

Several protein regions contribute to determine the nuclear and cytoplasmic localization of the influenza A virus nucleoprotein

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A systematic analysis was carried out to identify the amino acid signals that regulate the nucleo-cytoplasmic transport of the influenza A virus nucleoprotein (NP). The analysis involved determining the intracellular localization of eight deleted recombinant NP proteins and 14 chimeric proteins containing the green fluorescent protein fused to different NP fragments. In addition, the subcellular distribution of NP derivatives that contained specific substitutions at serine-3, which is the major phosphorylation site of the A/Victoria/3/75 NP, were analysed. From the results obtained, it is concluded that the NP contains three signals involved in nuclear accumulation and two regions that cause cytoplasmic accumulation of the fusion proteins. One of the karyophilic signals was located at the N terminus of the protein, and the data obtained suggest that the functionality of this signal can be modified by phosphorylation at serine-3. These findings are discussed in the context of the transport of influenza virus ribonucleoprotein complexes into and out of the nucleus.

Introduction

The genome of influenza A virus is divided into eight single-stranded RNA segments, which are packaged as ribonucleoprotein (RNP) complexes. The RNPs contain four proteins: the nucleoprotein (NP), which encapsidates the genomic RNA, and the three subunits of the polymerase complex (Lamb & Krug, 1996). In a natural infection, the incoming RNPs are delivered into the cytoplasm and are transported to the cell nucleus (Martin & Helenius, 1991*a, b*). The NP and polymerase proteins synthesized during a lytic infection move from the cytosol to the cell nucleus, where they are assembled into RNPs, which later exit the nucleus, since virus budding occurs at the cell membrane. The virus has evolved mechanisms to regulate this bidirectional transport of RNPs. In fact, it has been shown that each of the RNP protein components contains nuclear-targeting signals and that the M1 and NS2 proteins play a regulatory role in the RNP nucleo-cytoplasmic traffic events (Whittaker *et al.*, 1996; O'Neill *et al.*, 1998; Whittaker & Helenius, 1998; Portela *et al.*, 1999).

Protein import and export are signal-mediated events that occur through the nuclear pore complex. There are different

nuclear import and export pathways, each involving the interaction of the target protein with specific cell receptors. In this report, a protein sequence that acts as a signal for protein import will be referred to as a nuclear-import signal (NIS), whichever cell receptor is used for import into the nucleus. The term nuclear-localization signal (NLS) will only be used to designate the NIS that has been shown to bind to the NLS receptor (importin α and importin β ; also known as karyopherin α and karyopherin β), whereas the term nuclear-export signal (NES) will designate protein sequences that direct nuclear export, independently of the export pathway used. In addition to these transport signals, it has been shown that some proteins contain retention signals, either nuclear (NRS) or cytoplasmic (CRS), that also contribute to the determination of subcellular localization (all these aspects have been reviewed by Jans & Hübner, 1996; Nigg, 1997; Izaurralde & Adam, 1998; Görlich, 1998; Mattaj & Englmeier, 1998).

A full understanding of the mechanisms that regulate the nucleo-cytoplasmic transport of RNPs would require the identification of the signals that regulate the intracellular localization of isolated NP. However, despite several reports on this issue, the identification of these signals is far from complete. Davey *et al.* (1985) concluded, from experiments with *Xenopus* oocytes, that the region required for NP nuclear accumulation was located between residues 327 and 345.

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However, this conclusion was questioned by results showing that proteins containing substitutions or deletions within this region still accumulated in the nuclei of mammalian cells (Neumann *et al.*, 1997; Wang *et al.*, 1997; Digard *et al.*, 1999; Mena *et al.*, 1999). Moreover, it was also found that the NP contains an NLS within the 20 N-terminal residues of NP (O'Neill & Palese, 1995; Neumann *et al.*, 1997; Wang *et al.*, 1997). Unexpectedly, an NP derivative lacking the two karyophilic signals mentioned above localized mostly to the cell nucleus, indicating the presence of additional NLSs in the NP (Neumann *et al.*, 1997; Wang *et al.*, 1997). Furthermore, it has been reported recently that the NP contains an actin-binding domain, and it has been suggested that this domain could act functionally as a CRS to cause the cytoplasmic retention of RNPs later in infection (Digard *et al.*, 1999).

