Influenza virus NS1 protein alters the subnuclear localization of cellular splicing components

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Intranuclear structure was studied in influenza virus-infected cells by immunofluorescence microscopy with antibodies specific for fibrillarin, the splicing factor SC35 and the autoantigen p80 coilin. In the course of the infection, an increase in the number of coiled bodies was observed, with a parallel decrease in their size. In addition, the normal speckled pattern of the SC35 factor was altered to generate a more punctate distribution. However, no alteration was observed in the fibrillarin staining pattern. Since an alteration in the splicing of both viral and cellular mRNAs upon expression of influenza virus NS1 protein has been reported previously, the possible effects of NS1 expression on intranuclear structure were assayed. The increase in the coiled body numbers was not specific for the expression of NS1 protein, but alterations in the nuclear location of small ribonucleoprotein particles, as determined by immunofluorescence with an anti-Sm serum or the SC35 splicing factor, were produced by the sole expression of NS1 protein. These results correlate with the previously reported inhibition of splicing induced by NS1 protein expression and suggest an interaction of this influenza virus protein with the cellular splicing machinery.

Among negative-stranded RNA viruses, the influenza viruses are unique in using the nuclear compartment of the infected cell to transcribe and replicate their genomic RNAs (Herz *et al.*, 1981; López-Turiso *et al.*, 1990; Shapiro & Krug, 1988). In doing so, the virus takes advantage of the normal transcriptional mechanisms of the infected cell to develop its own gene expression programme. Thus, newly synthesized RNA polymerase II transcripts are used as donors of capped oligonucleotides to prime viral mRNA synthesis (Krug *et al.*, 1979) and the collinear transcripts of the smaller viral RNA segments are spliced to allow the expression of the NS2 and M2 proteins (Inglis *et al.*, 1979; Inglis & Brown, 1981; Lamb & Choppin, 1979; Lamb *et al.*, 1981). However, these splicing events are regulated to permit the concurrent expression of the NS1 and M1 proteins, which are produced from the collinear mRNAs and are also essential for the virus infection to proceed. Both *cis*- and *trans*-acting elements appear to mediate the control of these processes (Nemeroff *et al.*, 1992; Smith & Inglis, 1985; Valcárcel *et al.*, 1991) and the NS1 protein itself might be responsible for such regulation, since it alters the splicing pattern of NS1 mRNA and other cellular mRNAs (Fortes *et al.*, 1994). Furthermore, the expression of NS1 protein leads to a generalized nuclear retention of mRNAs (Fortes *et al.*, 1994; Qiu & Krug, 1994).

The splicing of pre-mRNA takes place by the concerted action of a number of small ribonucleoprotein particles (U1, U2, U4/U6 and U5 snRNPs), that assemble in an ordered fashion onto the mRNA precursor, and some soluble splicing factors (reviewed in Green, 1991). The spatial arrangement of these elements and their movements during the splicing process in the cell nucleus appears not to be random. Several intranuclear structures have been shown to accumulate snRNPs, splicing factors or nascent mRNAs: perichromatin fibrils and interchromatin granules are nuclear substructures in which both snRNPs and the soluble splicing factor SC35 are preferentially located (reviewed in Spector, 1993) and they might play an important role in cellular post-transcriptional processes. In addition, the structures now called coiled bodies (first identified as accessory bodies in neurons) are subnuclear domains present in most cell types which contain the p80 coilin autoantigen and accumulate high concentrations of snRNPs (Carmo-Fonseca *et al.*, 1991) as well as the U2AF splicing factor (Zhang *et al.*, 1992), but not the SC35 splicing factor (Spector *et al.*, 1991; Zhang *et al.*,

