

Complex structure of the nuclear translocation signal of influenza virus polymerase PA subunit

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The protein regions involved in the nuclear translocation of the influenza virus PA polymerase subunit have been identified by deletion analysis of the protein expressed from a recombinant simian virus 40. Two regions seem to play a role in the process: region I (amino acids 124 to 139) and region II (amino acids 186 to 247). A nucleoplasm-like nuclear translocation signal (NLS) has been identified in region I and an additional NLS appears to be present in region II, although no consensus targeting sequence can be detected. Alteration in any of the regions identified by short deletions completely prevented nuclear transport, whereas elimination of the regions I or II by large amino- or carboxy-terminal

deletions did not prevent nuclear targeting of the truncated protein. In addition, a point mutation at position 154 completely eliminated nuclear transport. A β -galactosidase fusion protein containing the 280 amino acid terminal region of the PA protein was partially transported to the nucleus and mutant PA proteins with a cytoplasmic phenotype could not be rescued by superinfection with influenza virus. These results suggest that the PA protein contains a functional nuclear targeting region which is required in influenza virus infection, with two independent NLSs, one in region I and the other in region II.

Introduction

Small proteins may passively diffuse into the nucleus, but transport of large ones is mediated by an energy-dependent, saturable system in which the nuclear pore plays a central role (for reviews, see García-Bustos *et al.*, 1991; Gerace, 1992; Goldfarb & Michaud, 1991; Silver, 1991). Proteins actively transported into the nucleus contain a specific sequence motif (NLS) which has been implicated in this process by deletion analysis, mutational inactivation or by its ability to target cytoplasmic proteins to the nucleus when molecularly fused to them. These signals, including the prototype NLSs in yeast MAT α 2 factor and simian virus 40 (SV40) T antigen (Hall *et al.*, 1984; Kalderon *et al.*, 1984), consist of a short stretch of basic amino acids, sometimes surrounding a hydrophobic core. Reiterated NLSs, of similar but not identical sequence, are present in some proteins, as in the polyoma virus T antigen (Richardson *et al.*, 1986) and the glucocorticoid receptor (Picard & Yamamoto, 1987). In the case of redundant or bipartite signals, mutations at one of them may impair but do not abolish the transport process, suggesting a modular structure of the NLS (Robbins *et al.*, 1991). Although the NLS is sufficient for a protein to be transported into the cell nucleus, it may

not be independent of the sequence context in which it is located because the NLS needs to be accessible for interaction with the transport machinery (Roberts *et al.*, 1987), and this accessibility can be modulated by intramolecular (Henkel *et al.*, 1992) or intermolecular masking (for review, see Schmitz *et al.*, 1991).

The genome of influenza virus consists of eight single-stranded RNA segments of negative polarity, each one in a ribonucleoprotein (RNP) complex with viral nucleoprotein (NP) and the three subunits of the polymerase (PB1, PB2 and PA) (for review, see Lamb, 1989). Transcription and replication of the viral RNA take place in the nucleus of the infected cells (Herz *et al.*, 1981). Each of the proteins in the RNP complex, as well as the non-structural (NS) proteins NS1 and NS2, accumulate in the nucleus of the cell, both in productive virus infection and when they are independently expressed from cloned versions of the genes (Greenspan *et al.*, 1985; Portela *et al.*, 1985 *a, b*; Smith *et al.*, 1987). The NLSs of some of these proteins have been identified: the NP has an acidic, non-canonical NLS (Davey *et al.*, 1985), whereas PB1, PB2 and NS1 proteins each contain a bipartite, basic NLS (Greenspan *et al.*, 1988; Mukai-gawa & Nayak, 1991; Nath & Nayak, 1990). The PA polymerase subunit is also a nuclear protein, as it

accumulates in the nuclear compartment when expressed from a vaccinia virus recombinant (Smith *et al.*, 1987) or from an SV40 recombinant (Nieto *et al.*, 1992). However, the transport to the nucleus of the PA subunit can be modulated by co-expressing PB1 or one of the NS proteins, suggesting a possible cooperation of other viral functions in the transport of the PA subunit (Nieto *et al.*, 1992).

In this report we have identified the protein regions responsible for the nuclear transport of the influenza virus PA polymerase subunit by deletion analysis as well as by the PA-mediated transport of a reporter protein. Our results suggest the existence of a complex organization including two NLSs, both required for the efficient transport of the full-length protein to the nucleus.