It is well documented that nuclear transport can be controlled by phosphorylation (Hunter & Karin, 1992; Jans & Hübner, 1996; Whittaker & Helenius, 1998). The NP is a phosphorylated protein (Privalsky & Penhoet, 1977; Kistner *et al.*, 1989) and we have demonstrated that the major phosphorylation site of the A/Victoria/3/75 NP is serine-3 (Arrese & Portela, 1996). The fact that this residue is conserved in practically all NPs sequenced to date (see Arrese & Portela, 1996, and references therein) and the observations that the N-terminal 13 amino acids of the NP include the binding site for members of the importin α family (O'Neill *et al.*, 1995; Wang *et al.*, 1997) and that inhibitors and activators of protein kinases can alter the subcellular localization of recombinant NP (Neumann *et al.*, 1997) suggest that phosphorylation/dephosphorylation of serine-3 may control some of the NP nuclear-traffic events. This hypothesis, however, remains to be demonstrated.

In this report we have carried out a systematic analysis to identify the regions involved in NP subcellular localization. Thus, we have analysed the intracellular localization of a number of NP derivatives, including deleted NP polypeptides, fusion proteins and proteins containing amino acid substitutions at the NP major phosphorylation site.

Methods

■ **Viral strains, cell lines and antibodies.** Vaccinia virus recombinant vTF7-3, which expresses the T7 RNA polymerase (Fuerst *et al.*, 1986), was kindly provided by B. Moss (NIH, Bethesda, MD, USA). MDCK, Vero and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum.

Monoclonal antibody M/58/p44/E, which recognizes the A/Victoria/3/75 NP protein (López *et al.*, 1986), and rabbit antisera raised against maltose-binding protein-fusion proteins containing the N-terminal 77 amino acids or the C-terminal 120 amino acids of the NP have been described previously (Arrese & Portela, 1996). For immunofluorescence analysis, the rabbit antisera were enriched in anti-NP antibodies by following the procedure described by Gu *et al.* (1994). Briefly, the antisera were passed through a column containing His-tagged NP and the bound antibodies were eluted by successive washes with 4 M MgCl₂ and with 50 mM diethylamine–15 mM NaCl. The eluted

antibodies were then dialysed against PBS. Anti-actin monoclonal antibody and anti-green fluorescent protein (GFP) polyclonal serum were purchased from Amersham and Clontech, respectively.

■ **Plasmids containing NP gene derivatives downstream of the T7 RNA polymerase promoter of vector pGEM-3.** Plasmid pGEM-NP, which contains a cDNA encoding the NP gene of the influenza A/Victoria/3/75 virus cloned downstream of the T7 RNA polymerase promoter of plasmid pGEM-3 (Promega), has been described previously (de la Luna *et al.*, 1989; Mena *et al.*, 1994). pGEM-NP derivatives encoding deleted NPs have also been described previously (Albo *et al.*, 1995). Plasmid $\Delta 13$ was prepared by oligonucleotide-directed mutagenesis as described by Mena *et al.* (1999). Plasmids NP-S3D and NP-S3E were prepared by site-directed mutagenesis, with plasmid pGEM-NP as the template, as described previously for plasmid NP-S3A (Arrese & Portela, 1996). These latter plasmids encode full-length NP proteins that contain Asp (NP-S3D), Glu (NP-S3E) or Ala (NP-S3A) instead of Ser at position 3 of the NP gene.

■ **Plasmids expressing GFP-NP fusion proteins.** Fusion proteins containing GFP were obtained by subcloning specific NP gene fragments into the plasmid vectors pEGFP-C1 or pEGFP-N1 (Clontech), which contain a jellyfish GFP gene optimized for maximum fluorescence downstream of a CMV promoter. The NP gene-specific fragments were obtained by PCR by using plasmid pGEM-NP as the template and two oligonucleotides that contained NP gene sequences. In addition to these sequences, one of the oligonucleotides contained an *Asp718* restriction site and the other oligonucleotide included, after the NP gene sequence, a stop codon and a restriction site for the enzyme *Xba*I. The PCR fragments obtained were digested with these restriction enzymes and cloned between the same restriction sites present in the vector pEGFP-C1 (Clontech) to yield fusion proteins that contained GFP at the N terminus of the chimeric polypeptide. The plasmids that encode fusion proteins GFP-GST1 and GFP-GST2 contained GFP followed by the N-terminal 80 or 160 residues of the bacterial glutathione S-transferase (GST) protein, respectively, and they were prepared by the same approach used to prepare the chimeric GFP-NP polypeptides.

