JOURNAL OF VIROLOGY, Apr. 1995, p. 2427–2433 Vol. 69, No. 4
0022-538X/95/$04.00+0

Influenza Virus NS1 Protein Enhances the Rate of Translation Initiation of Viral mRNAs

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Received 22 September 1994/Accepted 22 December 1994

The effect of NS1 protein on the efficiency of influenza virus mRNA translation was evaluated by determining the accumulation of nucleoprotein (NP) or M1 mRNAs in the cytoplasm of cells expressing either of these genes alone or in combination with the NS1 gene, as well as the total cell accumulation of NP or M1 protein. Coexpression of NS1, but not of NS2 protein, led to increases in the translation of these mRNAs in the range of 5- to 100-fold. This translation enhancement was specific for viral mRNAs, since the translation of neither *cat* nor *lacZ* mRNAs was affected by the coexpression of NS1 protein. The use of chimeric *cat* genes containing the 5′-extracistronic sequences of the influenza virus mRNAs corresponding to segment 2, 7, or 8 indicated that these sequences can in part account for the observed effect. The enhancement of viral mRNA translation mediated by NS1 protein was due to an increase in the translation initiation rate, since the sizes of NP-specific polysomes, but not those of *lacZ*-specific polysomes, was significantly higher in cells coexpressing NS1 protein than in those expressing only the NP gene.

Influenza viruses induce a series of alterations in the synthesis and metabolism of cellular macromolecules upon infection of susceptible cells in vitro. Thus, there is some decrease in cellular mRNA synthesis, probably because of the virus-induced cap-snatching activity. The nucleocytoplasmic transport of the cellular mRNAs is inhibited and/or cellular RNAs are degraded in the nucleus (24). The cellular mRNAs located in the cytoplasm are degraded during the infection, accounting, at least in part, for the arrest of cellular protein synthesis (1, 21). Inhibition of cellular protein synthesis has also been reported, even in the absence of substantial cytoplasmic mRNA degradation (24).

It has been proposed that influenza virus takes over the cellular translation machinery by impairment of a cellular initiation factor. The initiation factor involved should be other than eIF-2, since its inactivation by phosphorylation is prevented during the infection (23, 25, 28). The inactivated initiation factor might be the eIF-4E (p24) polypeptide, one of the components of the eIF-4F initiation factor, in view of its underphosphorylation in influenza virus-infected cells (9). This proposal is in line with the ability of influenza virus-specific protein synthesis to take place in cells previously infected with adenovirus (22), which blocks cellular protein synthesis by the same mechanism. On the other hand, influenza virus infection cannot proceed in poliovirus-infected cells, in which the protein p220, another component of the same initiation factor, is degraded (42). The translation shutoff induced by influenza virus has been studied by a transfection-infection protocol (12, 13). By such an approach, it has been shown that an RNA polymerase II transcript encoding a truncated version of the NP gene can be efficiently translated in infected cells and that the recognition of transfected gene mRNAs as viral depends on the 5′-terminal sequences of the mRNA.

The influenza virus NS1 protein is encoded by the collinear mRNA transcribed from the viral RNA segment 8. It mediates a generalized cellular mRNA retention in the nucleus (11, 36) and might act as a negative *trans* regulator of its own mRNA splicing (11, 41, 45). The NS1 protein is an RNA-binding protein (17, 18) that accumulates mainly in the nucleus, both during virus infection and when expressed from cloned DNA (16, 34). However, a fraction of NS1 protein is also found in association with polysomes (4, 27, and this report). Viral mutants affected in the NS1 cistron show a lack of late viral gene expression that might be interpreted as the consequence of an NS1 protein role in the regulation of viral gene expression or in viral RNA amplification (26, 40). Consistent with this proposal, Enami et al. (8) recently reported the stimulation by NS1 protein of the translation of an M-chloramphenicol acetyltransferase (CAT) chimeric mRNA.

In this report we show that expression of NS1 protein from cloned DNA leads to a large enhancement in the expression of influenza virus genes. This effect is specific for viral mRNAs, depends on their 5′-terminal extracistronic sequences, and is mediated at the translational level by increasing the rate of initiation of protein synthesis.

MATERIALS AND METHODS

Biological materials. The COS-1 cell line (14) was obtained from Y. Gluzman, and the Madin-Darby canine kidney (MDCK) cell line was purchased from the American Type Culture Collection. The cell cultures were grown in Dulbecco's modified Eagle medium containing 5% fetal calf serum. Influenza virus A/Victoria/3/75 was grown and titrated by plaque assay with MDCK cells (32). Monolayers of COS-1 cells were infected with influenza virus at a multiplicity of infection of 10 PFU per cell.

