Virus Research, 24 (1992) 65–75

VIRUS 00769

Nuclear transport of influenza virus polymerase PA protein *

Amelia Nieto [2], Susana de la Luna [1,2], Juan Bárcena [3], Agustín Portela [3], Juan Valcárcel [1,2,#], José A. Melero [3] and Juan Ortín [1,2]

[1] *Centro Nacional de Biotecnología (CSIC), Universidad Autónoma, Cantoblanco, Madrid, Spain,* [2] *Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Cantoblanco, Madrid, Spain and* [3] *Servicio de Biología Molecular, Centro Nacional de Microbiología, Instituto Carlos III, Majadahonda, 28220 Madrid, Spain*

(Received 21 October 1991; revision received and accepted 13 January 1992)

Summary

The subcellular distribution of influenza polymerase PA subunit has been studied using a SV40-recombinant virus (SVPA76), which allows the expression and accumulation of this protein in COS-1 cells. In contrast to the complete nuclear localization observed for the PA subunit several hours after influenza virus infection, when COS-1 cells were infected with the SVPA76 recombinant, the PA protein accumulated either in the nucleus, in the cytoplasm or was distributed throughout the cell. When cells were infected with the SVPA76 recombinant and superinfected with influenza virus, a clear increase in the proportion of cells showing nuclear localization of the PA protein was observed, suggesting that some *trans*-factor may be required to allow complete nuclear accumulation of the protein. Double infections using SVPA76 recombinant and either SVPB1 or SVNS recombinant viruses showed a complete correlation between expression of polymerase PB1 subunit or NS1 protein and nuclear localization of polymerase PA subunit. However, no such correlation was observed in the double infections of SVPA76 and SVNP recombinants. These results suggest that polymerase PB1

Correspondence to: J. Ortín, Centro Nacional de Biotecnología (CSIC), Cantoblanco, 28049 Madrid, Spain.

* Presented in part at the Eighth International Conference on Negative Strand Viruses, Charleston, SC, U.S.A., 15–20 September 1991.

Present address: Program in Molecular Medicine, University of Massachussets Medical Center, 373 Plantation street Worcester, MA 01605, U.S.A.

subunit and the non-structural proteins could be involved in the nuclear targeting or nuclear retention of influenza polymerase PA protein.

Influenza virus polymerase; PA subunit

Introduction

Influenza virus genome consists of eight single stranded RNA segments of negative polarity that encode ten proteins (Lamb, 1989). Their transcription and replication is carried out by complexes that include at least the three polymerase subunits (PB1, PB2 and PA) and the nucleoprotein (NP) (Braam et al., 1983; Horisberger, 1980; Huang et al., 1990). Both transcription and replication take place in the nucleus of the infected cells (Herz et al., 1981; Jackson et al., 1982; Lopez Turiso et al., 1990; Shapiro and Krug, 1988), where soluble complexes of the three polymerase subunits can be detected (Detjen et al., 1987).

Influenza virus nucleoprotein is transported into the nucleus when expressed from cloned DNA (Lin and Lai, 1983; Portela et al., 1985) and the sequences responsible for the nuclear accumulation have been identified (Davey et al., 1985). Likewise, all three polymerase subunits move into the nucleus during influenza virus infection (Akkina et al., 1987; Jones et al., 1986), as well as when expressed from vaccinia virus recombinants (Smith et al., 1987), but their kinetics of nuclear accumulation may not be identical (Akkina et al., 1987). The sequences responsible for the nuclear transport of PB1 and PB2 subunits have been shown to consist of two separate elements (Mukaigawa and Nayak, 1991; Nath and Nayak, 1990), as is also the case for NS1 protein (Greenspan et al., 1988).

In this report we have analyzed the localization of the PA subunit of influenza virus polymerase when expressed from a SV40 recombinant. Unexpectedly, a large proportion of the cells expressing PA protein showed a cytoplasmic localization. However, coexpression of PB1 or non-structural proteins, but not NP, correlated to a complete nuclear localization of PA protein.

Materials and Methods

Biological materials

Plasmid pBSL-4, used as intermediate vector, contains the SV40 genome late region, from the *Hin*dIII site (5171) to the *Bcl*I site (2770), cloned between the *Hin*dIII and *Bam*HI sites of Bluescript plasmid (Stratagene). All plasmids were maintained in *Escherichia coli* DH-5.