To obtain plasmid S3wt-GFP, an NP gene-specific fragment corresponding to the first 80 amino acids was obtained by PCR with two oligonucleotides that included restriction sites for the enzymes *Eco*RI and *Asp718*. The PCR product was then digested with these two restriction enzymes and cloned between the same two restriction sites present in the vector pEGFP-N1, so that a GFP-fusion polypeptide was obtained that contained the NP protein sequence at the N terminus. Plasmids containing the same NP fragment but with an Ala (S3A-GFP), Asp (S3D-GFP) or Glu (S3E-GFP) residue at position 3 were prepared by following similar procedures.

In all the plasmids that encoded chimeric proteins, the integrity of the NP gene sequence was confirmed by sequencing. The sequences of the oligonucleotides used for mutagenesis will be provided on request.

■ **Subcellular localization of NP derivatives expressed from pGEM-derived plasmids.** Cultures of Vero and/or COS-1 cells growing on glass coverslips were infected with vTF7-3 and transfected individually with the indicated pGEM-NP-derived plasmid and processed for indirect immunofluorescence as described previously (Mena *et al.*, 1999).

■ **Detection of GFP-fusion proteins by direct fluorescence.** COS-1 cells growing on coverslips were transfected individually with plasmids encoding GFP-fusion proteins by using liposomes. At 24 h post-transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, incubated in the presence of Hoechst

33258 dye (0.5 µg/µl) for 30 min and visualized with a fluorescence microscope.

■ **Nuclear and cytoplasmic fractionation.** The procedure described by Avalos *et al.* (1997) was used. Briefly, transfected COS-1 cells were lysed in a solution containing 1% Nonidet P-40, 1% Triton X-100 and 0.5% sodium deoxycholate. The cell lysate was then passed 20 times through a 25-gauge hypodermic needle and centrifuged at 1000 *g* for 5 min. The supernatant (cytoplasmic) and the pellet (nuclear) fractions were resuspended in SDS sample buffer and analysed by SDS-PAGE and Western blotting as described previously (Arrese & Portela, 1996).

Results and Discussion

There is no single NIS in the influenza A virus NP

To identify the NP regions involved in nuclear targeting, the subcellular localization of a number of deleted NP proteins was determined by indirect immunofluorescence (Fig. 1). In cultures transfected with the plasmid encoding the wild-type NP, cells that showed exclusively nuclear staining (pattern N), as well as cells that displayed predominantly nuclear labelling but also showed some cytoplasmic fluorescence (pattern N > C), were observed (representative examples of these patterns are shown in Fig. 1*b*). The same two patterns were observed with all deleted NP proteins tested (Fig. 1) and, since the deletions introduced in the NP protein covered the whole length of the protein, it was concluded, in agreement with previous data (Neumann *et al.*, 1997; Wang *et al.*, 1997), that the NP contains more than one signal involved in nuclear accumulation.

Identification of regions of NP involved in nuclear targeting of the protein

It was then decided to prepare and determine the subcellular localization of chimeric constructs containing different NP fragments fused to a reporter protein. This type of approach is routinely based on the use of reporter proteins that are too large to diffuse freely through nuclear pores. Hence, if the fusion protein is found in the nucleus it indicates the presence of an NIS in the fused protein fragment. However, the use of a large reporter protein is not appropriate for detecting protein regions that contain a functional NRS but lack an NIS. In this latter case, the chimeric protein would not be retained in the nucleus because the polypeptide would never reach this compartment due to its large size.

We decided to use GFP, a 27 kDa protein not known to contain any sequence involved in nuclear targeting, as the reporter protein. Due to its small size, GFP can diffuse passively through the nuclear pore complex and, in fact, the protein distributes uniformly throughout the cytoplasm and nucleus (pattern N + C) when expressed in mammalian cells (Ogawa *et al.*, 1995; Pines, 1995; Carey *et al.*, 1996). However, the intracellular distribution of GFP changes when it is fused to a protein containing a functional NLS or NES (Pines,