The preparation of antisera specific for NP and NS1 proteins has been described previously (11, 30).

The plasmid pSVa232N, in which expression of influenza virus proteins NS1 and NS2 is driven by simian virus 40 (SV40) genome late control region, and the construction of its mutant derivatives expressing only NS1 protein (pSVa232NS1) or NS2 protein (pSVa232NS2) have been described (11, 34). Plasmid pSVa963, which contains the influenza virus nucleoprotein (NP) segment cloned under the control of SV40 early promoter and polyadenylation signals (35), and the construction of hybrid plasmids expressing various combinations of the NP, NS1, and NS2 genes (pSVaNPNS, pSVaNPNS1, and pSVaNPNS2 [Table 1]) have been reported previously (11). Plasmid pSVa982, which expresses the RNA segment 7 cDNA cloned under the control of SV40 early promoter and polyadenylation signals, has been described (44).

Plasmid pSV2cat (15) was provided by W. Doerfler, and plasmid pPB2cat-9, which contains the coding region of the CAT (*cat*) gene flanked by influenza

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TABLE 1. Proteins expressed by the plasmids used in this report

Plasmid	Protein(s) expressed	Reference or source
pSVa232N	NS1, NS2	36
pSVa232NS1	NS1	11
pSVa232NS2	NS2	11
pSVa963	NP	35
pSVaNPNS	NP, NS1, NS2	11
pSVaNPNS1	NP, NS1	11
pSVaNPNS2	NP, NS2	11
pSVa982	M1, M2	44
pSVaMNS	M1, M2, NS1, NS2	This report
pSVaMNS1	M1, M2, NS1	This report
pSVaMNS2	M1, M2, NS2	This report
pSV2cat	CAT	15
pSVpb2cat	CAT	This report
pSVmcat	CAT	This report
pSVnscat	CAT	This report
pSV2catNS	CAT, NS1, NS2	This report
pSVpb2catNS	CAT, NS1, NS2	This report
pSVmcatNS	CAT, NS1, NS2	This report
pSVnscatNS	CAT, NS1, NS2	This report
pSV2catNS1	CAT, NS1	This report
pSVpb2catNS1	CAT, NS1	This report
pSVmcatNS1	CAT, NS1	This report
pSVnscatNS1	CAT, NS1	This report
pSVβgal	β-Galactosidase	Promega
pSVβgalNS	β-Galactosidase, NS1, NS2	This report
pSVβgalNS1	β-Galactosidase, NS1	This report

virus A/PR/8/34 (PR8) PB2 segment terminal sequences, was supplied by M. Krystal. Plasmids pIVACAT1-S (33) and pT3MCAT, containing the coding region of the *cat* gene flanked by either PR8 virus segment 8 or PR8 virus segment 7 terminal sequences, respectively, were provided by P. Palese. Plasmid pSVβgalactosidase (pSVβgal) was purchased from Promega.

To coexpress M1 and the NS proteins, the *Bgl*I fragment from plasmid pSVa982, containing RNA segment 7 cDNA, was used to substitute for the *Bgl*I fragment encoding T antigen in plasmids pSVa232N, pSVa232NS1, and pSVa 232NS2, generating plasmids pSVaMNS, pSVaMNS1, and pSVaMNS2, respectively (Table 1).

Three series of *cat* gene-containing plasmids, in which the *cat* gene coding region was either framed by the influenza virus PB2, M, or NS1 5′-extracistronic sequences or lacked any viral genetic information, were constructed. In every case, the *cat* gene was under SV40 early transcription control. To that aim, the blunt-ended *Xho*I-*Msc*I fragment of plasmid pPB2cat9, the blunt-ended *Xba*I-*Msc*I fragment of plasmid pIVACAT1/S, and the corresponding blunt-ended *Hga*I-*Msc*I fragment of pT3MCAT were cloned between the blunt-ended *Hin*dIII and the *Msc*I sites of pSV2cat plasmid, generating plasmids pSVpb2cat, pSVnscat, and pSVmcat, respectively. To coexpress these *cat* genes with the NS1 and NS2 proteins, the fragments *Bgl*I-*Hpa*I obtained from pSV2cat plasmid and the previous constructs were cloned into the corresponding sites of plasmid pSVa232N. Thus, plasmids pSVcatNS, pSVpb2catNS, pSVmcatNS, and pSVn scatNS were obtained. They are formally analogous to plasmid pSVaNPNS (11) and express the *cat* gene driven by the early SV40 promoter and the NS1 and NS2 proteins driven by the late SV40 promoter. Further, the NS2 or NS1 gene mutations present in plasmids pSVa232NS1 or pSVa232NS2 (11) were introduced in the above described series by swapping the *Stu*I-*Pst*I fragments (Table 1).