Methods

Biological materials. Plasmid pUPA76 (pUPA) (Nieto *et al.*, 1992) was a cDNA clone whose sequence had a Gly to Glu change at position 154 when compared to the published A/Victoria/3/75 PA gene sequence (de la Luna *et al.*, 1989). The PA gene cloned in plasmid pUPA was considered as wild-type (wt), in view of its biological activity in the chloramphenicol acetyltransferase expression system (de la Luna *et al.*, 1993). The structures of the intermediate vector pBSL-4, which contains the SV40 early region cloned into a Bluescript plasmid, and plasmid pSEPA, which has the PA cDNA cloned into the pBSL-4 vector, have been described (Nieto *et al.*, 1992). Plasmid pSV- β gal, which contains the β -galactosidase gene under the control of the SV40 early promoter, was purchased from Promega. All plasmids were maintained in *Escherichia coli* DH5 cells (Hanahan, 1983).

The COS-1 cell line (Gluzman, 1981) and the MDCK cell line were cultivated as described (Ortín *et al.*, 1980). Influenza virus strain A/PR/8/34 was grown in MDCK cells as described previously (Ortín *et al.*, 1980). Monoclonal antibodies (MAbs) specific for the PA protein were prepared by immunization of mice with the full-length protein expressed in *E. coli*. Their complete description will be presented elsewhere. Rabbit anti-RNP serum was prepared by immunization with purified viral RNP. Rabbit anti- β -galactosidase serum was obtained from J. G. Castaño.

DNA manipulations and cloning. DNA manipulations, including restriction enzyme digestions, restriction end-modifications and ligations were done according to standard procedures (Sambrook *et al.*, 1989). *E. coli* transformations were carried out as described (Hanahan, 1983). Standard DNA amplification reactions were performed as described (Innis & Gelfand, 1990).

Construction of mutants. A series of terminal deletion mutants (PA/C; PA/St; PA/Av; PA/Bg; PA/B; PA/S; PA/K; PA/D) and internal deletion mutants (PA Δ BS; PA Δ BA; PA Δ SB; PA Δ HS; PA Δ SA; PA Δ SH) (Fig. 1) were generated by digestion of recombinants pUPA or pSEPA (Nieto *et al.*, 1992) at restriction sites in the PA gene and the vector's polylinker. Their construction will be described in detail elsewhere.

Mutant PA Δ 3 was generated by PCR, by a modification of the procedure described by Jones & Howard (1990). Briefly, two separate amplification reactions were carried out on a template of pUPA DNA: the first one used as primers the M13 reverse primer (5' AACAGCTATGACCGTG 3') and oligonucleotide PA448d388

(5' TCTCAGATGTTACT 3'), the latter containing a deletion of nucleotides 395 to 441 in the PA gene sequence. The second amplification reaction was done with oligonucleotides PA388d448 (5' AGTAACATCTGAGA 3') and PA886.868 (5' AAAGCATCCATCAGAAGGAA 3'). The former primer had the same deletion as oligonucleotide PA448d388 but in the opposite polarity. Finally, a third amplification was performed using a mixture of the products of the previous amplifications as template and the oligonucleotides M13 reverse primer and PA886.868 as external primers. The final PCR product was digested with restriction nucleases *Sma*I and *Bst*BI and the relevant fragment was isolated from a preparative agarose gel and ligated to plasmid pSEPA digested with the same enzymes. The sequence of the cloned amplified region was checked by dideoxynucleotide sequencing. From each pSEPA mutant plasmid, SV40 recombinants were generated by transfection into COS-1 cells, as described (Nieto *et al.*, 1992). In addition, a spontaneous point mutant (PA154) was isolated in the cDNA cloning of the PA gene, which had a Glu to Gly change at sequence position 154.

Two different PA- β -galactosidase plasmids were constructed. Plasmid pSV- β Gal was digested with *Cla*I, blunt-ended by Klenow polymerase treatment and further digested with either *Hind*III or *Asp*718. The appropriate DNA fragment was isolated and ligated to the *Hind*III-*Stu*I (nucleotides 1 to 581) or *Asp*718-*Ava*II (nucleotides 1 to 861) PA DNA fragments obtained from pSEPA, respectively. The resulting plasmids, pA186- β Gal and pA280- β Gal, contained the genetic information for the first 186 or 280 amino acids of the PA protein, respectively, fused in-frame to a β -galactosidase polypeptide that lacked the amino-terminal 313 amino acids.