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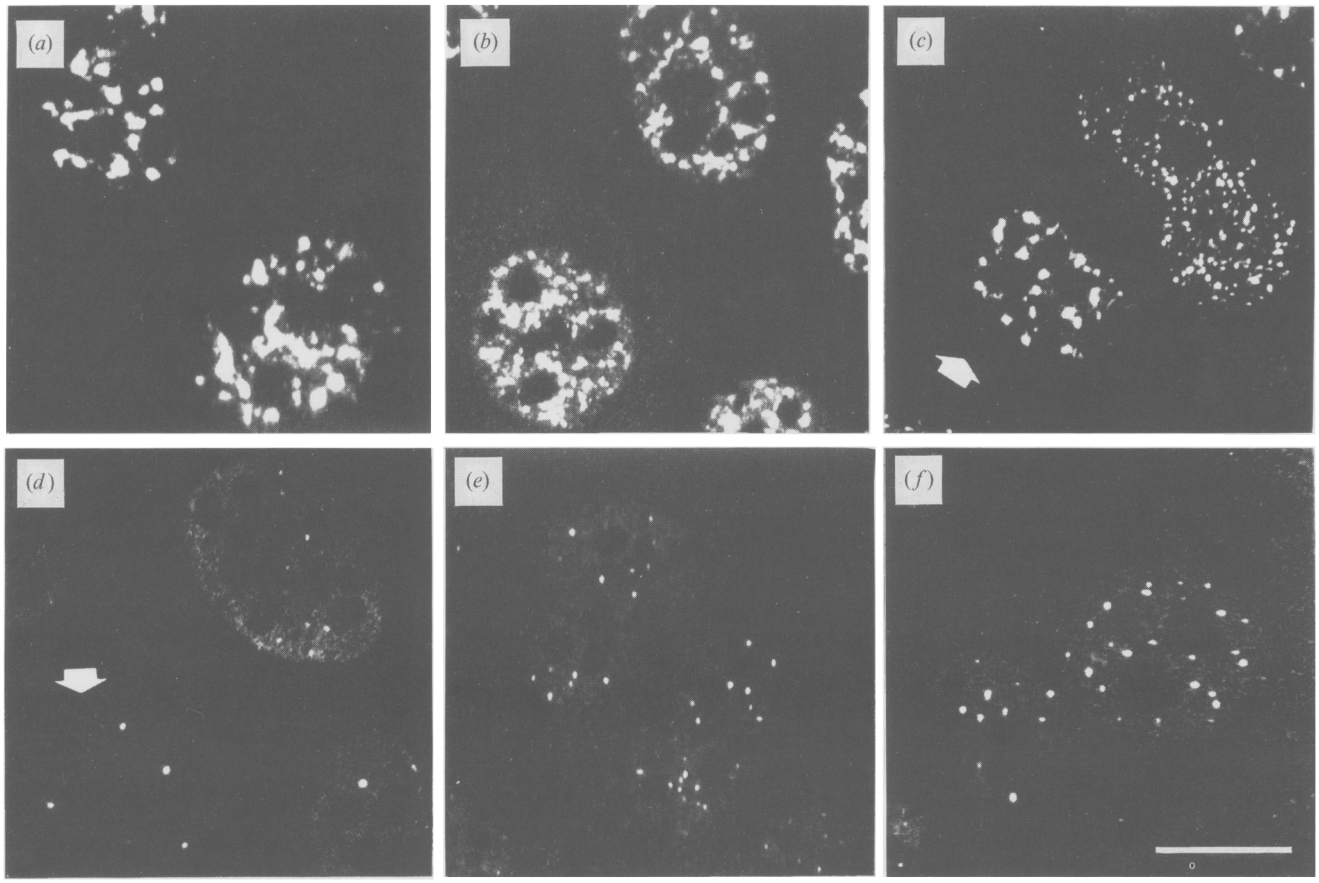

Fig. 1. Localization of nuclear antigens in influenza virus-infected cells. Cultures of MDK cells were infected with influenza virus (strain A/Victoria/3/75) at an m.o.i. of 0·5 p.f.u. per cell (*b–f*) or mock-infected (*a*). At 3 (*d*), 5 (*e* and *b*), 7 (*f*), or 20 (*c*) h p.i., the cultures were fixed and incubated with anti-SC35 (*a–c*) or anti-coilin (*d–f*) monoclonal antibodies and anti-NP serum (*a–f*). The bound antibodies were revealed with FITC-conjugated rabbit anti-mouse antibody and Texas Red-conjugated goat anti-rabbit antibody. Only the FITC-specific fluorescence, denoting the SC35 or coilin antigens, is shown. The uninfected cells (not labelled with anti-NP antibodies) are indicated by arrows. Bar marker represents 10 μm.