The MDCK and CV-1 cell lines were obtained from the American Type Culture Collection. The COS-1 cell line (Gluzman, 1981) was obtained from Y. Gluzman. Influenza viruses A/Victoria/3/75 (VIC) and A/PR/8/34 (PR8) were grown in

MDCK cells as reported previously (Ortín et al., 1980). Recombinant vaccinia virus VA-C expressing A/PR/8/34 PA protein (Smith et al., 1987) was obtained from G. Smith.

Monoclonal antibodies specific for the PA subunit were prepared using as antigen the full-length protein expressed in *E. coli*. Their complete description will be presented elsewhere (Bárcena et al., in preparation). Rabbit anti-NS1 sera were provided by P. Palese and J. Young. Rabbit anti-NP serum was prepared by immunization with purified viral RNPs. Peptide MPB1/1 (positions 70–81 of the VIC virus PB1 sequence; de la Luna et al., 1989) was synthesized on a lysine core as described (Tam, 1988), using the FMOC chemistry. Anti-peptide serum was prepared by intradermal immunization of rabbits with purified peptide mixed with CFA, followed at monthly intervals by two intramuscular injections of IFA-peptide mixture.

Molecular cloning and gene expression

DNA manipulations including restriction enzyme digestions, DNA ligations and *E. coli* transformations were done by standard procedures (Sambrook et al., 1989).

SV40 recombinant viruses were obtained by transfection of the corresponding SVX DNAs into COS-1 cells using the DEAE-dextran method (Lai and Nathans, 1974). Culture supernatants of the transfected cells were used to produce high titre virus stocks by infection of COS-1 cells.

Protein labelling was carried out at 60 hpi or 5 hpi in cells infected with SV40 recombinants or influenza virus, respectively. Cell monolayers were washed and incubated for 1 h with methionine-free DMEM medium. [^{35}S]Met was then added and incubation was continued for 1 h. After washing in PBS, total cell extracts were prepared in loading buffer and analyzed by polyacrylamide gel electrophoresis as described (Studier, 1972).

Immunofluorescence

Influenza virus infections were done at a m.o.i. of 10 pfu per cell either in MDCK or COS-1 cells; CV-1 cells were infected with VA-C virus at a m.o.i. of 30 pfu per cell; SV40 recombinant virus infections were done in COS-1 cells using a m.o.i. of 1–5 pfu per cell. Monolayers of mock infected or virus infected cells were fixed at different times after infection with methanol at −20°C for 20 min and stored in phosphate buffer saline at 4°C until use. Fixed cells were incubated with PA-specific monoclonal antibodies (culture supernatants) and, in double staining experiments, with either rabbit anti-MPB1/1 peptide serum (dilution 1:1000), rabbit anti-NS1 protein serum (dilution 1:200) or rabbit anti-NP serum (dilution 1:200), for 1 h at room temperature. The cells were then washed with PBS and incubated with Texas red-labelled sheep anti-mouse immunoglobulin antibodies (dilution 1:200). In double staining experiments, fluorescein-conjugated donkey anti-rabbit immunoglobulin antibodies and the nuclear Hoechst dye (0.5 μg/ml) were also used. Dilutions of sera were done in PBS-2% BSA. After further

washing in PBS, preparations were mounted in Mowiol and photographed in a Zeiss fluorescence microscope. Pictures of each preparation were taken with identical exposure times.

Results and Discussion

Expression of polymerase subunits in COS-1 cells

The cDNAs corresponding to VIC influenza virus RNA segments 1, 2, 3 and 5 were synthesized and cloned into pUC18 plasmid as described previously (de la Luna et al., 1989) The appropriate inserts were subcloned into the pBSL intermediate vector to obtain the pSEX-plasmid series. After removing the bacteria-derived sequences from these recombinants by *Xba*I digestion (Fig. 1), the SV40 recombinant DNAs were circularized and transfected into COS-1 cells. SV40

