carboxy-terminus of Rev, Rev M10 (a dominant-negative mutant of Rev), or Rev amino acids 1–69 (Rev*) (Figure 2A). Rev* is a truncated mutant lacking the effector domain. The activities of Rev and its derivatives were assayed using the reporter plasmid pDM128 (Hope *et al.*, 1990). Unspliced transcripts, from which chloramphenicol acetyl transferase (CAT) is expressed, are only transported to the cytoplasm and translated in the presence of functional Rev. These transcripts contain a Rev-response element (RRE) through which Rev acts.

A Rev-NEP fusion protein induced CAT activity to a level similar to wild-type Rev, demonstrating that the NEP does not interfere with the normal function of Rev (Figure 2B). Similar levels of CAT activity were produced in cells which co-expressed the NEP protein fused to the Rev effector mutant M10 or the truncated Rev protein Rev*, demonstrating that NEP contains an effector domain of its own which can substitute for the NES of Rev. Neither unfused Rev M10 nor the truncated Rev* induce CAT

expression in pDM128-transfected cells. In addition, wild-type NEP cannot induce expression of CAT. This demonstrates that NEP does not generally alter the ability of unspliced RNAs to be transported across the nuclear pore, or itself act through the RRE contained within the CAT transcript.

Mapping of the NEP effector domain

To analyze further the effector function of NEP, we used Rev-dependent expression of CAT from pDM128 to map a functional domain on the NEP. A nested set of deletions of NEP was fused to the carboxy-terminus of Rev* and was examined for the ability to induce CAT expression (Figure 3A). All fusion proteins which contain amino acids 1–30 of NEP induced CAT expression to levels similar to Rev*-NEP, while constructs which lack the first 30 amino acids induced no CAT activity above background. Therefore, the first 30 amino acids of NEP contain an effector domain which can functionally replace the NES of Rev.