Much with the same strategy, the β-galactosidase gene present in plasmid pSVβgal was transferred to plasmid pSVa232N by ligating fragment *Stu*I-*Bsm*I from the former into the corresponding sites of the latter. In addition, the mutation in the NS2 gene was introduced by the procedure described above. Thus, plasmids pSVβgalNS and pSVβgalNS1 were obtained (Table 1).

For riboprobe synthesis, plasmids pGNP (11), pGcat, and pGβgal were used. Plasmid pGcat was constructed by ligation of fragment *Eco*RI-*Bgl*II, containing the *cat* gene 3′ end from plasmid pPB2cat-9, into pGEM4 digested with *Eco*RI and *Bam*HI. Likewise, plasmid pGβgal was constructed by ligating the *Bst*XI (treated with T4 DNA polymerase)-*Eco*RI fragment from plasmid pSVβgal into *Sma*I- and *Eco*RI-digested pGEM4 vector. An M1-specific cDNA probe was prepared by random priming (10) on an isolated segment 7 *Nco*I DNA fragment (positions 353 to 632 in the sequence).

DNA manipulation. DNA manipulations, including restriction, modifications of restriction ends, ligations, and cloning were carried out by standard techniques (38), with *Escherichia coli* DH-5 as a host. The mutations in the NS1 and NS2 genes present in several constructions were checked by dideoxy termination sequencing with specific primers (39).

Transfection. Cultures of COS-1 cells were transfected with plasmid DNA by the liposome-mediated method with Lipofectin (Gibco-BRL), according to the manufacturer's suggestions.

RNA isolation. Cytoplasmic RNA was isolated and poly(A)$^+$ RNA was purified exactly as previously described (11). For riboprobe synthesis, plasmid pGcat was digested with *Nco*I and transcribed with T7 RNA polymerase. Likewise, plasmids pGNP and pGβgal were digested with *Sca*I and *Hin*dIII, respectively, and transcribed with SP6 RNA polymerase in the presence of [^{32}P]CTP (400 μCi/pmol). The negative-polarity RNA transcripts were treated with RNase-free DNase I (Boehringer) and gel purified after ethanol precipitation. RNase protection assays were carried out as previously described (11, 45). For dot blot hybridization, RNA was incubated for 15 min at 65°C in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–7.5% formaldehyde and applied to a nylon filter with a dot blot apparatus. The nylon blots were baked for 1 h at 80°C and hybridized with the corresponding probes as previously described (11).

Protein analyses. Western blot (immunoblot) analysis was carried out with total cell extracts. Cell monolayers were washed with ice-cold phosphate-buffered saline (PBS) and lysed in electrophoresis buffer. Proteins were resolved by electrophoresis in sodium dodecyl sulfate (SDS)–10% or 7.5 to 15% gradient polyacrylamide gels and then transferred to nitrocellulose membranes (Bio-Rad) as described elsewhere (7). After being blocked with 3% bovine serum albumin in PBS buffer and incubated with the corresponding antibodies, the specific bands were visualized with peroxidase-coupled secondary antibodies and by enhanced chemiluminescence.

Cell extracts for CAT assays were prepared 60 h after transfection, and CAT activity was analyzed by the phase extraction method, as described previously (6). For β-galactosidase activity determination, the cell extracts were prepared and the enzymatic activity was carried out as previously described (38). Protein concentration was determined by the bicinchoninic acid method.

Polysome analysis. Cytoplasmic extracts were prepared as previously described (31). The extracts were layered onto 7 to 47% linear sucrose gradients on top of a 70% sucrose cushion in IB buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris · HCl, pH 8.5) and resolved by centrifugation in a Beckman SWTi-41 rotor at 38,000 rpm for 1 h 40 min at 1°C. Gradient fractions of 500 μl were collected. For RNA preparation, an aliquot of each fraction was incubated for 1 h at 37°C with 100 μg of proteinase K per ml in the presence of 1% SDS. The mixture was phenol extracted and ethanol precipitated. In order to ensure that the RNA in the fractions was DNA free, the RNA was subjected to digestion with RNase-free DNase I and further purified by phenol extraction and ethanol precipitation.