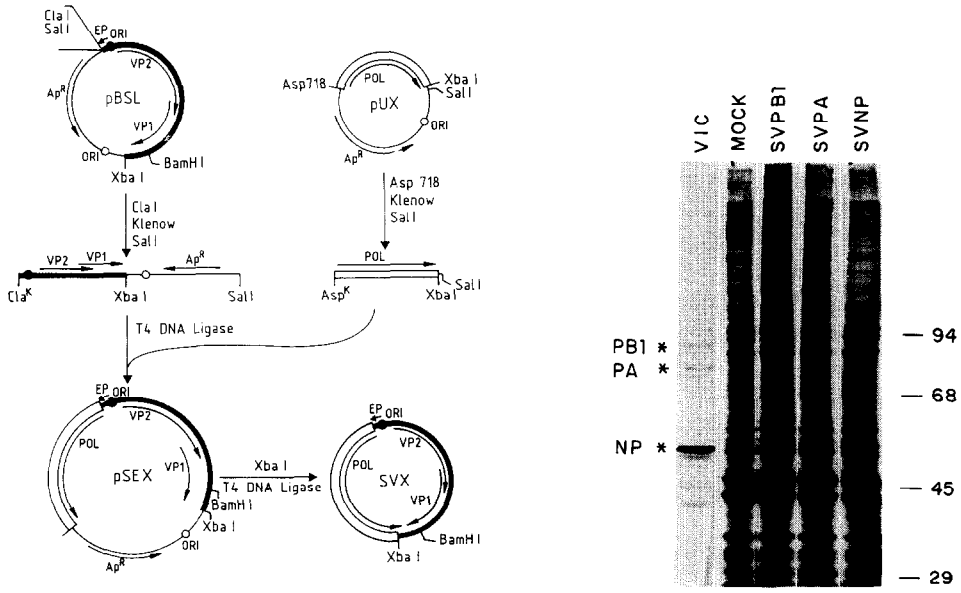

Fig. 1. Expression of influenza virus genes in COS-1 cells. Plasmids of the pSEX serie were constructed by transferring segment 1, 2, 3 and 5 cDNAs from the corresponding pUC18 recombinants (pUX serie), to plasmid pBSL, Bluescript-derived DNA was climinated by *Xba*I digestion. After recircularization, the SVX DNAs were transfected into COS-1 cells to amplify the SV40 recombinants. Mock-infected, influenza virus-infected or SV40 recombinant-infected COS-1 cells were labelled with [^{35}S]methionine, total cell extracts were prepared and analyzed by polyacrylamide gel electrophoresis as described under Materials and Methods. Left panel: (black bar), SV40 DNA; (line) Bluescript DNA; (white bar), Influenza virus cDNA. Right panel: MOCK, VIC, SVPB1, SVPA and SVNP indicate the electrophoretic patterns obtained with extracts from uninfected cells or cells infected by influenza virus (Victoria strain), SVPB1, SVPA, and SVNP recombinant viruses, respectively. Numbers to the right (in kDa) indicate the position of molecular weight markers.

pseudovirus stocks were collected and amplified by serial infections in COS-1 cells. Segment 8 was cloned into vector pBSV9 by oriented cDNA synthesis and circularization, as described (Portela et al., 1985).

The expression of the cloned genes was studied by pulse labelling SV40 recombinant-virus infected cells. The electrophoretic analyses of the labelled protein extracts obtained are shown in Fig. 1 and indicate the presence of bands with apparent molecular weights identical to those obtained in COS-1 cells infected with VIC virus. The identification of the PB1, PA and NP proteins expressed as bona fide influenza virus gene products was confirmed by their immunologic detection with specific antibodies (see below).

Subcellular distribution of polymerase PA subunit in influenza or SV40-recombinant virus infected cells

Cultures of COS-1 cells were infected with VIC virus or with SVPA76 recombinant. At several times after infection the cells were fixed and processed for indirect immunofluorescence using PA subunit-specific monoclonal antibodies. The results obtained are presented in Figs. 2 and 3. Early after influenza virus infection PA protein could be detected in the cytoplasm of the cells, when other nuclear influenza virus proteins as PB1, NP or NS1 were already localized in the nuclei (Fig. 2). This observation is in agreement with previous results (Akkina et al., 1987) and suggests that the transport of the PA subunit to the nucleus may be regulated during infection. Six hours post-infection, the PA protein accumulated in the nucleus and the immunofluorescence pattern indicates that, even at 10 hpi, it did not leave this cellular compartment. This behaviour contrasts with that observed for other nuclear proteins like NP and NS1 that appeared distributed all over the cell at those very late times post-infection.