Comparison of different NESs with amino acids 1–30

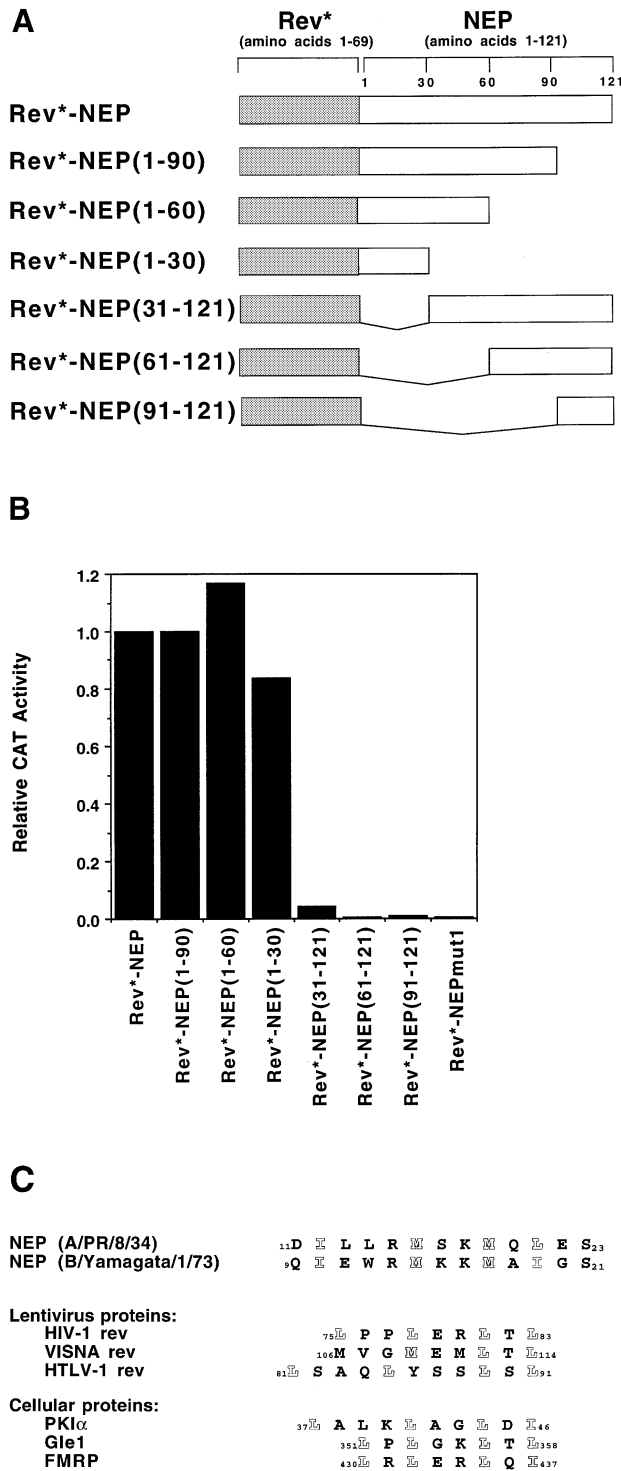


Fig. 3. The effector domain of NEP maps to its amino terminus. (A) Schematic representation of the nested NEP deletions expressed as protein fusions to amino acids 1–69 of Rev (Rev*). For constructions, see Materials and methods. (B) CAT assays were performed as described in the legend to Figure 2. CAT values are normalized to levels induced by wild-type Rev protein. (C) The amino-terminal NEP sequence of influenza A virus is shown aligned with defined NES sequences. Highlighted residues in the influenza A/PR/8/34 NEP sequence were mutagenized to alanine to create NEPmut1. For comparison, the homologous sequence from influenza B/Yamagata/1/73 is included. Important hydrophobic residues for NES function are also highlighted. Sources of sequence information: Rev, Fischer *et al.*, 1995; Visna Rev, Meyer *et al.*, 1996; adenovirus E4 34 kDa, Dobbelstein *et al.*, 1997; PKI, Wen *et al.*, 1995; and Gle1, Murphy and Wentz, 1996.

of the NEP enabled us to identify residues which might be critical for its NES function (Figure 3C). The NEP contains several hydrophobic amino acids (isoleucine, leucine and two methionines), which can be aligned with the critical residues within several defined NESs. In fact, this domain is highly conserved among NEP proteins of all influenza A and B virus strains. The isoleucine at position 12 of the NEP may be important since it is conserved between influenza A and B viruses and can be aligned with a critical leucine residue at position 37 of the PKI α NES (Wen *et al.*, 1994). In contrast, the leucine at position 13 of the influenza A virus NEP, which can be aligned with Leu75 of HIV-1 Rev, is not conserved in influenza B viruses and may not be important to NES function.

To determine whether these NEP residues are critical for the function of the domain, the methionines at positions 16 and 19 and the leucine at position 21 were changed simultaneously to alanines (Rev*-NEPmut1, Figure 3B). No CAT was expressed from pDM128 when these residues were mutated, demonstrating that one or more of these residues are important to the function of the Rev*-NEP fusion protein in the assay. In addition, when these residues were altered in pLexA-NEP and tested in the yeast two-hybrid system, NEP no longer interacted with Rab, yRip1, yNup100 or yNup116 (data not shown). Therefore, the NEP amino-terminal effector domain is similar both in sequence and functional activity to known NESs of viral and cellular origin (Figure 3C).

The amino-terminus of NEP contains a nuclear export signal

Since the amino-terminus of the NEP could functionally replace the effector domain of Rev, we examined directly whether this domain functions as an NES and promotes the nuclear export of a heterologous protein to which it is linked. A synthetic oligopeptide corresponding to NEP amino acids 11–23 (Figure 3C) was cross-linked to glutathione-S transferase (GST). GST was used as a reporter molecule to study NES function since it contains neither an NLS nor an NES of its own (Meyer *et al.*, 1996). We estimate that each cross-linked GST molecule (GST-x-NEP) contained 2–9 oligopeptides, as judged by altered mobility on an SDS–polyacrylamide gel (data not shown).