RESULTS

Increased expression of viral genes in cells expressing NS1 protein. The expression of influenza virus NS1 protein leads to a generalized nuclear retention of cellular mRNAs (11). In the course of these analyses, the expression of various viral genes, coexpressed with NS1 protein by RNA polymerase II-dependent transcription from cloned DNAs, was determined by Western blot or by immunoprecipitation. The observation was made that, despite these viral mRNAs being essentially retained in the nucleus, the level of the corresponding proteins did not diminish. To study further this phenomenon, experiments in which influenza virus NP was expressed as a single viral protein or coexpressed with NS1 protein were performed. To this end, COS-1 cells were transfected with plasmid pSVa963, expressing NP, or with plasmids pSVaNPNS, pS VaNPNS1, or pSVaNPNS2, which express NP in combination with NS1 and NS2 proteins, NS1, or NS2 proteins, respectively (Table 1). Aliquots of the same cultures were used to (i) determine the accumulation of NP mRNA in the cytoplasmic fractions of the transfected cells and (ii) determine the accumulation of NP. The results are presented in Fig. 1. Consistent with our previous observations, the accumulation of NP mRNA in the cytoplasm was much greater in cells expressing the NP gene alone or with NS2 protein (Fig. 1A) than in those also expressing NS1 protein (Fig. 1A, slots NPNS and NPNS1). However, the accumulation of NP protein was similar in every culture expressing NP (Fig. 1B). The half-life of the NP was not altered when it was synthesized in the presence or absence of NS1 protein, thereby eliminating the possibility that the increase in its accumulation was due to an increased stability of the protein itself (data not shown). The results in Fig. 1A and

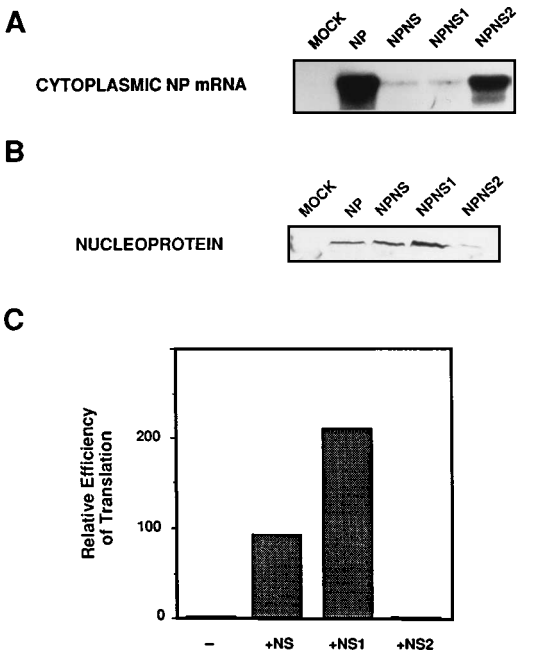

FIG. 1. NS1 protein enhances translation of NP mRNA. Cultures of COS-1 cells were transfected with plasmids pSVa963 (NP), pSVaNPNS (NPNS), pSVaNPNS1 (NPNS1), and pSVaNPNS2 (NPNS2) or were mock transfected (MOCK). The cells were harvested and split into two portions, one of which was processed for cytoplasmic poly(A)$^+$ mRNA isolation and the other of which was processed for total protein extraction, as described in Materials and Methods. The RNA preparations were analyzed by RNase protection assay, and the protein fractions were examined by Western blot, with NP-specific probes and antibodies, respectively. (A) NP-specific RNase protection band (388 nucleotides) obtained from cells transfected by the indicated plasmids. (B) NP-specific Western blot signals obtained from cells transfected by the indicated plasmids. (C) The signals in panels A and B were quantitated by microdensitometry, and their ratios (normalized for the value obtained for the NP sample) are indicated.