When PA protein was expressed from the SVPA76 recombinant, some of the cells showed nuclear localization, while in others the PA subunit accumulated in the cytoplasm or was distributed throughout the cell (Fig. 3). The percentage of cells with cytoplasmic PA protein localization ranged between 20 and 60% in different infections. These data suggest that PA protein contains a nuclear transport signal, but when expressed as a single influenza virus specific protein, its accumulation in the nucleus is inefficient. The differences in the distribution patterns observed in influenza virus and SVPA76 recombinant infected cells suggest that, for an efficient accumulation of PA protein in the nucleus to occur, some other viral function(s) are required.

The results obtained with the SVPA76 recombinant are in contradistinction to those published earlier (Smith et al., 1987), using a vaccinia-PA (VA-C) recombinant. In fact, using VA-C recombinant, we have confirmed that the PA subunit could be found preferentially in the nuclei of the infected cells, although cytoplasmic fluorescence was also apparent. The same pattern of subcellular distribution could be observed with PA76 clone when expressed from a pGEM vector using the vaccinia virus-T7 expression system (Fuerst et al., 1987) (data not shown). This would suggest that the vaccinia expression system acts as a helper for the PA

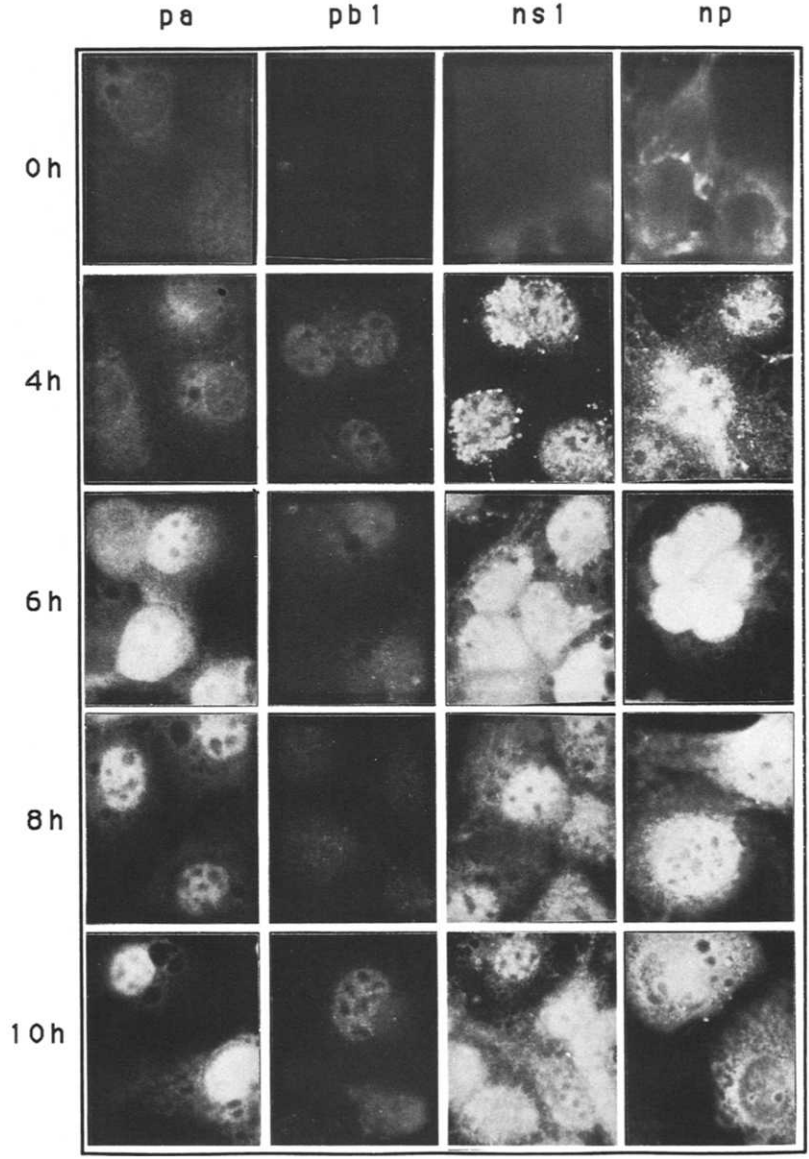

Fig. 2. Localization by indirect immunofluorescence of influenza virus-specific proteins in COS-1 infected cells. Cells were fixed at various times post-infection and stained with either PA-specific monoclonal antibodies (pa), anti-MPB1/1 peptide serum (pb1), anti-NS1 protein serum (ns1) or anti-NP serum (np) as described under Materials and Methods. Numbers to the left indicate the time after influenza virus infection in hours.