GST-x-NEP quickly migrated to the cytoplasm after microinjection into the nuclei of Madin–Darby bovine kidney (MDBK) cells (Figure 4A and C). After injection, cells were incubated at 37°C for 30 min prior to fixation and detection of injected antigens by indirect immunofluorescence. In each experiment, a control mouse monoclonal antibody was co-injected so that the site of injection could be monitored. It was shown previously that immunoglobulins lack signals for transport between the nucleus and cytosol, and remain confined to the compartment in which they are injected (Guiochon-Mantel *et al.*, 1991; Wen *et al.*, 1994; Meyer *et al.*, 1996). In Figure 4C and D, two cells were injected: one in the cytoplasm (small arrow) and one in the nucleus (large arrow). However, GST-x-NEP is completely cytosolic in both cells 30 min after injection (Figure 4C). Therefore, we conclude that the NEP protein contains an NES within amino acids 11–23. As a control, a second synthetic peptide, in which NEP amino acids 16, 19 and 21 (the residues highlighted

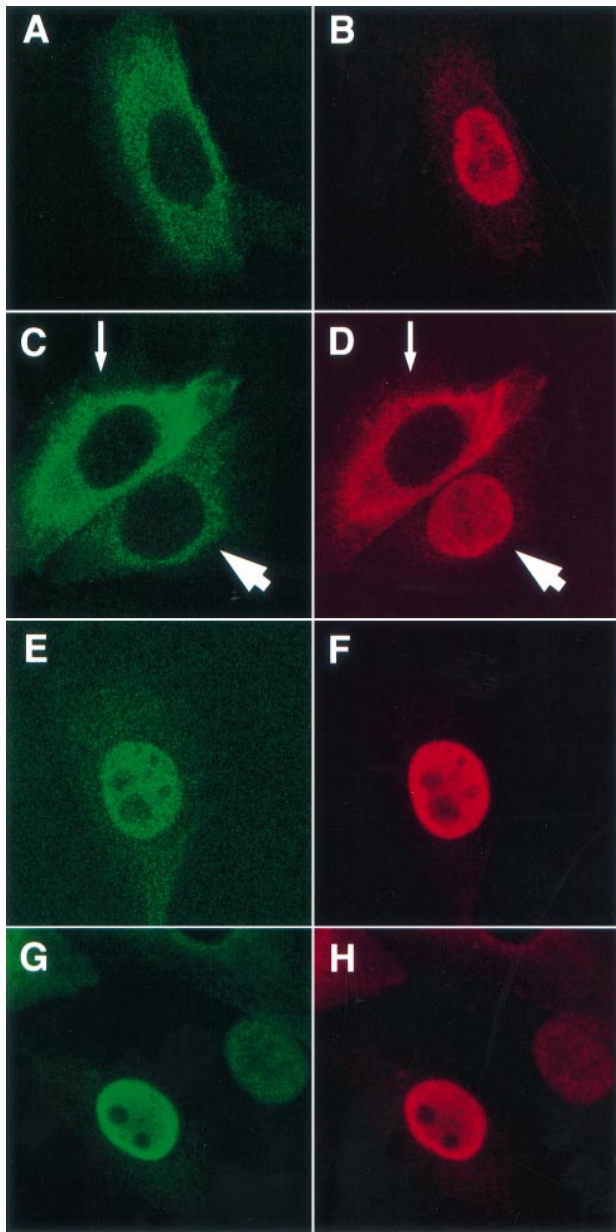


Fig. 4. The amino-terminus of NEP contains a nuclear export signal. GST, which was conjugated to the wild-type NS2 NES oligopeptide CDILLRMSKMQLS (A–D) or the mutant NS2 oligopeptide CDILLRASKAQAES (E–H), was microinjected into the nuclei of MDBK cells. All cells were co-injected with the mouse monoclonal antibody HT103 in order to monitor the compartment of injection. After incubation for 30 min at 37°C, cells were fixed and examined for the localization of injected GST by indirect immunofluorescence and visualized by confocal microscopy. The large and small arrows in (C) and (D) indicate cells which were injected in the nucleus and cytoplasm, respectively. Conjugated peptides were detected using rabbit antiserum directed against GST and a fluorescein-conjugated secondary antibody (A, C, E and G); the control peptide HT103 was detected with a Texas red-conjugated anti-mouse Ig serum (B, D, F and H).

in Figure 3C) were changed to alanines, was tested in this assay system (Figure 4E–H). This mutant peptide did not promote the nuclear export of GST, to which it was cross-linked. As is the case for the HIV-1 Rev protein, amino acid substitutions which disrupt nucleoporin binding also destroyed the NES activity of NEP.