Immunofluorescence. Cultures of COS-1 cells were infected with the SVPA recombinant viruses at an m.o.i. of 1 to 5 p.f.u./cell or transfected with 5 μ g of β -galactosidase plasmids, using lipofectin (BRL) according to the manufacturer's recommendations. At 48 h post-infection (p.i.) or 60 h post-transfection (p.t.), the cells were fixed with methanol at -20°C and stored in PBS. Fixed cells were incubated with PA-specific MAbs (culture supernatant), β -galactosidase-specific serum (1:500 dilution), anti-RNP serum (1:500 dilution) or control antibodies, in PBS-2% BSA for 1 h at room temperature. After washing with PBS, cells were stained with a 1:500 dilution of Texas red-labelled donkey anti-rabbit immunoglobulin antibodies, a 1:200 dilution of Texas red-labelled sheep anti-mouse immunoglobulin antibodies and/or a 1:500 dilution of fluorescein-labelled donkey anti-rabbit immunoglobulin antibodies in PBS-2% BSA, for 1 h at room temperature. Finally, the preparations were washed with PBS, mounted in Mowiol (Aldrich) and photographed in a Zeiss fluorescence microscope. All the recombinants were tested by immunofluorescence in at least three different experiments.

Results

Subcellular distribution of wt and mutated forms of the PA polymerase subunit in SV40 recombinant-infected cells

When COS-1 cell cultures were infected with an SV40 recombinant encoding the PA subunit of the influenza virus polymerase, it was observed that the cells showed nuclear localization of the protein, as detected by indirect immunofluorescence analysis with PA-specific MAbs (Nieto *et al.*, 1992). Although the transport to the nucleus was not complete in all cells, this result indicated that the PA protein contains its own NLS.

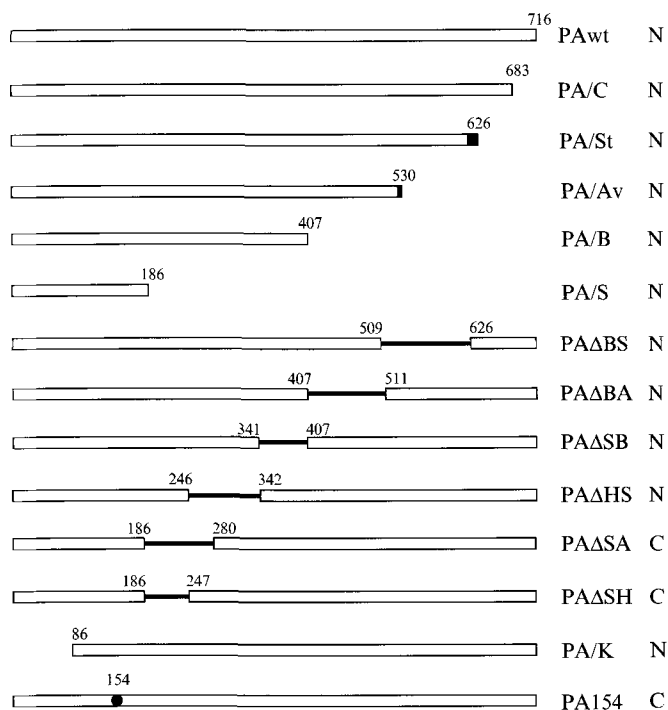


Fig. 1. Structure and the phenotypes of PA protein deletion mutants. Numbers indicate the amino acid positions delimiting the deletions produced. Letters to the right indicate whether the phenotype of each mutant is nuclear (N) or cytoplasmic (C) (see text for a detailed definition of each phenotype). In addition to the deletion mutants, the point mutant PA154, which has a Glu to Gly change at position 154, is included.

In order to localize the PA sequences responsible for its transport to the nucleus, a series of 14 deletion mutants was constructed (Fig. 1). It included mutants deleted at the amino or at the carboxy terminus, as well as mutants with internal deletions. Cultures of COS-1 cells were infected with either wt SVPA recombinant virus, each of the SVPA deletion mutants or the SVPA154 spontaneous point mutant described in Methods. The subcellular distribution of the PA protein expressed in these cells was studied by indirect immunofluorescence. In cells infected with wt SVPA recombinant virus, nuclear location of PA protein was detected in a variable proportion of the cells, ranging from 70 to 90 % in different experiments, but some cells showed either cytoplasmic or nuclear and cytoplasmic staining. This phenotype is regarded as nuclear inasmuch as the PA protein is located exclusively at the nucleus in at least some of the infected cells. The nuclear location of PA protein, but not cytosolic location, correlated with chromatin condensation and aberrant nuclear morphology, as seen by simultaneous Hoechst staining (Nieto *et al.*, 1992, and data not shown). Most of the deletion mutants showed an immunofluorescence pattern indistinguishable from that presented by the wt PA protein.