protein nuclear accumulation. The alternative possibility, i.e., that the SV40 expression system would be defective in nuclear transport is unlikely, since PB1, NP and NS1 proteins accumulate into the nucleus when expressed from the

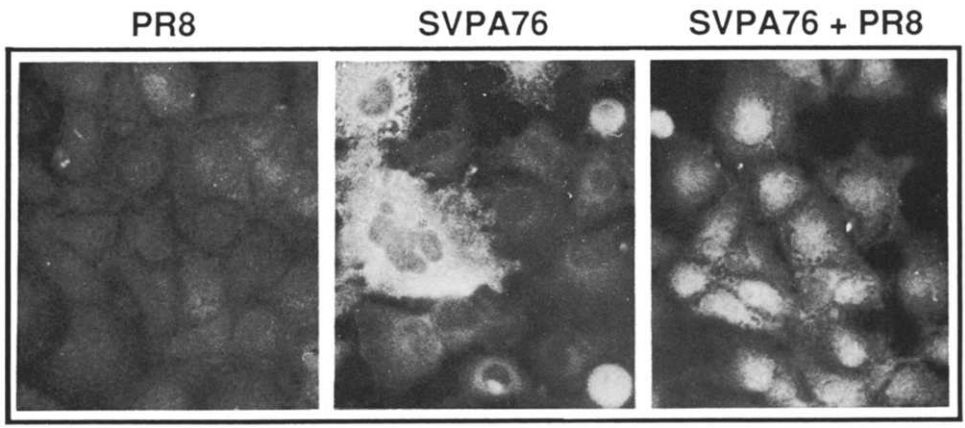

Fig. 3. Localization of PA protein in cells infected with SVPA76 recombinant: effect of influenza virus superinfection. COS-1 cell cultures were infected with the PR8 strain of influenza virus for 12 h (PR8) or with SVPA76 recombinant for 48 h (SVPA76). Some of the SVPA76 recombinant-infected cultures were then superinfected with the PR8 strain of influenza virus for 12 more hours (SVPA76+PR8). After these incubation times the cells were fixed and the PA protein was localized by indirect immunofluorescence using a PA-specific monoclonal antibody that poorly recognizes the PA protein of PR8 strain, as described under Materials and Methods.

corresponding SV40 recombinants (see below). The mechanisms responsible for the differences observed when the PA protein was expressed by either a SV40 or a vaccinia virus system are not clear, but the strong cellular shutoff induced by vaccinia virus may be relevant in this context. Thus, the activity of nuclear proteases may decrease as a consequence of this shut-off and hence the concentration of nuclear PA protein may be higher than in SV40 recombinant infected cells.

It could be argued that the molecular clone that we have expressed in SVPA76 recombinant is a fortuitous mutant, defective in nuclear transport. However, this is unlikely since two independent molecular clones (PA76 and PA105) had identical nucleotide sequence. Moreover, the PA protein expressed from SVPA76 recombinant was fully functional, since this recombinant was able to induce the synthesis of CAT enzyme from a PB2-CAT chimeric vRNA when it was transfected into cells coinfected with the corresponding PB1, PB2 and NP SV40 recombinants (data not shown).