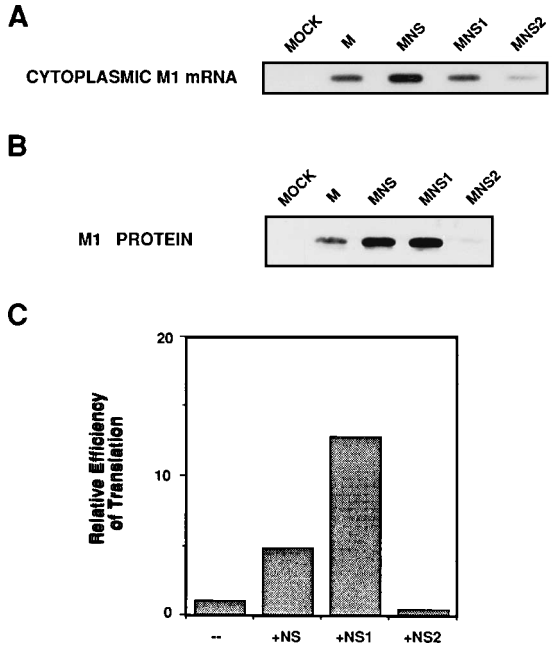

FIG. 2. NS1 protein enhances translation of M1 protein mRNA. Cultures of COS-1 cells were transfected with plasmids pSVa982 (M), pSVaMNS (MNS), pSVaMNS1 (MNS1), and pSVaMNS2 (MNS2) or were mock transfected (MOCK). The cells were processed for mRNA isolation and protein extraction, as described in the legend to Fig. 1. The RNA preparations were analyzed by slot blot hybridization, and the protein fractions were examined by Western blot, with an M1-specific probe and antibody, respectively. (A) M1-specific slot blot hybridization signal obtained from cells transfected by the indicated plasmids. (B) M1-specific Western blot signals obtained from cells transfected by the indicated plasmids. (C) The signals in panels A and B were quantitated by microdensitometry, and their ratios (normalized for the value obtained for the M sample) are indicated.

B were quantitated by microdensitometry of the RNA- and protein-specific bands and are represented in Fig. 1C as the relative efficiency of translation (ratio of the corresponding protein to RNA signals) for the different combinations of expressed genes. These results indicate that the expression of the NP gene in the presence of NS1 protein is in the range of 100-fold-more efficient than in its absence. To ascertain the generality of the observed effect on other viral mRNAs, similar experiments, expressing M1 protein either individually or in combination with NS1 and/or NS2 proteins, were carried out. Cultures of COS-1 cells were transfected with plasmid pSVa982, which expresses M1 and M2 proteins (44), or with plasmids pSVaMNS, pSVaMNS1, or pSVaMNS2, which express M1 protein in various combinations with NS1 and/or NS2 proteins (Table 1). The accumulations of M1 mRNA and M1 protein were determined as described above for NP, and the results are presented in Fig. 2. The ratio of the protein (Fig. 2A) to mRNA (Fig. 2B) accumulations is expressed as relative efficiency of translation in Fig. 2C. Increases in range from 5- to 13-fold were observed in the presence of NS1 protein. For both NP and M1 protein expression, the inductions observed by NS1 protein coexpression were greater than those obtained by combined coexpression of NS1 and NS2 proteins. It is not clear at present whether this observation reflects a true biological effect of NS2 protein or, rather, is a consequence of a decrease in the level of NS1 protein expression.

Expression of NS1 protein does not affect the translation of nonviral mRNAs. To ascertain whether the translational enhancement detected with NP or M1 proteins as reporters was also found when genes not related to influenza virus were used, the accumulation of either CAT or β-galactosidase activities was determined when these genes were expressed singly or in combination with NS1 protein. Cultures of COS-1 cells were transfected with plasmid pSV2cat or pSVβgal, leading to the individual expression of each gene, or with plasmid pSVcatNS, pSVcatNS1, pSVβgalNS, or pSVβgalNS1 (Table 1), allowing the expression in the same cell of either gene in combination with NS1 protein or with both NS1 and NS2 proteins. The accumulation of *cat* or *lacZ* mRNAs was measured by dot blot hybridization of poly(A)$^+$ cytoplasmic RNA with gene-specific probes, while the accumulation of the corresponding proteins was tested by determination of their enzymatic activities in total cell extracts. Both analyses were carried out with aliquots of the same transfected cultures, to avoid the experimental variation of the transfection step (Fig. 3). Nuclear retention of either *cat* or *lacZ* mRNAs was also detected when the genes were coexpressed with NS1 protein (Fig. 3A, slots +NS and +NS1), but contrary to the results obtained for NP protein, the accumulation of CAT or β-galactosidase activities essentially paralleled those of their respective cytoplasmic mRNAs (Fig. 3B). After quantitation of the dot hybridization, the relative efficiency of translation (ratio of enzymatic activity to cytoplasmic mRNA) of β-galactosidase mRNA was not affected by NS1 protein expression (Fig. 3C, cf. Fig. 1C). A slight increase in the translation efficiency of CAT mRNA (Fig. 3C) that might be related to the nucleotide sequence homology detected between the pSV2cat transcript leader and that of NP