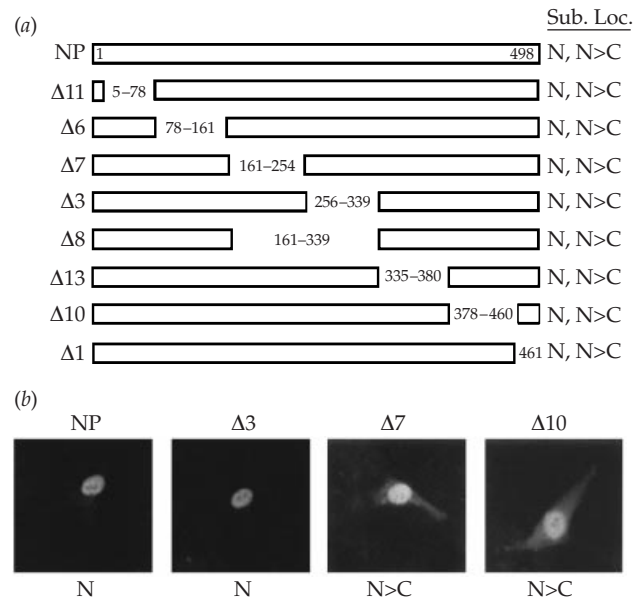


Fig. 1. Subcellular localization of NP deleted proteins. (a) Schematic representation of wild-type NP and NP deleted proteins expressed from recombinant plasmids. The subcellular localization of the NP derivatives is indicated on the right (Sub. Loc.; see text for details). (b) Intracellular localization of the recombinant NP proteins as determined by indirect immunofluorescence. Vero cells were infected with vTF7-3, transfected with the indicated pGEM-NP-derived plasmids and analysed by indirect immunofluorescence as indicated in Methods. The pictures show stained cells representative of the two NP subcellular distribution patterns observed.

1995; Carey *et al.*, 1996), and we observed that a fusion protein (GFP-NP0) containing the full-length NP attached to the C terminus of GFP was localized exclusively to the cell nucleus (Fig. 2).

It was observed that GFP-fusion proteins containing the first 80 or 160 amino acids of the bacterial GST protein displayed the same staining pattern (N + C) as the control GFP (Fig. 2), indicating that these three proteins could diffuse into the nucleus because of their small size. Considering the observation made above about large reporter proteins, it was decided to prepare fusion proteins containing NP fragments of about 160 amino acids or less, so that the proteins could enter passively into the nucleus. We reasoned that, if the NP fragment fused to the GFP contained an NIS and/or an NRS, the protein would be detected preferentially in the cell nucleus. We were aware that by using such a strategy it would not be possible to distinguish between proteins containing an NIS or an NRS, because the proteins would display the same nuclear accumulation phenotype in both cases. Despite this potential drawback, it was considered that using small and similar-sized proteins could be a useful approach to characterize the NP signals involved in subcellular accumulation, since previous studies have shown the presence of several of these signals in NP and some of these studies have obtained conflicting results (see Introduction).