Microinjected anti-NEP antibodies block influenza virus RNP export

No function or activity was assigned previously to the NEP protein during the influenza virus life cycle. NEP appears to be a structural component of the virus particle in which it is bound to the viral matrix protein M1 (Lamb *et al.*, 1978; Richardson and Akkina, 1991; Yasuda *et al.*, 1993). Participation of M1 in the transport of influenza virus RNP to the cytoplasm was shown previously (Whittaker *et al.*, 1996a): antibodies directed against M1, which were microinjected into cells infected by influenza virus, blocked the cytoplasmic accumulation of nucleoprotein and RNP at late times after infection. Because NEP but not M1 interacted with cellular nucleoporins, we wished to determine whether the function of the NEP protein was to promote the nuclear export of viral RNP. NEP (and M1) are late proteins: they accumulate to levels sufficient for detection by indirect immunofluorescence 4 h after infection (data not shown). Prior to NEP and M1 accumulation, the fluorescent signal due to NP is exclusively nuclear. Afterwards, the cytosol accumulates increasing amounts of NP.

Affinity-purified polyclonal antibodies directed against the NEP were microinjected into the cytosol of MDBK cells. Microinjected cells subsequently were infected by influenza A virus; antibodies directed against NEP should sequester newly translated NEP as NEP accumulates. At 8 h post-infection, cells were fixed and examined for the presence or absence of injected antibody, and for the subcellular localization of the NP protein; localization of the NP protein in the cytoplasm was assumed to represent cytosolic accumulation of RNP (Whittaker *et al.*, 1996a). Antibodies directed against NEP inhibited the cytoplasmic accumulation of influenza virus NP/RNP (Figure 5A and B, large arrows); adjacent uninjected cells had considerable amounts of NP in the cytoplasm (small arrows). Injected cells were identified by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig serum (Figure 5B). A fluorescent signal was seen in both the cytoplasm and the nucleus. Fluorescence of both the nucleus and the cytoplasm indicates either (i) that both compartments were injected, or (ii) that a portion of antibodies bound to NEP were piggybacked into the nucleus by NEP. Inhibition of NP/RNP transport was not due to the injection process since non-immune antibodies did not inhibit transport of NP/RNP to the cytoplasm (data not shown). Therefore, we postulate that the NEP mediates the nuclear export of influenza virus RNP at late times after infection.

Discussion

We have identified the influenza virus NEP as a virus-encoded nuclear export factor and present a model suggesting that NEP mediates the nuclear export of the viral RNPs at late times in the infectious cycle. The experimental evidence is 4-fold. First, we found using the yeast two-hybrid system that NEP binds to cellular nucleoporins. Second, the NEP can functionally replace the effector domain of HIV-1 Rev in a CAT-based reporter assay involving the nuclear export of RNA transcripts. Third, the effector domain was mapped to the N-terminal amino acids of NEP; specifically, a nuclear export function was