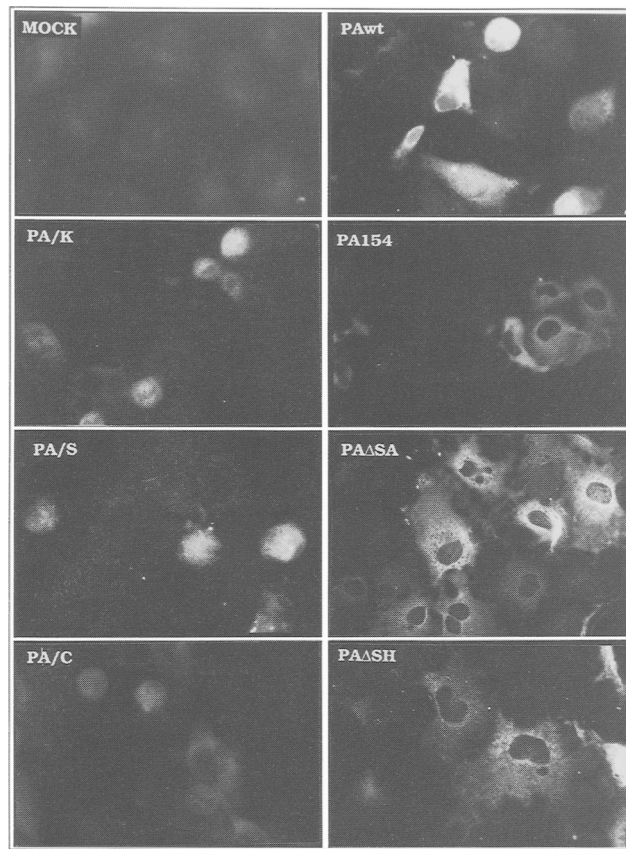


Fig. 2. Subcellular localization of PA protein deletion mutants. Cultures of COS-1 cells were either mock-infected or infected with wt or mutant SVPA recombinant viruses. At 48 h p.i. the cultures were fixed and processed for immunofluorescence using PA12 MAb, as described in Methods. The exposure times were identical for all samples. The detailed definitions of nuclear and cytoplasmic phenotypes are described in the text.

This phenotype is demonstrated in Fig. 2 by mutants SVPA/K, SVPA/S and SVPA/C. In contrast, deletion mutants SVPAΔSA and SVPAΔSH, as well as point mutant SVPA154, showed a characteristic pattern of exclusively cytoplasmic staining. The phenotypes of the deletion mutants are summarized in Fig. 1 and, taken together, indicate that (i) protein sequences included in the region of residues 186 to 274 are essential for the transport of the PA subunit to the nucleus and (ii) additional sequences relevant for the nuclear transport of the PA subunit might be located in the region of amino acids 1 to 186, most probably between positions 86 and 186, in view of the nuclear phenotype of mutants PA/K and PA/S. Passive diffusion of PA/S mutant protein into the nucleus is a possible alternative explanation.

In order to identify more precisely the sequences within the regions 86 to 186 and 187 to 247 that could be responsible for the transport of the PA protein to the nucleus, two additional mutants were constructed, PAΔ3

		Phenotype
124	139	
GVTRREVHIYYLEKANKIKSENTHIHIFSFTGEEMATKADYTLD		
Nucleoplasmin-like sequence		
123	140	
GVT.....SENTHIHIFSFTGEEMATKADYTLD		
		PAΔ3 C
155		
MATKADYTLD		
		PA/D N/C

Fig. 3. The sequence of wt PA protein in the region of bases 121 to 164 is shown. A sequence analogous to the nucleoplasmin NLS (positions 124 to 139) is framed. The sequences of deletion mutants PAΔ3 and PA/D and point mutant PA154 are included. The letters to the right indicate whether the phenotype of each mutant is nuclear (N), nucleocytoplasmic (N/C) or cytoplasmic (C).