Effect of influenza virus infection on the localization of the PA subunit expressed from the SVPA76 recombinant

In order to study if any viral *trans* factors could be responsible for the efficient nuclear localization of the PA subunit observed in influenza virus infected cells, COS-1 cells were infected with the SVPA76 recombinant and 48 h postinfection the cells were superinfected with the PR8 strain of influenza virus. Twelve hours thereafter, the cultures were fixed and the localization of the VIC virus PA subunit was carried out by immunofluorescence using a monoclonal antibody which poorly

recognized the PR8 strain PA protein. The results are shown in Fig. 3. In the cells infected with PR8 influenza virus, the pattern of PA protein subcellular localization observed (studied with a cross-reactive monoclonal antibody) was identical to

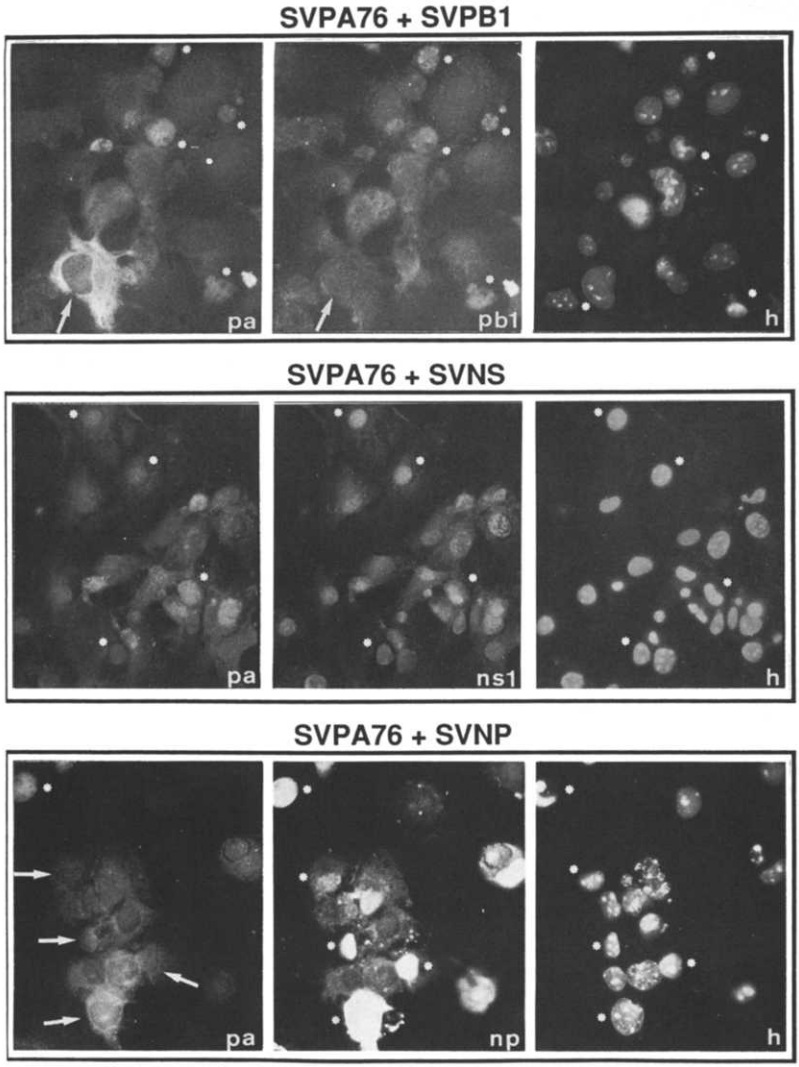

Fig. 4. Effect of specific influenza virus proteins in the subcellular localization of the PA polymerase subunit. Cultures of COS-1 cells were double-infected with SVPA76 recombinant and other SV40 recombinants expressing either PB1 (SVPA76 + SVPB1), the non-structural proteins (SVPA76 + SVNS) or nucleoprotein (SVPA76 + SVNP). Forty eight hours post-infection the cells were fixed and influenza virus proteins were localized by indirect immunofluorescence using PA-specific monoclonal antibodies (pa) and either anti-MPB1/1 peptide serum (pb1), anti-NS1 protein serum (ns1) or anti-NP serum (np). In all cases, nuclei were visualized by staining with the Hoechst nuclear dye (h), as described under Materials and Methods. Arrows in the right panels show cells with cytoplasmic staining of PA protein, whereas asterisks indicate nuclear staining.

that obtained in cells infected with the VIC strain (data not shown), i.e., the antigen is found in the nuclei of the cells. When the cells were infected with the SVPA76 recombinant and superinfected with PR8 virus, a dramatic increase in the nuclear localization of VIC virus PA protein was observed (Fig. 3). These results strongly suggest that some influenza specific or induced protein is involved in the nuclear targeting or in the nuclear retention of polymerase PA protein.