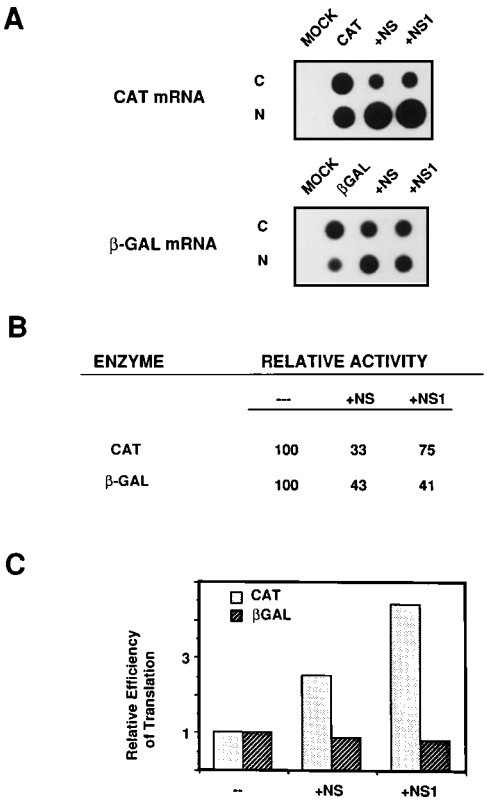

FIG. 3. NS1 protein does not alter the translation efficiency of *cat* or *lacZ* mRNAs. Cultures of COS-1 cells were transfected with plasmids pSV2cat (CAT), pSVβgal (βGAL), pSVcatNS (+NS), pSVcatNS1 (+NS1), pSVβgalNS (+NS), and pSVβgalNS1 (+NS1) or were mock transfected (MOCK). The cells were harvested and split into two portions, one of which was fractionated into nuclei and cytoplasm and processed for poly(A)$^+$ RNA isolation and the other of which was processed for total protein extraction, as described in Materials and Methods. The RNA preparations were analyzed by dot blot hybridization with *cat*- or *lacZ*-specific probes, and the protein fractions were used for determination of enzymatic activity. (A) Hybridization signals obtained in the cytoplasmic (C) or nuclear (N) RNAs of cells transfected with the indicated plasmids. (B) Relative enzymatic activity expressed as a percentage of that obtained with extracts from cells not expressing NS proteins. (C) The signals in panel A were quantitated by microdensitometry, and their ratios to the activities indicated in panel B (normalized for the value obtained for the CAT or β-galactosidase sample) are indicated.

mRNA was observed. Thus, six of the eight positions in the sequence GGTAGATA, conserved among the 5′-extracistronic sequences of NP and M1 mRNAs, are homologous to the CAT mRNA leader (Fig. 4). These sequences are also partially conserved in the 5′-extracistronic regions of NS and PB2 mRNAs. Therefore, it can be concluded that the observed

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CAT    ...GCAAAAAGCTTGGCGAGATTTTCAG..
          ||||||  ||  ||||  |||
NP     ...AGCAAAAGCA.GGGTAGATAATCAC..
          ||||||||||  |||||||| | |
M      ...AGCAAAAGCA..GGTAGATATTGAA..
          |||||||||  ||| || |  ||
NS     ...AGCAAAAGCA.GGGT.GACAAAGAC..
          ||| ||||||  |||  |  |
PB2    ...AGCGAAAGCA..GGTCAATTATATT..
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FIG. 4. Comparison of 5′-extracistronic sequences. The sequences of the leader regions corresponding to the mRNAs of segments 5 (NP), 7 (M), 8 (NS), and 2 (PB2) were compared with that of the leader region of the *cat* gene in construct pSV2cat, with the GAP program of the Genetics Computer Group package.

effects of NS1 protein on translation efficiency are virus specific.