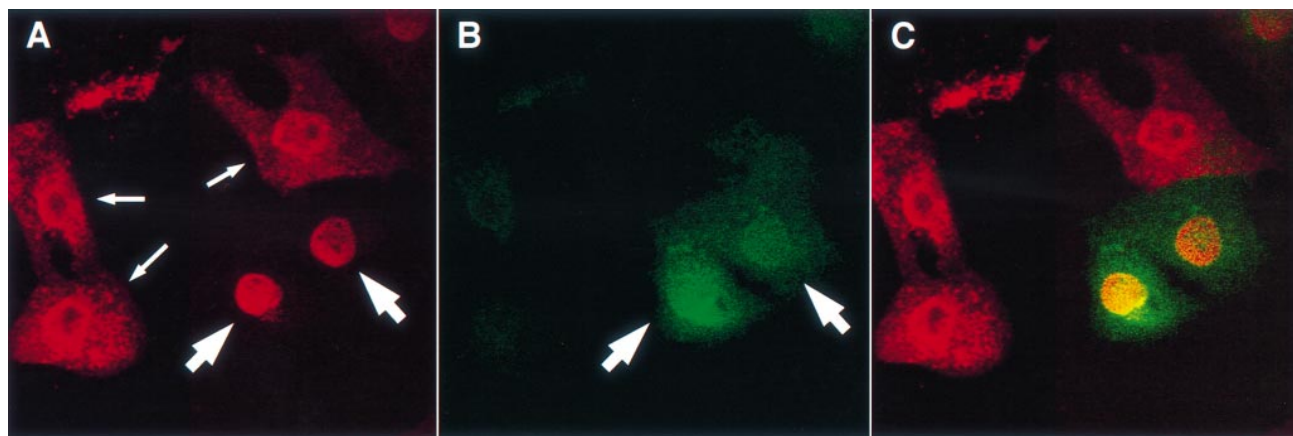


Fig. 5. Antibodies directed against NEP inhibit cytoplasmic accumulation of NP late in the influenza virus infectious cycle. Affinity-purified antibodies directed against the NEP were microinjected into MDBK cells 24 h after seeding on glass coverslips. At 12 h post-injection, cell monolayers were infected for 30 min at a multiplicity of 0.5 infectious particles per cell. Infected cells were incubated at 37°C for 8 h, at which time they were fixed, permeabilized and probed for (A) influenza virus NP and (B) injected anti-NS2 antibodies. NP was detected in two steps using the monoclonal antibody HT103 followed by a Texas red-linked goat anti-mouse Ig serum. Injected antibodies were detected in one step with fluorescein-conjugated goat anti-rabbit-Ig serum. (C) The superpositioning of (A) and (B).

found to be associated with amino acids 11–23 since the corresponding peptide promoted the nuclear export of a heterologous reporter protein to which it was linked. Fourth, antibodies directed against NEP when microinjected into the nucleus of infected cells prevented the export of NP and RNP.

In the influenza virus-infected cell, the NEP localizes to the nucleus (Greenspan *et al.*, 1985). Previously, the NEP was found in the virion in low quantities, but no function was attributed to it (Lamb *et al.*, 1978; Richardson and Akkina, 1991; Yasuda *et al.*, 1993). In the virion, the NEP is bound to the viral matrix protein M1, and during subfractionation of viral particles the NEP co-purifies with the M1 (Yasuda *et al.*, 1993). This interaction has been duplicated in the yeast two-hybrid system (Ward *et al.*, 1995; our unpublished data), and in filter binding assays (Yasuda *et al.*, 1993). Binding of NEP to M1 may be critical for the nucleocytoplasmic transport function of the NEP protein. Participation by M1 in the nuclear export of influenza virus RNP was demonstrated by Helenius and colleagues, and nuclear M1 was shown to be required for viral RNPs to be exported (Martin and Helenius, 1991). Furthermore, antibodies directed against M1 also inhibited RNP export when microinjected into infected cells (Whittaker *et al.*, 1996a). Despite this indirect evidence for a role for M1 in nuclear export, we were unable to show that a functional interaction of M1 and NEP is required for this process. M1 is unable to interact with cellular nucleoporins in the yeast two-hybrid system, suggesting that M1 is not a viral factor which interacts directly with the cellular transport machinery. Although the failure of M1 to interact with FG-containing nucleoporins in the yeast two-hybrid system could be due to trivial reasons, such as failure of LexA-M1 to localize to the yeast nucleus, our other two-hybrid system data suggest that it is NEP which binds to and acts as the adaptor molecule between the NPC and M1-containing RNP complexes which form in the nucleus (Figure 6). Although previous results using the ts51 mutant of influenza virus suggested that the M1 need not be physically associated with the viral RNP during nuclear export (Rey and Nayak,

1992), our model would favor a direct role for the M1 in the export of the viral RNA. Also, the earlier data could easily be explained if small amounts of M1 (not detected under the assay conditions) were sufficient to allow the NEP to pull viral RNP through the nuclear pore. Finally, it has been shown that although the mutant M1 accumulates in the nucleus, it shuttles between the nucleus and the cytoplasm (Whittaker *et al.*, 1996a), and thus could participate in nuclear export of viral RNA. On the other hand, our data do not address directly the role of M1 in the nuclear export of RNP, and it is possible that the NEP directly interacts with RNA or (modified) RNP to facilitate export (Figure 6).

The HIV-1 Rev and the influenza virus NEP appear to have similar functional domains. Both of these proteins interact with the NPC through FG-repeat-containing nucleoporins. In fact, the relative strength of the interaction between NEP and the nucleoporins Rab/hRip1, yRip1 yNup100 and yNup116 is similar to that between Rev and the same nucleoporins. Also, mutations which abolish Rev and NEP NES function abolish their ability to act as nuclear export factors. Despite the limited number of nucleoporins tested, we believe that these results are significant and may suggest that there is a hierarchy of the strength of protein–protein interactions between NESs and the nucleoporin FG-domains, and that the different binding activities may promote directionality of the export processes.