Trans effect of specific influenza virus gene products in the subcellular distribution of the polymerase PA subunit

The evidence presented above, supporting the notion that an influenza virus induced protein is involved in the nuclear accumulation of the polymerase PA subunit, prompted us to carry out double infection experiments in which COS-1 cells were coinfected with the SVPA76 recombinant and other SV40-recombinants able to express PB1, NP or the non-structural proteins. The results of double infections with SVPA76 plus SVPB1 or SVPA76 plus SVNS recombinants showed an absolute correlation between PB1 or NS1 protein expression and nuclear localization of polymerase PA subunit (Fig. 4), as well as an increase in the proportion of cells showing nuclear accumulation of the PA subunit. This effect was not as complete as in the case of influenza virus-SVPA76 doubly infected cells, due to the lower multiplicity of infection attainable with SV40 recombinants. In contrast, no correlation was observed when cells were double infected with SVPA76 and SVNP recombinants (Fig. 4).

Conclusions

The results presented above indicate that the PA protein can be transported to the nucleus of the cell, even when expressed in the absence of other influenza virus proteins, in accordance with previous results (Smith et al., 1987). However, the nuclear accumulation observed was not complete when a SV40 recombinant was used, unless other viral proteins were present (Fig. 3). These results suggest that, although the PA protein may move into the nucleus via active transport mediated by a nuclear targeting signal, other viral factors may play a role in its accumulation in this cellular compartment, in agreement to its delayed localization in the nucleus in the influenza virus infection cycle (Fig. 2) (Akkina et al., 1987). Several mechanisms may be conceived for such a helper function: (i) physical association to a viral or virus-induced protein that is efficiently transported to the nucleus; (ii) virus-mediated post-translational modification of the PA protein to increase its transport; (iii) intranuclear binding that would increase PA protein retention in the nucleus; and (iv) metabolic stabilization of intranuclear PA subunit, i.e. binding to a virus-specific or modification by a virus-specific protein that would lead to an increased half-life of the PA subunit in the nucleus. It is difficult at present to

decide which of these mechanisms is really operating, specially since the involvement of PB1 subunit and any of the non-structural proteins (Fig. 4) suggests that more than one of them may play a role in the process.

The involvement of PB1 protein is not unexpected, in view of its presence in a complex with PB2 and PA in the nucleus (Detjen et al., 1987) and therefore a retention and/or stabilization mechanism would seem reasonable. In fact, preliminary results suggest a similar involvement of PB2 subunit (data not shown). The mechanism by which the RNA segment 8 genes mediate PA protein nuclear accumulation is far less obvious. It should be first pointed out that, although NS1 protein has been used as a marker for segment 8 expression, we can not be certain at present whether NS1 or NS2 protein is involved. With this caveat in mind, it is tempting to speculate that the phenotype of *ts* mutants in the NS1 cistron, compatible with a lack of vRNA synthesis (Shimizu et al., 1982), may be in relation with the genetic interaction with the PA gene. In this context, it is also worth mentioning the intriguing phenotype shown by a mutant in the NS2 cistron in which a specific accumulation of PA defective vRNA is observed (Odagiri and Tobita, 1990). Taken together, these results suggest a role for the non-structural protein(s) in modulating the PA protein function in influenza virus-infected cells.

Acknowledgements

We are grateful to Dr. C.R. Howard for training us in the peptide synthesis techniques. We thank A. Beloso, J. Rebelles and F. Ocaña for their excellent technical assistance and G. Smith, J. Young and P. Palese for providing the VAC-C recombinant and anti-NS1 sera, respectively. J.B. was a predoctoral fellow of the Plan de Formación de Personal Investigador. This work was supported by Comisión Interministerial de Ciencia y Tecnología, grants BIO88-1091 and BIO89-0545, and by an institutional grant of Fundación Areces to the CBM.

References

Akkina, R.K., Chambers, T.M., Londo, D.R. and Nayak, D.P. (1987) Intracellular localization of the viral polymerase proteins in cells infected with influenza virus and cells expressing PB1 protein from cloned cDNA. J. Virol, 61, 2217–2224.

Braam, J., Ulmanen, I. and Krug, R.M. (1983) Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. Cell 34, 609–18.