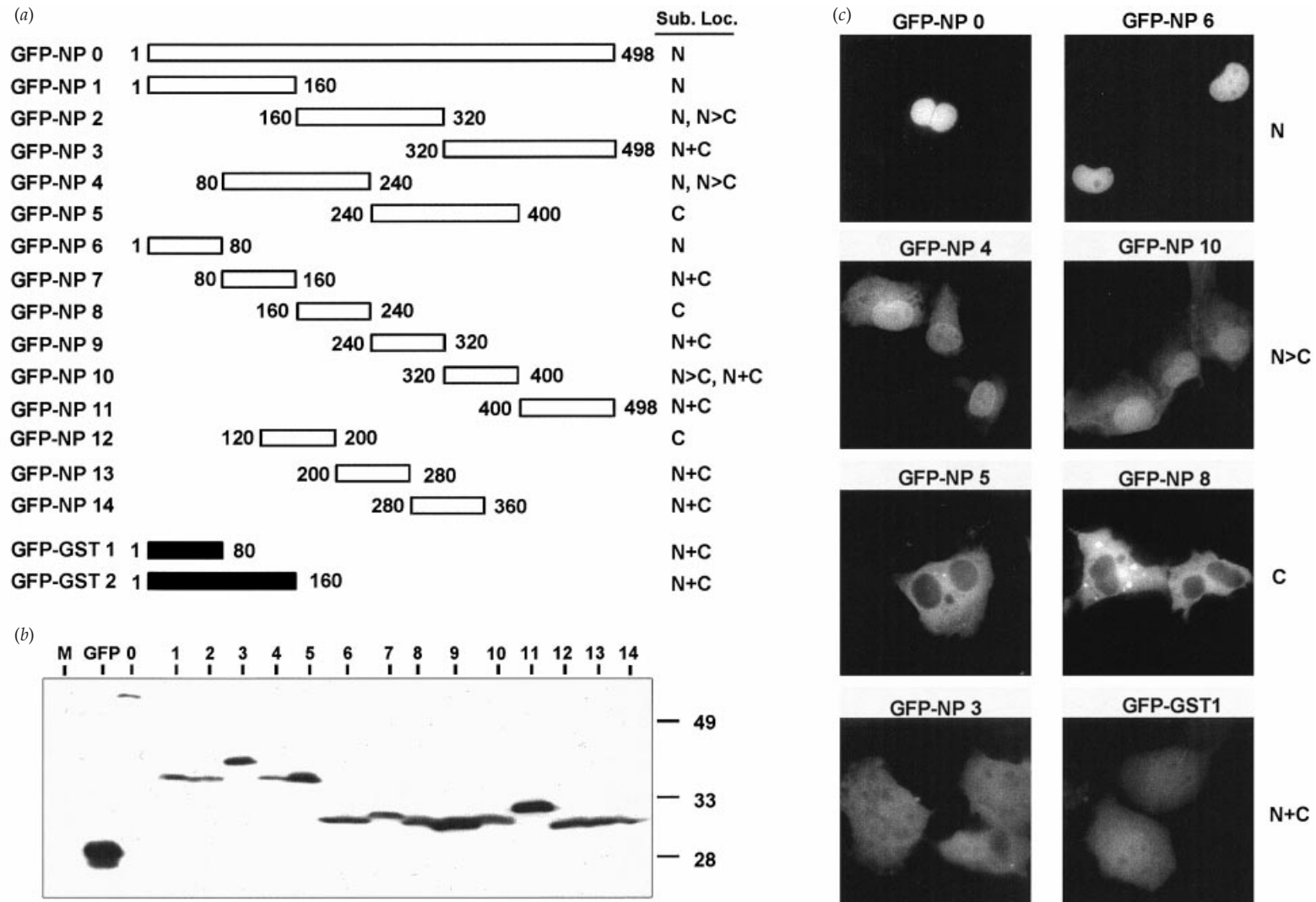


Fig. 2. Expression and subcellular distribution of GFP-NP fusion proteins. (a) Schematic representation of the GFP-fusion proteins. For each of the proteins, only the NP (open rectangles) or the GST (filled rectangles) fragments are shown. The subcellular localization (Sub. Loc.) of the chimeric proteins is indicated on the right. Mutants GFP-NP2, GFP-NP3, GFP-NP4, GFP-NP5 and GFP-NP8 showed granular fluorescence in some cells [as shown for protein GFP-NP8 in (c)]. (b) COS-1 cell extracts prepared from cells individually transfected with the plasmids indicated in (a) were analysed by Western blotting with an anti-GFP serum and enhanced chemiluminescence. Lanes M and GFP correspond to cell extracts from mock-transfected cells and from cells transfected with the plasmid expressing wild-type GFP, respectively. Numbers above the other lanes correspond to the GFP-NP derivatives (0–14) indicated in (a). The positions of molecular mass markers are indicated on the right in kDa. (c) Representative examples of the four subcellular localization patterns observed for the GFP-NP fusion proteins. COS-1 cells were transfected with the plasmid indicated, fixed 24 h later and visualized with a fluorescence microscope as indicated in Methods.

Firstly, five fusion proteins (GFP–NP1 to GFP–NP5) were prepared that spanned the whole length of NP and that contained the NP fragments at the end of the GFP gene (Fig. 2*a*). It was then demonstrated that the fusion proteins were of the predicted size and that they were efficiently expressed in transfected COS-1 cells (Fig. 2*b*). As summarized in Fig. 2*a*), four cellular staining patterns were observed: the three patterns described above (N, N > C and N + C) and a fourth pattern that corresponded to cells displaying exclusively cytoplasmic fluorescence (pattern C). From these results, it was considered that the NP contained at least two signals involved in nuclear accumulation, one located within the N-terminal 160 amino acids and the other one in the region (residues 80–320) spanned by proteins GFP–NP2 and GFP–NP4.

To delineate further the signals involved in NP subcellular localization, nine more recombinant GFP-fusion proteins (GFP–NP6 to GFP–NP14) were prepared (Fig. 2*a*). Eight of these proteins contained 80 amino acid-long NP inserts and one included the C-terminal 99 residues of NP. All recombinant proteins accumulated efficiently in transfected cells (Fig. 2*b*) and their subcellular distributions are summarized and illustrated in Fig. 2*a, c*.