Further similarity between the nucleoporin-binding domains of NEP and Rev was demonstrated in Rev functional assays: the NEP NES can substitute for the Rev effector domain in Rev–NEP fusion proteins, confirming that NEP contains an NES. It should be noted that in this assay system the Rev RNA-binding domain provided the specificity for transport of RRE-containing transcripts, and NEP alone was not able to promote the nuclear export of unspliced CAT mRNA. This is in contrast to the herpes simplex virus Us11 protein, which does not require fusion to the RNA-binding region of Rev in order to interact with and induce transport of unspliced RRE-containing RNAs (Diaz *et al.*, 1996). The transport activity

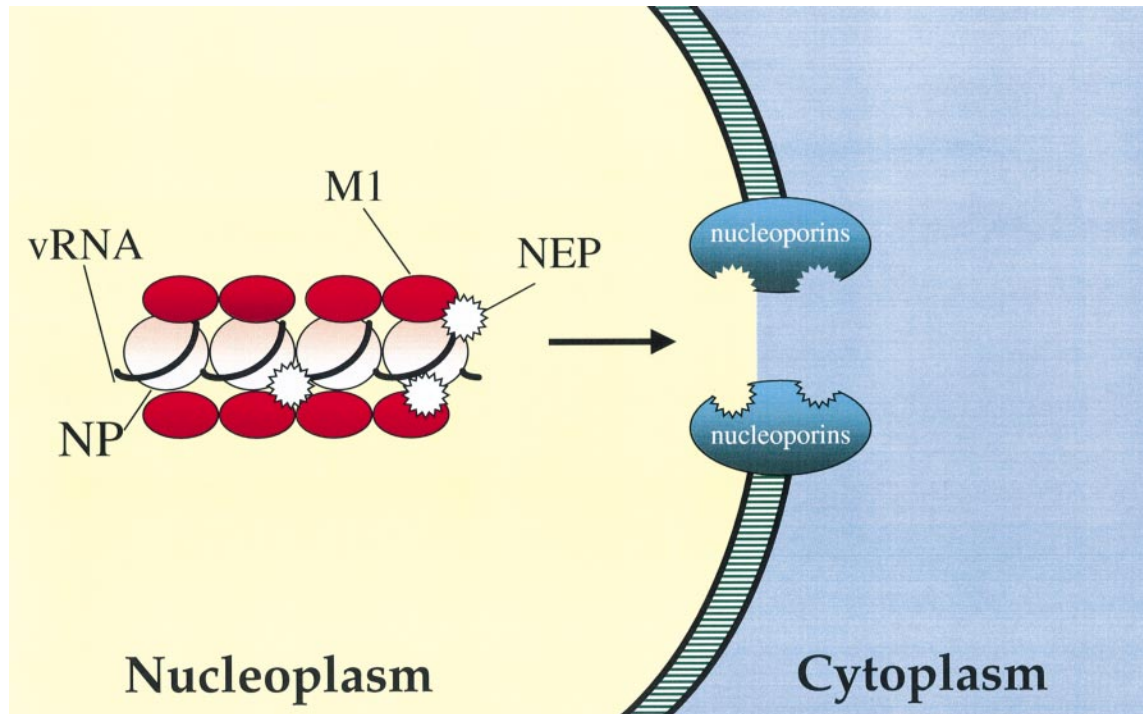


Fig. 6. Model of influenza virus ribonucleoprotein export from the nucleus. Influenza virus genomic RNA is associated with multiple copies of the viral nucleoprotein (pink), and the viral protein NEP (star) is required for transport of RNP from the nucleus to the cytoplasm. The NEP may act as a bridge between nucleoporins (protein constituents of the nuclear pore complex) and the RNP complex. Interaction of NEP with nuclear ribonucleoprotein complexes may be mediated by viral M1 (red) or other viral components. The stoichiometry of the NEP during RNP nuclear export is not known. We postulate that the NES of NEP interacts with multiple nucleoporins at multiple sites during the transport of influenza virus RNA through the nuclear pore complex.