1992). Since the coiled bodies are dynamic structures whose number and content changes with the metabolic state of the cell and the cell cycle, it seems that they could be important loci for the recycling of the snRNPs and other splicing factors (reviewed in Lamond & Carmo-Fonseca, 1993).

Since the sole expression of influenza virus NS1 protein leads to profound alterations in the normal splicing and nucleocytoplasmic transport events (Fortes *et al.*, 1994), we set out to examine the possible changes that NS1 protein expression could induce in intranuclear structures. To that aim, the localization of several well-characterized relevant nuclear antigens, including the splicing factor SC35, the coiled body autoantigen coilin and the nucleolar protein fibrillarin, was studied by immunofluorescence. These studies were carried out first in virus-infected MDCK cells, to ascertain their relevance in the context of the normal virus infection. Cells were infected with influenza virus (strain A/Victoria/3/75) at an m.o.i. of 0·5 to 10 p.f.u. per cell, or were mock-infected. Low m.o.i.s were used to obtain infected and uninfected cells in the same field. At different times post-infection (p.i.), the cultures were washed with PBS, fixed for 10 min in 3·7% paraformaldehyde then washed with PBS and permeabilized for 15 min in 0·5% Triton X-100 in CSK buffer (100 mM-NaCl, 300 mM-sucrose, 3 mM-$MgCl_2$, 1 mM-EGTA, 10 mM-PIPES pH 6·8). The cells were incubated with anti-SC35, anti-fibrillarin or with anti-coilin monoclonal antibodies (at 1:1 dilution) and anti-nucleoprotein (NP) serum as fluorescent marker of infection (at a 1:400 dilution), for 1 h at room temperature and washed with PBS. The bound antibodies were revealed with FITC-conjugated rabbit anti-mouse antibody (1:50 dilution) or Texas Red-conjugated goat anti-rabbit antibody (1:100 dilution), by incubation for 1 h at room temperature. After washing with PBS, the preparations were mounted with Mowiol and photographed using a compact confocal microscope