***cis* signals present in the 5′-extracistronic regions are involved in the NS1 protein effect on viral mRNA translation.** The viral specificity of the translational enhancement suggests the involvement of *cis* signals located in the viral mRNAs. Since the constructs expressed in COS-1 cells did not contain the viral 3′-extracistronic regions (35), the presumptive signals could be located in the 5′-extracistronic region, in the coding sequences, or in both. These alternatives were studied by measuring the translational enhancement observed on the expression of constructs in which the 5′-extracistronic regions (from the first nucleotide to the ATG of each segment) of the influenza virus segment 2, 7, or 8, encoding PB2, M1+M2, or NS1+NS2 proteins, respectively, substituted for the leader sequence of the *cat* gene in plasmid pSV2cat (plasmid pSVpb2cat, pSVmcat, or pSVnscat [Table 1]). To study the effect of the NS1 protein coexpression, a second series of plasmids which, in addition to the *cat* gene, expressed the NS1 protein or the NS1 and NS2 proteins from the SV40 late promoter (plasmids pSVpb2catNS, pSVmcatNS, pSVnscatNS, pSVpb2catNS1, pSVmcatNS1, and pSVnscatNS1 [Table 1]) was prepared. The accumulation of *cat* mRNA was determined by dot hybridization of cytoplasmic poly(A)$^+$ RNA, and the accumulation of CAT was carried out by measuring the CAT activity in total cell extracts from the same transfected cultures (Fig. 5A and B). The quantitation of these results showed that expression of NS1 protein leads to an enhancement of about 10-fold in the efficiency of translation (ratio of the CAT activity to RNA signals) (Fig. 5C). Thus, it can be concluded that the signals to drive the NS1 effect on translation are, at least in part, located in the 5′-extracistronic sequences of these viral mRNAs.

Expression of NS1 protein induces an increase in the initiation rate of NP mRNA translation in vivo. The increase of viral mRNA translation observed by coexpression of NS1 protein could be exerted at the initiation and/or at the elongation rates of translation. These possibilities were analyzed by studying the size of viral mRNA-specific polysomes. An increase in their size would indicate a stimulation in the translation initiation, whereas an enhancement in elongation rate would lead to a decrease in the average polysome size. Thus, cytoplasmic extracts of cells transfected with plasmids pSVa963, pSVaN PNS1, or pSVaNPNS2 or cells infected with influenza virus were fractionated by centrifugation on sucrose gradients as described in Materials and Methods. The RNA from each fraction was isolated, and the NP-specific mRNA was determined by dot hybridization. The results are presented in Fig. 6 as a histogram showing the distribution of the NP RNA in material sedimenting at less than 80S (F; gradient fractions 1 to 5), at 80S (M; gradient fractions 6 to 10), as small polysomes (SP; gradient fractions 11 to 16), or as large polysomes (LP; gradient fractions 17 to 24). Most of the NP mRNA from cells transfected with plasmid pSVa963 was in the F or M fraction (Fig. 6, upper panel). On the contrary, a large proportion of the NP mRNA localized to fractions SP and LP in influenza virus-infected cells (Fig. 6, upper panel). Similar results were obtained by coexpression of NP with NS1 protein. Thus, a transfer of NP mRNA from the S plus M to the SP plus LP fractions was clearly apparent (Fig. 6, upper panel). On the contrary, coexpression of NS2 protein did not alter the polysome pattern of NP mRNA (data not shown). The true nature of polysomal structures in the fast-sedimenting material shown in Fig. 6 was demonstrated by treatment of the cytoplasmic fraction with EDTA before centrifugation or treatment of the cells with puromycin before fractionation. With EDTA treat-