From the results obtained with all the fusion proteins, it was concluded that the NP contains at least three karyophilic signals. One of them was mapped to the first 80 amino acids of the protein, since GFP–NP6 but not GFP–NP7 displayed the same nuclear (N) staining pattern as GFP–NP1. This signal probably corresponds to the NLS identified within the N-terminal 20 residues of NP (Wang *et al.*, 1997; Neumann *et al.*, 1997). The second of the signals involved in the nuclear phenotype of NP was that located between residues 80 and 320, which is the NP region covered by proteins GFP–NP2 and GFP–NP4. It has been impossible to narrow the location of this signal down since none of the five recombinant polypeptides that included short fragments of this NP region localized preferentially to the nucleus. It is, however, suggested that the karyophilic signal within this region would be located within the 80 amino acids (residues 160–240) shared by proteins GFP–NP2 and GFP–NP4, since Weber *et al.* (1998) showed the presence of an NIS in the NP region extending from residues 198 to 216. A third signal involved in NP nuclear localization was located between residues 320 and 400, since most of the cells (70%) expressing protein GFP–NP10 displayed a clear N C pattern. This NP region includes the signal identified in *Xenopus* oocytes (Davey *et al.*, 1985) and this report shows, for the first time, that this region includes a karyophilic signal that is also active in mammalian cells.

Does the NP contain an NES and/or a CRS?

Strikingly, it was observed that three fusion proteins (GFP–NP5, GFP–NP8 and GFP–NP12) were excluded from the nuclei of transfected cells (Fig. 2*a, c*), a result that strongly suggests the presence of an NES and/or CRS in the NP protein.

Considering the NP sequences included in these cytosolic fusion proteins, it is concluded that the NP contains at least two signals that drive NP cytoplasmic accumulation. One signal resides between residues 240 and 400 and the other one is tentatively assigned to the region extending from amino acid 160 to 200, which corresponds to the NP fragment shared by cytosolic proteins GFP–NP8 and GFP–NP12. Further experiments are required to determine whether these regions include an NES and/or a CRS. It is worth noting that the NP contains an actin-binding domain that may act functionally as a CRS (Digard *et al.*, 1999), since NP proteins with mutations that lower the affinity for actin display increased nuclear accumulation. Since the studies carried out by Digard *et al.* (1999) indicate that this domain is around residue 340 of the NP, it is tempting to speculate that the most C-terminal of the two NP signals that drive cytoplasmic accumulation may correspond to the domain involved in binding actin (Digard *et al.*, 1999).

Interestingly, according to the location of the NP signals involved in the subcellular localization of the protein, each of the signals that causes cytoplasmic accumulation is in close proximity to a karyophilic signal. It is thus tempting to speculate that these two types of signals may overlap, so that the same protein domain could confer bidirectional transport of the corresponding protein across the nuclear envelope, as has been reported for the ~ 40 amino acid domains M9, KNS and HNS present in hnRNP A1, hnRNP K and HuR, respectively (Siomi & Dreyfuss, 1995; Michael *et al.*, 1995, 1997; Fan & Steitz, 1998).

The close proximity of the cytoplasmic accumulation signals and the NISs probably explains in part the apparently contradictory results obtained with some of the GFP-fusion proteins. For example, the nuclear protein GFP–NP2 and the cytoplasmic protein GFP–NP8 would contain, according to the data mentioned above, both a karyophilic and a cytoplasmic accumulation domain. To explain the different subcellular localization of these two proteins, it is proposed that the karyophilic signal is properly folded and exposed in protein GFP–NP2 so that it dominates over the cytoplasmic accumulation signal (which may also be partially masked) whereas, because of conformational effects, the opposite would be true for protein GFP–NP8.

Amino acid substitutions at residue 3 of the NP alter the functionality of the N-terminal NLS

It was decided to investigate the possible role of phosphorylation of serine 3 in modulating nuclear translocation of NP. Thus, we prepared plasmids encoding full-length NP containing substitutions at position 3 that were supposed to mimic the unphosphorylated (mutation S3A) and the negatively charged, phosphorylated (mutations S3D and S3E) forms of the protein. The wild-type NP and the three serine-altered mutant proteins accumulated to similar levels in transfected cells (Fig. 3*a*) and displayed indistinguishable fluorescence patterns, which included cells showing exclusively