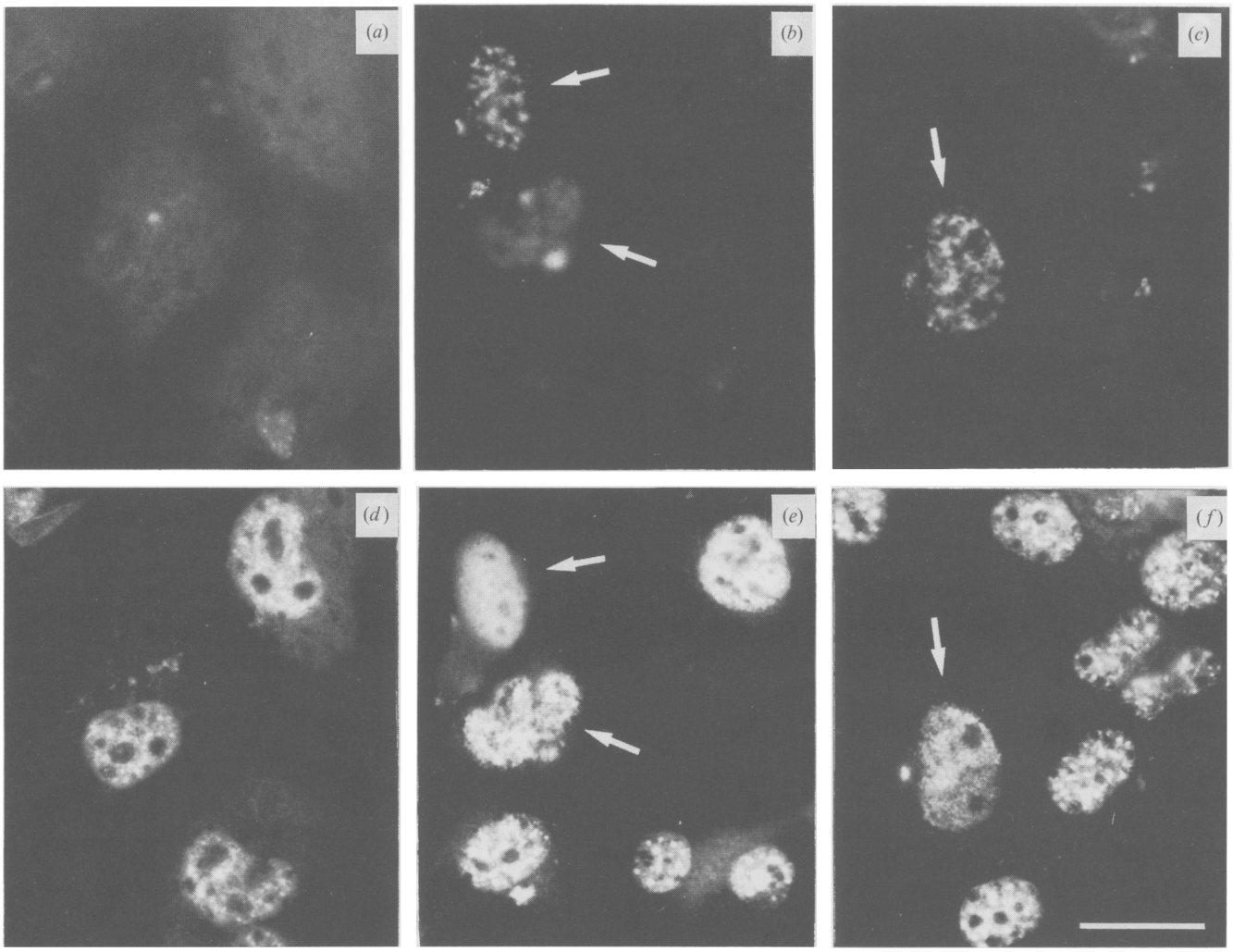

Fig. 2. Localization of snRNPs in cells expressing influenza virus NS1 protein. COS-1 cell cultures, transfected with pSVa232N (*b*, *e*), pSVa232NS1 (*c*, *f*) or mock-transfected (*a*, *d*), were fixed at 72 h post-transfection and analysed by double immunofluorescence using rabbit anti-NS1 serum (*a–c*) or human anti-Sm serum (*d–f*). The bound antibodies were revealed with Texas Red-labelled donkey anti-rabbit immunoglobulin antibodies or FITC-labelled goat anti-human immunoglobulin antibodies. The arrows indicate NS1-expressing cells. (*a*, *d*), (*b*, *e*) and (*c*, *f*), respectively, show identical fields in the preparation. Bar marker represents 5 μm.

(EMBL; Heidelberg), with excitation wavelengths of 488 nm (for FITC) or 520 nm (for Texas Red). The results are presented in Fig. 1. The regular pattern of speckles, as determined by staining for the SC35 splicing factor, was dramatically altered (Fig. 1*a–c*). Thus, the number of speckles increased and their structure became disorganized as the infection proceeded, up to extreme cases as shown in Fig. 1(*c*). Similar results were obtained when COS-1 cells were used instead of MDCK cells (data not shown). In addition, the number of coiled bodies per nucleus (2–6 in normal uninfected cells) increased stepwise in the course of the infection cycle. More than 20 coiled bodies per nucleus were observed by 5–7 h p.i. (Fig. 1*d–f*). This increase in the number of coiled bodies was paralleled by a decrease in their size, in such a way that the total amount of coilin, as determined by western blot analysis, did not change (data not shown). However, these alterations were not the consequence of a generalized breakdown of the nuclear structure, since the staining pattern of fibrillarin was not modified during the infection (data not shown). The changes described above are not without precedent in other virus infections of cells. The snRNPs are redistributed upon infection with herpes simplex virus type 1 (HSV-1), from the standard speckled pattern to a punctate distribution (Martin *et al.*, 1987) and colocalize with the immediate early IE63 gene product (ICP27) when it is expressed by transfection (Phelan *et al.*, 1993), while staining of the coiled bodies