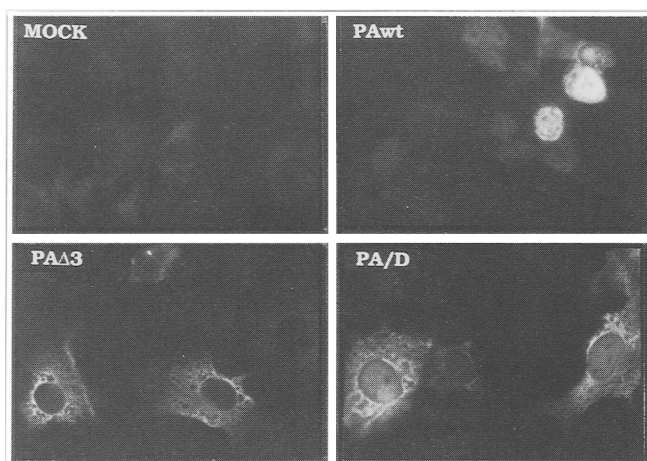


Fig. 4. Subcellular localization of PA protein mutants. Cultures of COS-1 cells were infected with the mutants described in Fig. 3, fixed and processed for immunofluorescence as in Methods and the legend to Fig. 2. The staining of cultures infected with PA/D recombinant virus was carried out with a mixture of PA-specific MABs. The exposure times were identical for all samples.

and PA/D (Fig. 3). Mutant PAΔ3 lacked amino acids 124 to 139 in the PA protein, a sequence homologous to the consensus nucleoplasmin-like NLS (Dingwall & Laskey, 1991; Robbins *et al.*, 1991). The deletion of this sequence led to a mutant PA protein with a cytoplasmic localization, since COS-1 cells infected with mutant SVPAΔ3 showed exclusively cytoplasmic staining when analysed by immunofluorescence (Fig. 4). This result suggests that the nucleoplasmin-like sequence, present in the region of amino acids 124 to 139 of the PA protein, constitutes a functional NLS.

Next, we investigated whether the nucleoplasmin-like NLS identified between positions 124 and 139 was the only nuclear targeting sequence present in PA protein. To this end, mutant PA/D was constructed, which lacks the amino-terminal 154 amino acids in the PA sequence

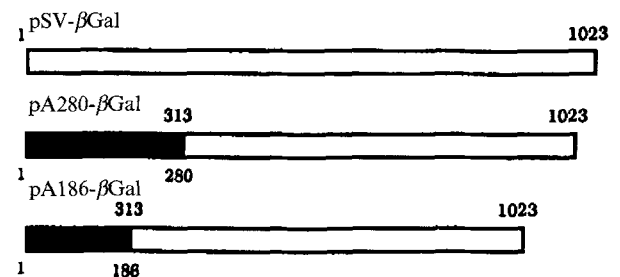
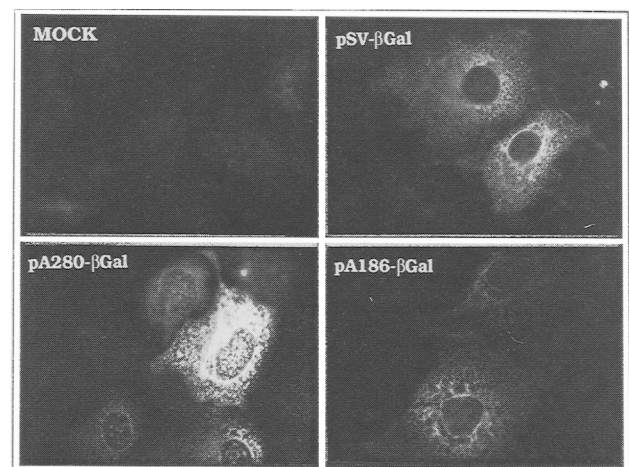


Fig. 5. PA-mediated transport of β -galactosidase. Cultures of COS-1 cells were transfected with either pSV- β Gal, pA186- β Gal, pA280- β Gal, or mock-transfected. At 48 h p.t., the cells were fixed and stained with anti- β -galactosidase serum and Texas red-tagged donkey anti-rabbit antibodies, as described in Methods. The diagram at the bottom of the figure indicates the structure of the proteins expressed. Open bars denote β -galactosidase sequences and filled bars indicate PA sequences. The numbers above and below the bars show the sequence positions of β -galactosidase and PA protein residues, respectively.

bearing this NLS. This mutant protein showed both cytoplasmic and nuclear localization when analysed in COS-1 cells infected with SVPA/D recombinant (Fig. 4). This result indicates that an additional NLS exists downstream from position 154, probably within the