of NEP is likely to be specific for influenza virus RNP, and this specificity appears to reside in peptide sequences downstream of the NES. Indeed, an M1-binding domain has been mapped to the carboxy-terminal 70 amino acids of NEP (Ward *et al.*, 1995). Unlike Rev, NEP has no inherent RNA-binding activity; contact of NEP with influenza virus RNA is likely to be mediated by the protein M1 (Figure 6). Thus, in this respect, the NEP may be similar to adenovirus E4-34 kDa protein, which contains an NES but contacts viral mRNA through another virus protein E1B-55 kDa (Dobbelstein *et al.*, 1997). However, in contrast to the RNA transporters of lenti- and adenoviruses, NEP mediates the export of non-polyadenylated, non-messenger viral genomic RNA.

In addition to NEP, the viral NS1 protein also bound to Rab/hRIP1. However, NS1 failed to bind to other nucleoporins tested. NS1 is a viral protein which regulates mRNA transport and inhibits export of viral and cellular mRNAs (Alonso-Caplen and Krug, 1991, 1992; Fortes *et al.*, 1994; Qiu and Krug, 1994). Whether the NS1-Rab interaction is relevant to mRNA retention by NS1 is not clear but is presently under investigation.

In summary, the influenza virus NEP possesses a Rev-like nuclear export function. It is an adaptor, which allows for the binding of viral RNP and nucleoporins and most likely for the transport of RNPs through the NPC. Unlike Rev, the NEP does not bind directly to its viral RNA but most probably recognizes the viral RNA as an RNP (Figure 6).

Materials and methods

Yeast two-hybrid constructions and screen

Saccharomyces cerevisiae EGY48 (*MAT α trp1 ura3 his3 LEU::pLEX-Aop6-LEU2*); pEG202, pRFHM1 and pSH18-34 were kindly provided by Dr R.Brent (Harvard Medical School) and have been described elsewhere (Gyuris *et al.*, 1993; Zervos *et al.*, 1993). Two-hybrid constructs encoding yRip1 (amino acids 151–275), yNup100 (amino acids 278–539), yNup1 (amino acids 438–737) and yNup116 (amino acids 459–672) in pJG4-5 were provided by Dr M.Rosbash (Brandeis University) (Stutz *et al.*, 1995). pVP16/Rab was provided by Dr B.Cullen (Duke University) (Bogerd *et al.*, 1995). pLexA-NP and pLexA-NS1 were described previously (O'Neill and Palese, 1995; Wolff *et al.*, 1996). Two-hybrid bait plasmids containing influenza virus cDNAs derived from the A/PR/8/34 strain (Young *et al.*, 1983; Greenspan *et al.*, 1985) were constructed in pEG202. PCR-amplified influenza virus open reading frames were cloned between *EcoRI* and *SalI* restriction sites (PA), *EcoRI* and *XhoI* sites (NEP and M1), *NotI* and *XhoI* sites (PB1), or *BamHI* and *XhoI* sites (PB2). Assay of β -galactosidase expression from pSH18-34 in yeast cells transformed with various combinations of bait and prey plasmids was performed as previously described (Gyuris *et al.*, 1993; Zervos *et al.*, 1993).

Mammalian expression vectors

For tissue culture expression of NEP, Rev, the Rev M10 mutant protein and derivative fusion proteins, cDNAs were subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Rev, Rev M10 and amino acids 1–69 of Rev (Rev*) were cloned between *KpnI* and *EcoRI* restriction sites. NEP and the nested set of NEP deletions were subcloned between the *EcoRI* and *XhoI* sites. pcRev and pcM10, from which these constructs were derived, were kindly provided by B.Cullen (Duke University) (Malim *et al.*, 1989a). The reporter plasmid pDM128 was kindly provided by Tristram Parslow (University of California, San Francisco) (Hope *et al.*, 1990).