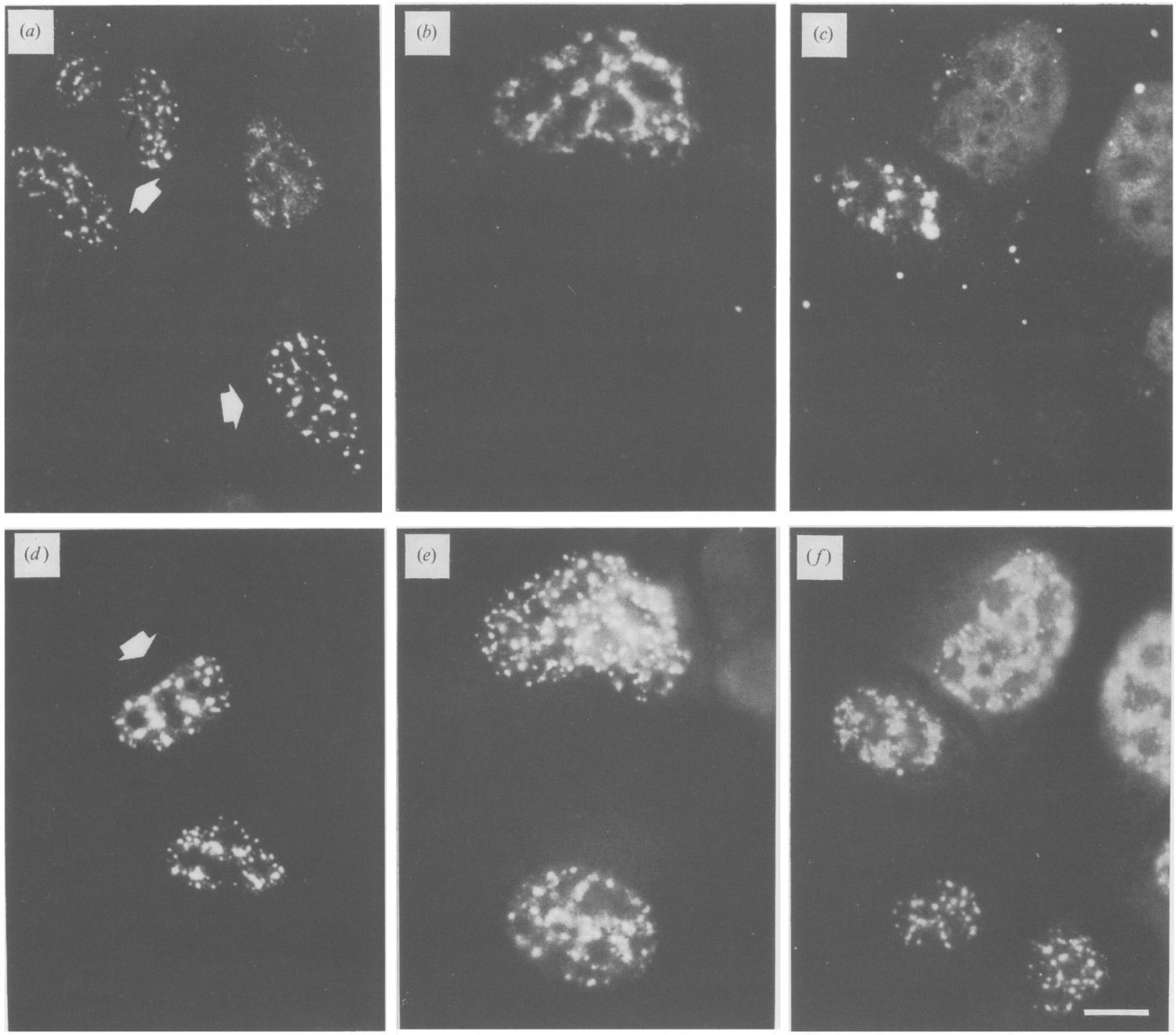

Fig. 3. Localization of splicing factor SC35 in cells expressing influenza virus NS1 protein. Cultures of COS-1 cells were infected with influenza virus (strain A/Victoria/3/75) (*a*) or transfected with plasmid pSVaNPNS (*b*, *c*, *e* and *f*) or plasmid pSVaNPNS2 (*d*). At 7 h p.i. (*a*) or 48 h post-transfection (*b–f*), the cultures were fixed and incubated with anti-SC35 monoclonal antibody (*a*, *d*, *e* and *f*) or anti-NS1 serum (*b* and *c*) and anti-NP serum (*a* and *d*). The bound antibodies were revealed with FITC-conjugated rabbit anti-mouse antibody and Texas Red-conjugated goat anti-rabbit antibody. (*b* and *f*) and (*c* and *f*) correspond to the same field. Only the FITC-specific fluorescence is shown in (*a*) and (*d*). The cells not transfected or not infected (not labelled with anti-NP antibodies) are indicated by arrows. Bar marker represents 5 μm.

disappear (Phelan *et al.*, 1993). A similar situation could be observed upon infection of permissive cells by adenovirus, i.e. the snRNPs accumulate at distinct sites close to (Bridge *et al.*, 1993) or identical to the centres of DNA replication (Jiménez-García & Spector, 1993), but staining of the coiled bodies was no longer detectable (Bridge *et al.*, 1993). It is noteworthy that the pattern of alterations observed after influenza virus infection is essentially opposite to that described for HSV or adenovirus infection. This fact might be related to the requirement of the latter for active mRNA splicing in the infected cell whereas influenza virus NS1 protein inhibits cellular and viral mRNA splicing (Fortes *et al.*, 1994).

In view of the biochemical evidence for the NS1 protein-induced alterations in the splicing and transport (Fortes *et al.*, 1994) and the changes induced in the intranuclear structure upon influenza virus infection (Fig. 1), cultures of COS-1 or HeLa cells were transfected

with a variety of plasmids expressing NS1 protein or NS2 protein, as a control. Cell cultures grown on coverslips were transfected with 1 μg of plasmid complexed with lipofectin (Gibco) at a 1:2 ratio, according to the recommendations of the supplier. After 12 h, the cultures were washed with medium and then fresh medium supplemented with 5% fetal bovine serum was added. At 36, 48 or 60 h post-transfection, the cells were fixed and processed for immunofluorescence as described above. In the first series of experiments, the nuclear distribution of the Sm antigen was studied by use of plasmid pSVa232N (Portela *et al.