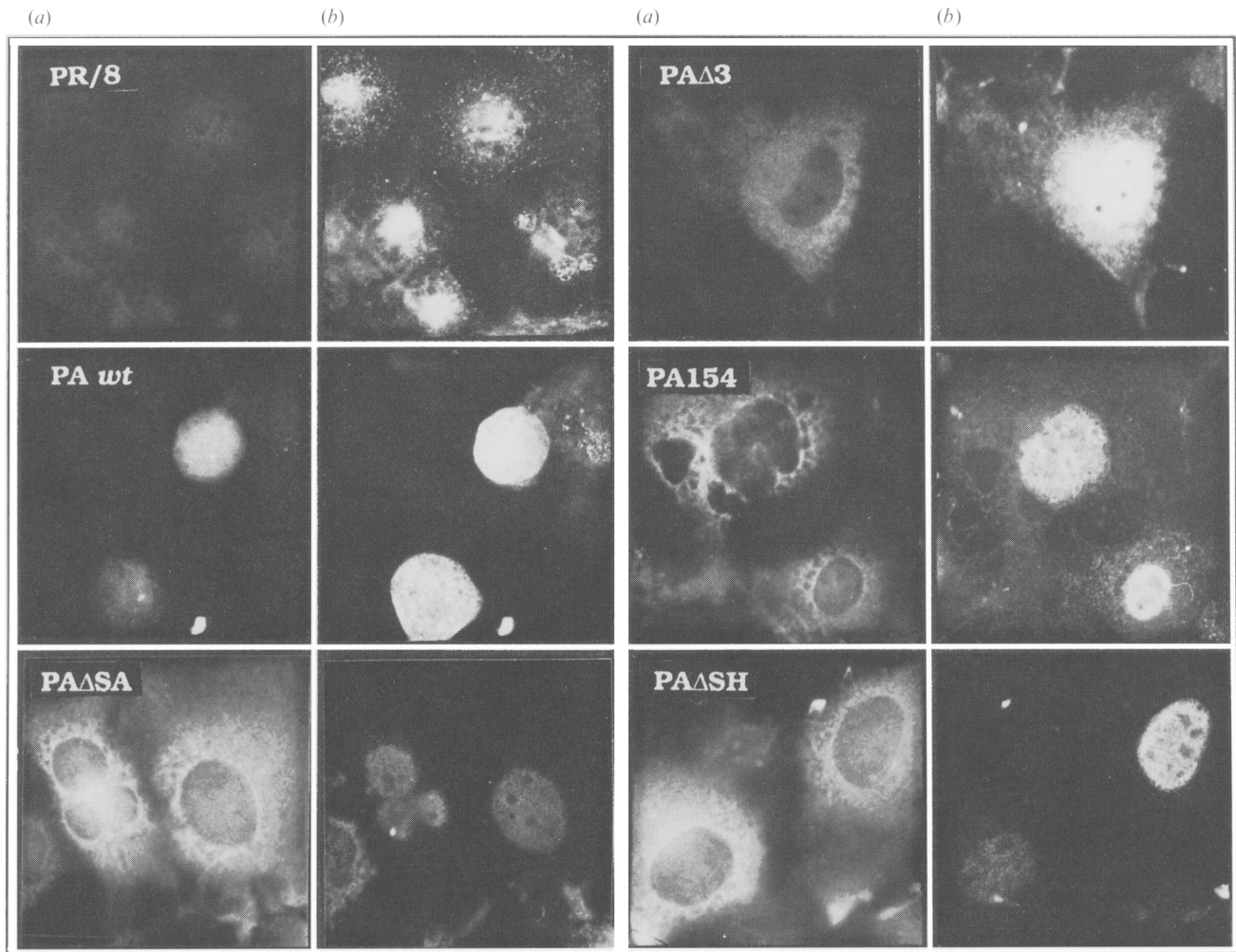


Fig. 6. Localization of mutant PA proteins in cells superinfected with influenza virus. Cultures of COS-1 cells were infected with wt SVPA or mutants SVPA Δ SA, SVPA Δ 3, SVPA154 or SVPA Δ SH. At 48 h p.i., the cells were superinfected with the A/PR/8/34 strain of influenza virus at an m.o.i. of 10 p.f.u. cell and were fixed 8 h later. The cultures were processed for double immunofluorescence with PA10 MAb (panels *a*) and anti-RNP serum (panels *b*), as described in Methods. Panels labelled PR/8 correspond to cultures infected with influenza virus but not infected with SV40 recombinant virus. The same areas of each preparation are shown as detected by staining with each of the antibodies.

region of amino acids 186 to 247 previously mapped. The comparison of the phenotypes of mutant PA/D (Fig. 4) and mutant PA/K (Fig. 2) suggests that this additional NLS is less efficient than that located at positions 124 to 139.