Transfections and CAT assays

Plasmid transfections were performed using the cationic liposomal reagent DOTAP (Boehringer-Mannheim) according to the manufacturer's

instructions. Briefly, duplicate 3.5 cm dishes of 293 cells, which were 60% confluent, were transfected with 2 µg of pDM128 and 3 µg of an effector plasmid expressing Rev, NEP or a Rev-NEP fusion protein as described in the text. Transfected cells were harvested after 48 h. Lysates were prepared by freeze-thawing three times in 100 µl of 0.25 mM Tris, pH 7.5, followed by microcentrifugation for 5 min at 4°C. Aliquots were then assayed for CAT activity, which was then quantitated by beta scanning of phosphorylated and non-phosphorylated chloramphenicol separated by thin-layer chromatography.

Cross-linking of oligopeptides to a reporter protein

GST was expressed from pGEX-2TK in *Escherichia coli* BL21 (Novagen) and was purified from induced cultures on glutathione-Sepharose (Pharmacia) as previously described (O'Neill and Palese, 1995). Two oligopeptides [NS2 NES (CDILLRMSKMQLES) and NS2mut1 (CDILLRASKAQAES)] were synthesized at the Mt. Sinai Oligopeptide Synthesis Facility. Peptides were conjugated to GST through an amino-terminal cysteine residue, which is not part of the NS2 sequence. Oligopeptide sequences correspond to NS2 amino acids 11–23. In order to activate GST for cross-linking to peptides, 2.8 mg of GST in 1 ml was mixed with 2 mg of sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce Chemical Co.) for 1 h at room temperature. Unincorporated cross-linker was removed by chromatography on G50-Sepharose. Approximately 1 mg of GST in 600 µl was incubated with 3 mg of oligopeptide for 12 h at 4°C. GST conjugates were dialyzed against phosphate-buffered saline (PBS) to remove unincorporated oligopeptides, and were stored in small aliquots at –70°C.

Affinity-purified antibodies

GST was expressed from pGEX-2TK (Pharmacia) in *E. coli* BL21 and affinity purified on glutathione-Sepharose. NEP was expressed as a hexahistidine-tagged protein in *E. coli* BL21 from the vector pET28a (Novagen) and affinity purified on Ni-NTA-agarose (Qiagen). Expression and purification of proteins were performed according to protocols provided by the manufacturer of the respective protein affinity matrices. Antisera were raised by immunization of rabbits with 300 µg of affinity-purified protein in complete Freund's adjuvant followed by two booster injections with 150 µg in incomplete Freund's adjuvant at 3 week intervals. Affinity-purified antibodies were prepared from 5 ml of rabbit immune serum by adsorption to NEP or GST, which had been cross-linked to CNBr-activated Sepharose 4B according to the recommendations of the manufacturer (Pharmacia, Inc.). Binding, washing and elution of bound antibodies were performed according to standard protocols (Harlow and Lane, 1988).

Microinjection of cells

MDBK cells were cultured in reinforced minimal essential medium with 10% fetal bovine serum. Cells were seeded to glass coverslips 24 h prior to injection and 36 h prior to infection. Individual cells were microinjected using a micromanipulator and transjection apparatus from Eppendorf.

Oligopeptide-conjugated GST and a control monoclonal antibody [HT103 (Dr J.L.Schulman, Mt Sinai)] were mixed, diluted to a final concentration of 1 mg/ml each and injected into the nuclei of MDBK cells. After injection, cells were incubated at 37°C for 30 min, and were then fixed and permeabilized for 20 min in 2.5% formaldehyde/0.25% Triton X-100/PBS. Injected antigens were detected by indirect immunofluorescence. GST was detected in two steps using a rabbit polyclonal serum directed against GST and an FITC-linked goat anti-rabbit Ig serum. HT103 was detected in one step with Texas red-linked goat anti-mouse Ig serum. Injected cells were visualized by confocal laser microscopy.

Affinity-purified anti-NEP antibodies and protein A-purified normal rabbit immunoglobulins were dialyzed against PBS (pH 7.4) and diluted to a final concentration of 1.3 mg/ml. Antibodies were microinjected into MDBK cells 24 h after seeding on glass coverslips. At 12 h post-injection, cell monolayers were infected at 37°C for 30 min at a multiplicity of 0.5 infectious particles per cell. Eight hours after infection, cells were fixed and permeabilized as described above. The influenza virus nucleoprotein was detected in two steps using the monoclonal antibody HT103 and a Texas red-linked goat anti-mouse Ig serum. Injected antibodies were detected in one step with an FITC-linked goat anti-rabbit Ig serum.

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