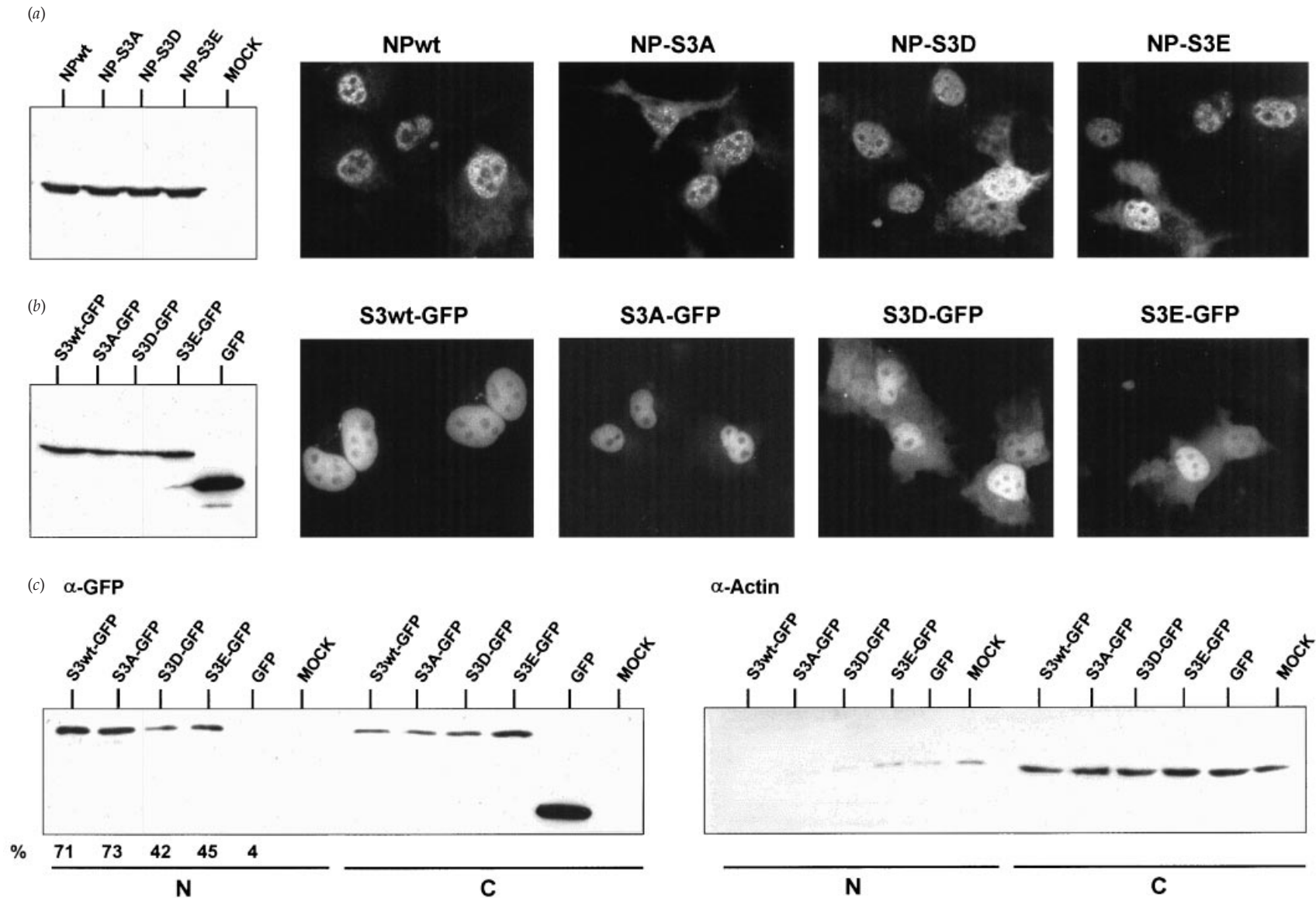


Fig. 3. Subcellular distribution of NP derivatives containing substitutions at residue 3 of NP. (a) COS-1 cells were infected with vTF7-3, transfected with the plasmids indicated (which encode full-length NP proteins) and, at 24 h post-infection, cell cultures were examined by Western blotting with an anti-NP serum (left panel) or by indirect immunofluorescence with an anti-NP monoclonal antibody. (b) COS-1 cells were transfected with the plasmids indicated (which encode GFP-fusion polypeptides) and, 24 h later, cell cultures were processed and examined by Western blotting (left panel) with an anti-GFP serum or by direct fluorescence. (c) COS-1 cells were transfected with the plasmids described in (b) and, 48 h after transfection, the cultures were harvested and fractionated into nuclear (N) and cytoplasmic (C) fractions. The extracts were then resolved by SDS-PAGE and transferred to Immobilon-P (Millipore). The membrane was sequentially developed with an anti-GFP serum (α -GFP; left panel) and an anti-actin monoclonal antibody (α -Actin; right panel) by using enhanced chemiluminescence. The percentage of NP protein that was found in the nuclear fraction for each of the GFP-NP proteins analysed is indicated (as determined by densitometric analysis).