*, 1986), which expresses NS1 and NS2 proteins, and its mutant form pSVa232NS1 (Fortes *et al.*, 1994), which expresses only NS1 protein owing to inactivation of the NS2 cistron. Transfection with NS1-expressing plasmids led to two distinct patterns of NS1 protein nuclear distribution; a diffuse staining and a punctate decoration (see Fig. 2*b* and Fig. 3*c* below). Irrespective of the NS1 protein nuclear pattern, expression of NS1 protein alone or in conjunction with NS2 protein resulted in the disappearance of the typical pattern of Sm antigen and its substitution by a more diffuse, generalized staining of the nucleus (Fig. 2; compare NS1-expressing cells in (*e*) and (*f*) with those non-expressing cells in (*d*). In addition, when the NS1 protein was expressed in the context of the influenza virus infection, a similar, although attenuated effect could be observed. On the other hand, no differences with the standard pattern of Sm antigen were observed in cells expressing only NS2 protein (data not shown).

Next, the location of the SC35 splicing factor was studied in cells expressing NS1 protein. To that end, plasmids pSVaNPNS, pSVaNPNS1 and pSVaNPNS2 (Fortes *et al.*, 1994) were used. They are derivatives of plasmid pSVa232N that also express the influenza virus nucleoprotein. This antigen was used as a fluorescence marker for transfection. The expression of NS1 protein was associated with a change in the distribution of the splicing factor SC35. The normal speckled pattern disappeared and was substituted by a diffuse staining all over the nucleus with an altered punctate pattern both in cells infected with influenza virus (Fig. 3*a*) and in transfected cells (Fig. 3*e*,*f*). These alterations were observed both in cells in which NS1 protein showed a diffuse staining and in those with a punctate NS1 protein distribution. However, when the cultures were transfected with plasmid pSVaNPNS2, no alteration in the SC35 speckled pattern was detected (Fig. 3*d*), indicating that the observed effects were indeed a specific consequence of NS1 protein expression and not a non-specific alteration due to the transfection itself. Moreover, neither transfection with pSVaNPNS, nor with pSVaNPNS2 plasmids, induced any change in the regular pattern of fibrillarin staining, supporting the notion that the expression of these viral proteins does not alter the general intranuclear structure (data not shown).