Davey, J., Dimmock, J. and Colman, A. (1985) Identification of the sequence responsible for the nuclear accumulation of the influenza virus nucleoprotein in *Xenopus* oocytes. Cell 40, 667–675.

de la Luna, S., Martínez, C. and Ortín, J. (1989) Molecular cloning and sequencing of influenza virus A/Victoria/3/75 polymerase genes: sequence evolution and prediction of possible functional domains. Virus Res. 13, 143–55.

Detjen, B.M., St. Angelo, C., Katze, M.G. and Krug, R.M. (1987) The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. J. Virol. 61, 16–22.

Fuerst, T.R., Earl, P.L. and Moss, B. (1987) Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. 7, 2538–2544.

Gluzman, Y. (1981) SV40 transformed simian cells support the replication of early SV40 mutants. Cell 23, 175–182.

Greenspan, D., Palese, P. and Krystal, M. (1988) Two nuclear location signals in the influenza virus NS1 nonstructural protein. J. Virol. 62, 3020–3026.

Herz, C., Stavnezer, E., Krug, R.M. and Gurney, T. (1981) Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. Cell 26, 391–400.

Horisberger, M.A. (1980) The large P proteins of influenza A viruses are composed of one acidic and two basic polypeptides. Virology 107, 302–305.

Huang, T.S., Palese, P. and Krystal, M. (1990) Determination of influenza virus proteins required for genome replication. J. Virol. 64, 5669–73.

Jackson, D.A., Caton, A.J., McCready, S.J. and Cook, P.R. (1982) Influenza virus RNA is synthesized at fixed sites in the nucleus. Nature 296, 366–368.

Jones, I.M., Reay, P.A. and Philpott, K.L. (1986) Nuclear location of all three influenza polymerase proteins and a nuclear signal in polymerase PB2. EMBO J. 5, 2371–2376.

Lai, C.Y. and Nathans, D. (1974) Mapping temperature-sensitive mutants of simian virus 40: rescue of mutants by fragments of viral DNA. Virology 60, 466–475.

Lamb, R.A. (1989) The genes and proteins of influenza viruses. In R.M. Krug (Ed.), The Influenza Viruses, pp. 1–87. Plenum, New York.

Lin, B.C. and Lai, C.J. (1983) The influenza virus nucleoprotein synthesized from cloned DNA in a simian virus 40 vector is detected in the nucleus. J. Virol. 45, 434–8.

Lopez Turiso, J.A., Martínez, C., Tanaka, T. and Ortín, J. (1990) The synthesis of influenza virus negative-strand RNA takes place in insoluble complexes present in the nuclear matrix fraction. Virus Res. 16, 325–37.

Mukaigawa, J. and Nayak, D.P. (1991) Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. J. Virol. 65, 245–253.

Nath, S.T. and Nayak, D.P. (1990) Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1) Mol. Cell. Biol. 10, 4139–45.

Odagiri, T. and Tobita, K. (1990) Mutation in NS2, a non-structural protein of influenza A virus, extragenically causes aberrant replication and expression of the PA gene and leads to generation of defective interfering particles. Proc. Natl. Acad. Sci. U.S.A. 87, 5988–5992.

Ortín, J., Nájera, R., López, C., M.D. and Domingo, E. (1980) Genetic variability of Hong Kong (H3N2) influenza viruses: spontaneous mutations and their location in the viral genome. Gene 11, 319–331.

Portela, A., Melero, J.A., Martínez, C., Domingo, E. and Ortín, J. (1985) Oriented synthesis and cloning of influenza virus nucleoprotein cDNA that leads to its expression in mammalian cells. Virus Res. 4, 69–82.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shapiro, G.I. and Krug, R.M. (1988) Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. J. Virol. 62, 2285–90.

Shimizu, K., Mullinix, M.G., Chanock, R.M.C. and Murphy, B.R. (1982) Temperature sensitive mutants of influenza A/Udorn/72(H3N2) virus. I. Isolation of temperature sensitive mutants some of which exhibit host dependent temperature sensitivity. Virology 117, 38–44.

Smith, G.L., Levin, J.Z., Palese, P. and Moss, B. (1987) Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. Virology 160, 336–345.

Studier, F.W. (1972) Bacteriophage T7. Genetic and biochemical analysis of this simple phage gives information about basic genetic processes. Science 176, 367–376.

Tam, J.P. (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. U.S.A. 85, 5409–5413.