PA protein sequences can direct the transport of a reporter protein to the nucleus

To address the question of whether the regions mapped as essential for PA protein translocation were capable of targeting a cytosolic reporter protein to the nucleus, two chimeric plasmids were constructed in which the β -galactosidase gene was fused to two different 5'-terminal regions of the PA gene encoding either the first 186

or 280 amino acids of the polymerase subunit (plasmids pA186- β Gal and pA280- β Gal). When cultures of COS-1 cells were transfected with the original pSV- β Gal plasmid, an exclusively cytosolic localization of β -galactosidase was observed. Similar results were obtained with fusion protein containing the amino-terminal 186 amino acids of PA protein (plasmid pA186- β Gal; Fig. 5). In contrast, a distribution in both cytoplasm and nucleus was obtained with the PA- β -galactosidase fusion protein expressed from recombinant pA280- β Gal (Fig. 5). These results indicate that the amino-terminal 280 amino acids of the PA polymerase subunit are not only required for its nuclear transport, but are sufficient to direct the translocation, at least in part, of a reporter protein to the nucleus.

Influenza virus infection cannot rescue PA mutant proteins

In order to check the physiological role of the PA protein NLSs during the standard influenza virus infection, wt PA protein and mutant proteins PA Δ 3, PA Δ 5A, PA Δ SH and PA154 were expressed in COS-1 cells by infection with the corresponding SV40 recombinants. At 48 h p.i., the cells were superinfected with the A/PR/8/34 strain of influenza virus and the cultures were fixed 8 h later. The intracellular location of the A/Victoria/3/75 PA protein expressed from SV40 recombinants was investigated by immunofluorescence using PA10 MAb, which recognizes the PA antigen from A/PR/8 virus poorly, whereas influenza virus infection was followed by immunofluorescence with anti-RNP serum. The results are shown in Fig. 6. For every mutant studied, the location of PA protein was indistinguishable whether expressed as a single influenza virus protein or synthesized in the environment of an influenza virus infection (compare Fig. 2, 4 and 6). The phenotype of mutant SVPA/D could not be studied because the PA/D mutant protein was not recognized by the PA10 MAb. Since the half-life of PA protein expressed from a SV40 recombinant is approximately 2 h (de la Luna, 1989), most of the protein detected by immunofluorescence was synthesized after influenza virus infection and yet was not cotransported to the nucleus by other viral proteins.

Discussion

Several types of NLS have been described, but in its most general form, it is composed of two clusters, the first one having two basic amino acids and the second formed by a set of three basic amino acids out of five residues, separated by a spacer of variable length in the range of 10 residues (Robbins *et al.*, 1991). This bipartite signal, known as the nucleoplasmin-like NLS, is present in more than 50% of the known nuclear proteins, including steroid hormone receptors, transcription factors, polymerases and topoisomerases, histone binding proteins such as nucleoplasmin and N1, and viral proteins such as influenza virus polymerase subunits PB1 and PB2 (Dingwall & Laskey, 1991). Small changes in the distance between the two basic domains in the bipartite NLS can be tolerated, but there is growing evidence for the requirement of a correct location of these two elements relative to each other, in order to achieve efficient nuclear transport.

The results presented in this paper suggest a complex structure for the signal responsible for nuclear transport of the polymerase PA subunit. The search for protein sequences involved in the targeting process has revealed the existence of two different regions of the protein,

Nucleoplasmin	R K P A A T K K A G Q A . K K K K
N1	K R K T E E E S P L R D K D A K K
PA protein (type A)	124 R R . E V H I Y Y L E K A N K I K 139
PA protein (type B)	125 K G . . L A D D Y F W K K K E K L 139
PA protein (type C)	109 R K . . A D D S Y F Q S K F G K L 123

Fig. 7. The sequence of the nucleoplasmin-like NLS of type A influenza virus PA subunits is shown in relation to those of nucleoplasmin and N1 proteins (Dingwall & Laskey, 1991) and to the homologous regions in the sequences of influenza virus type B and C PA subunits. The residues relevant for nuclear targeting activity in nucleoplasmin and N1 proteins are framed.

regions I and II (Fig. 1), both of which contribute to the wt nuclear phenotype. Region I comprises amino acids 124 to 139, where a nucleoplasmin-like NLS has been identified. This putative NLS is completely conserved among all type A influenza virus PA proteins sequenced to date. A similar nucleoplasmin-like motif can also be identified in the corresponding sequence regions of the PA subunits of type B and type C viruses, although the spacer is somewhat shorter (Fig. 7). Region II is defined by deletion Δ SH and includes amino acids 186 to 247. Finally, position 154 appears to be very important for nuclear transport of the PA protein, probably by allowing a proper relative position of regions I and II.