On the other hand, when the cultures transfected with plasmid pSVaNPNS were stained with anti-coilin serum (at a 1:5000 dilution) and anti-NP monoclonal antibody (M58p51G; López *et al.*, 1986) (at a 1:1 dilution), an increase in the number of coiled bodies per nucleus was observed, but this effect was not consistently found in every transfected cell and, in addition, it was also detected in cells transfected with control pSVaNPNS2 plasmid (data not shown). Therefore, the augmentation in coiled body numbers induced after virus infection could not be ascribed to the expression of any specific viral gene in particular and was most probably due to a general stimulation of gene expression induced by either infection or transfection, as described before (Lamond & Carmo-Fonseca, 1993).

Transfection of COS-1 cells with a plasmid expressing a rat tropomyosin gene induced a recruitment of splicing factors to the new sites of transcription (Jiménez-García & Spector, 1993), as shown for adenovirus- and herpesvirus-infected cells (Bridge *et al.*, 1993; Martin *et al.*, 1987). It appears that the overexpression of genes requiring splicing by either transfection of autoreplicative plasmids or by virus infection determines a redistribution of snRNPs by altering their normal functional cycle in the nucleus. On the contrary, infection by influenza virus led to the disappearance of the speckled pattern of splicing factor SC35 (Figs 1*a–c* and 3*a*). This result is consistent with the reduced requirement for splicing during productive infection by influenza virus. However, inhibition of splicing with specific U1 or U6 oligonucleotides induced the accumulation of SC35 splicing factor into large, rounded structures in the nucleus (O'Keefe *et al.*, 1994), suggesting that different steps in the splicing pathway are affected by NS1 protein and these oligonucleotides.

The observed change in the nuclear organization in COS-1 cells transfected with pSVaNPNS plasmid is in line with the inhibition of splicing detected as a consequence of the NS1 protein expression (Fortes *et al.*, 1994) and is suggestive of an interaction of NS1 protein with components of the splicing machinery. The NS1 protein is an RNA-binding protein which has been shown to interact with virion-sense influenza RNA (Hatada & Fukuda, 1992; Hatada *et al.*, 1992) and with poly(A) (Qiu & Krug, 1994). None of these interactions appear to explain the effects of NS1 protein expression on mRNA splicing and on the intranuclear distribution of its machinery. Other splicing factors, either RNA or protein molecules, could still be targets for NS1 protein binding. Experiments are in progress to determine the cellular targets for NS1 protein action. The NS1 protein expression led to alterations in the splicing of cellular

mRNAs and to the accumulation of splicing intermediates, as detected by RNAse protection assays of *in vivo*-isolated RNAs (Fortes *et al.*, 1994). From the loss of the pre-mRNA specific protection band and the increase in the accumulation of the intron-plus-second exon protection band, it could be suggested that NS1 protein enhances the formation of the spliceosomal complex but inhibits the second step in the splicing reaction. Thus, it could interfere with the function of the U2, U4/U6 and/or the U5 snRNPs which may be involved in the catalytic steps of splicing (Newman & Norman, 1992) or with the activity of the mammalian counterpart of the PRP16 factor, a non-snRNP yeast protein with RNA-dependent ATPase activity acting in the spliceosome before the second splicing step (Schwer & Guthrie, 1991).

The influenza virus NS1 protein is a small, multifunctional protein, able to act at several distinct steps in the virus–host cell interaction and the virus gene expression. Thus, in addition to the reported activities of NS1 protein in the alteration of normal cell RNA splicing (Fortes *et al.*, 1994) and mRNA nucleocytoplasmic transport (Fortes *et al.*, 1994; Qiu & Krug, 1994), it appears to be involved in a specific enhancement of viral mRNA translation (de la Luna *et al.*, unpublished results). Elucidation of the ways in which NS1 protein carries out so many different functions is a very interesting challenge for future work.

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