nuclear staining and cells showing the N C pattern (Fig. 3a). The three mutant proteins were also tested for their functionality in a system in which expression of a synthetic influenza virus-like CAT RNA is achieved in COS-1 cells that express the NP and the three subunits of the viral polymerase from cloned cDNAs (Mena *et al.*, 1994). As previously demonstrated for the S3A mutant (Arrese & Portela, 1996), it was observed that the two proteins containing acidic substitutions were also functional in the system and yielded CAT activities ranging from 50 to 90% of that obtained with the wild-type NP (data not shown). These results indicated that neither the subcellular distribution of the full-length protein nor NP activity, as regarding its role during replication/transcription of the viral genome, are modified drastically by the mutations introduced at position 3.

Despite the above results, it is possible that substitutions at amino acid 3 of NP altered the activity of the N-terminal NLS but that these effects were not observed due to the additional karyophilic signals present in the full-length NP protein. Thus, GFP-fusion proteins were prepared that included the 80 N-terminal NP residues and Ser (S3wt-GFP), Ala (S3A-GFP), Asp (S3D-GFP) or Glu (S3E-GFP) at position 3. Unlike those described above, these fusion proteins contained GFP fused to the C terminus of the NP fragment. As can be seen in Fig. 3(b), the four proteins were expressed in transfected cells, although protein S3D-GFP accumulated to a lower level (70%) than that reached by the other fusion proteins. When the expressing cultures were observed by direct fluorescence, proteins S3wt-GFP and S3A-GFP were detected exclusively in the cell nucleus, whereas proteins containing acidic substitutions showed a unique and clear N C phenotype. These patterns were the same whether the cells were examined at 24 or 48 h post-transfection. To analyse the subcellular distribution of these proteins by a different approach, cell extracts from transfected cultures were fractionated into nuclear and cytoplasmic fractions following the procedure described by Avalos *et al.* (1997). Under these fractionation conditions, only trace amounts of cellular actin (< 10%) were found in the nuclear fraction whereas practically all the GFP (> 95%) was found in the cytosolic fraction (Fig. 3c). The GFP-fusion proteins containing Ser or Ala at position 3 were detected preferentially in the nuclear fraction (> 70%), whereas the fusion proteins containing Asp or Glu at residue 3 distributed equally between nucleus and cytoplasm (Fig. 3c). The results obtained with S3wt-GFP and S3A-GFP suggest that there was some leakage of the fusion proteins into the cytosolic fraction during the fractionation procedure, since these proteins were exclusively nuclear in intact cells. A similar situation applies to the GFP protein, since the protein displayed an N + C pattern when examined by direct fluorescence but it was detected almost exclusively in the cytoplasmic fraction after the fractionation procedure. Despite this, the results obtained from the fractionation approach were consistent with those observed in intact cells and indicated that GFP-fusion proteins containing

acidic substitutions at residue 3 showed a higher cytosolic/nuclear ratio than S3wt-GFP and S3A-GFP.

Considering that the charged acidic amino acids may mimic the phosphorylated serine at position 3, the results suggest that phosphorylation of this residue impairs the functionality of the N-terminal NLS of NP. The situation may be similar to that found for the simian virus 40 (SV40) T antigen (and other proteins), in which phosphorylation diminishes the rate of protein nuclear import, probably as a consequence of reducing the affinity of the NIS for its cellular receptor (Jans & Hübner, 1996; Rihs *et al.*, 1991). The diminished activity of the NP N-terminal NLS may be relevant during certain stages of the virus life-cycle. For example, late in infection when the RNPs have been exported to the cytoplasm, a diminished functionality of the N-terminal NLS would contribute to the prevention of re-entry of the RNPs into the nucleus.

Summary

The results presented here, together with the previous studies mentioned above, emphasize the complexity of the signals that regulate nucleo-cytoplasmic transport of NP. The picture emerging is that NP contains three signals involved in nuclear accumulation and two signals that lead to cytoplasmic accumulation. Considering that masking/unmasking of these signals during the infectious cycle follows a strict temporal order, we postulate that phosphorylation and interactions of the NP protein with other factors (RNA, actin and other viral proteins) play a critical role in regulating the ordered exposure of the NP subcellular signals.

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