Removal of regions I or II, or mutation of Glu to Gly at position 154, brings about a dramatic change in the subcellular distribution of the protein, since each of these mutant proteins is unable to accumulate in the nucleus (Fig. 2 and 4). In contrast, mutants SVPA/S and SVPA/D (in which large deletions have removed region II or region I respectively) express proteins that localize to the nucleus, although less efficiently in the latter mutant (Fig. 2 and 4). These results support the notion that PA protein contains at least two NLSs, one tentatively identified as a nucleoplasmin-like signal in region I and the other located carboxy-terminally of position 155, most probably within region II. The nature of the NLS in region II remains unclear, however, since no consensus NLS-like motif could be detected in this sequence. Furthermore, only the complete amino-terminal area of the PA protein, including regions I and II (amino acids 1 to 280), but not that comprising only region I (amino acids 1 to 186), was able to direct the translocation of β -galactosidase to the nucleus (Fig. 5). The fact that the transport was not complete is not surprising, in view of the size of the reporter protein and its capacity to oligomerize.

The presence of true NLSs in the PA polymerase subunit does not imply their ability to function in the course of influenza virus infection. Indeed, evidence for

a role of the PB1 subunit and an NS protein in the complete PA nuclear localization has been presented previously (Nieto *et al.*, 1992). Thus, simple cotransport of PA protein by binding to other viral nuclear proteins could be inferred. If that were the case, the cytoplasmic phenotype of the mutant PA proteins should be rescued by superinfection with influenza virus. However, none of those mutant proteins showed a nuclear phenotype when expressed in the context of an influenza virus-infected cell (Fig. 6). Therefore, we conclude that PA contains sequences responsible for nuclear targeting during influenza virus infection, although their action might be modulated by other viral proteins (see below).

The body of results obtained can be interpreted in the following way: in native PA protein, regions I and II must have a precise orientation relative to each other to permit a suitable interaction with the nuclear location signal receptor(s). Region I is postulated to undergo a stronger interaction with NLS receptors than region II. Removal of region II or region I (mutants PA/S and PA/D) would still allow an interaction with the nuclear receptor, and hence nuclear translocation could take place, as far as this is the consequence of large deletions in the protein. However, alteration of these regions by small deletions in the PA protein (mutants PA Δ SH and PA Δ 3) may result in incorrect folding of the mutant protein, thereby masking the still-present NLS. We thus speculate that regions I and II belong to separate domains in the protein. Point mutations at position 154 also appear to alter the structure of the protein, eliminating the interaction with NLS receptor(s). This point mutant may identify a possible 'hinge' region in the molecule, although the possibility that they would impair a non-canonical NLS cannot be eliminated at present. It is also possible that the sequences around position 154 together with region II might form a long, non-canonical NLS analogous to the one described for GAL-4 transcription factor (Silver *et al.*, 1988).

The polymerase PA subunit complexes to PB1 and PB2 subunits to carry out the transcription and replication of viral RNA. However, the newly synthesized PA protein accumulates in the cytoplasm at times in the infection cycle at which PB1 and PB2 subunits localize to the nucleus (Akkina *et al.*, 1987; Nieto *et al.*, 1992). Since the PA protein is able to accumulate in the nucleus when expressed in the absence of other influenza virus proteins (Nieto *et al.*, 1992; Smith *et al.*, 1987), its delayed transport is suggestive of cytoplasmic retention of individually expressed PA protein. The observation that influenza virus superinfection allows for a more complete nuclear localization of a wt PA subunit expressed from a SV40 recombinant virus (Nieto *et al.*, 1992) indicates that other virus-specific genes are involved in the process. In this regard, the complete nuclear accumulation of PA

protein when co-expressed with either PB1 or one of the viral NS proteins (Nieto *et al.*, 1992) suggests that nuclear retention might be enhanced by complex formation with other polymerase subunits or by virus-induced post-translational modification of the PA protein.

In conclusion, nuclear accumulation of the influenza virus polymerase PA subunit is mediated by an unusual protein structure in which at least two NLSs, apparently located in different domains of the protein, seem to participate. This structural complexity might be related to the PA protein's delayed translocation to the nucleus in the influenza virus infectious cycle, and to the involvement of other viral gene products in the transport process.

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