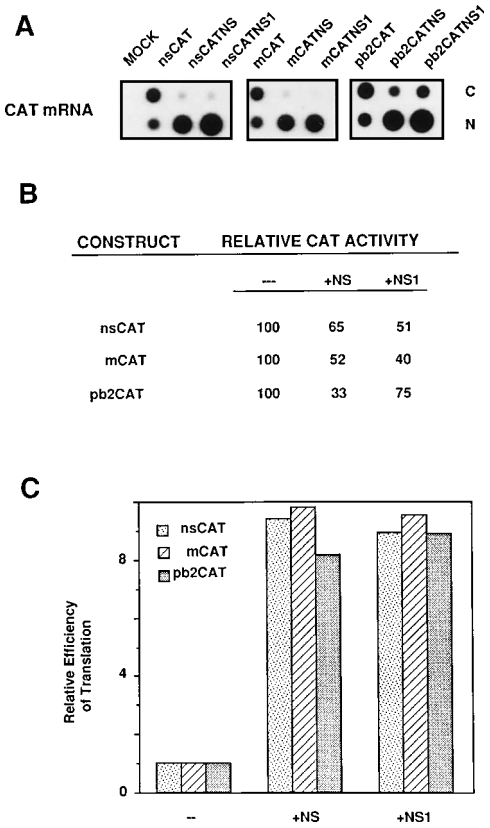

FIG. 5. Effects of the 5′-extracistronic sequences on the NS1 protein-mediated enhancement of translation. Cultures of COS-1 cells were transfected with plasmids pSVnscat (nsCAT), pSVnscatNS (nsCATNS), pSVnscatNS1 (nsCATNS1), pSVmcat (mCAT), pSVmcatNS (mCATNS), pSVmcatNS1 (mCATNS1), pSVpb2cat (pb2CAT), pSVpb2catNS (pb2CATNS), and pSVpb2 catNS1 (pb2CATNS1) or were mock transfected (MOCK). Cells were harvested and split into two portions, one of which was fractionated into nuclei and cytoplasm and processed for poly(A)$^+$ RNA isolation and the other of which was processed for total protein extraction, as described in Materials and Methods. The RNA preparations were analyzed by dot blot hybridization with a *cat*-specific probe, and the protein fractions were used for determination of enzymatic activity. (A) Hybridization signals obtained in the cytoplasmic (C) or nuclear (N) RNAs of cells transfected with the indicated plasmids. (B) Relative enzymatic activity expressed as a percentage of that obtained with extracts from cells not expressing NS proteins. (C) The signals in panel A were quantitated by microdensitometry, and their ratios to the activities indicated in panel B (normalized for the value obtained for the CAT sample) are indicated.

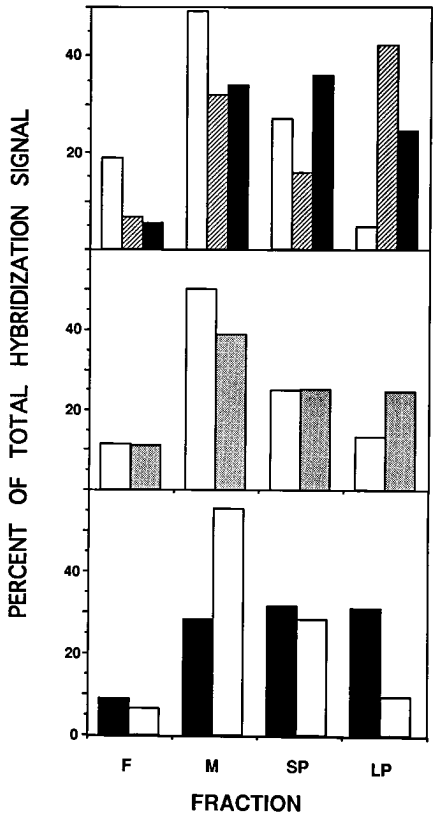

FIG. 6. Effects of NS1 protein expression on the size of NP- or *lacZ*-specific polysomes. Cultures of COS-1 cells were infected with influenza virus; transfected with plasmids pSVa963, pSVaNPNS1, pSVaNPNS2, pSVβgal, and pSVβgalNS1; or were mock transfected. At 5 h postinfection or 60 h posttransfection, the cells were collected and the cytoplasmic fractions were prepared. The polysomes were separated by centrifugation, and the RNA from each fraction was isolated as described in Materials and Methods. A sample of RNA from each fraction was hybridized to an NP- or a *lacZ*-specific probe, and the signals were quantitated by microdensitometry. The results are presented as a percentage of the total hybridization signal obtained from each gradient. Bars F, M, SP, or LP include the signals obtained in gradient fractions 1 to 5, 6 to 10, 11 to 16, or 17 to 24, respectively. (Upper panel) Results from cells infected with influenza virus (▨) or expressing NP alone (□) or in combination with NS1 protein (■). The results obtained by coexpressing NP and NS2 proteins were indistinguishable from those shown for NP. No hybridization signals were obtained from mock-transfected cells. (Middle panel) Results from cells expressing β-galactosidase alone (□) or in combination with NS1 protein (▨). (Lower panel) Results from cells coexpressing NP and NS1 protein (■) or from cytoplasmic preparations treated with EDTA, obtained from cell cultures transfected in parallel (□).

ment, the NP-specific mRNA, which sedimented with large polysomes in cells transfected with plasmid pSVaNPNS1, moved to more slowly sedimenting fractions (Fig. 6, lower panel). Similar results were obtained after puromycin treatment of the transfected cells (data not shown). It is worth pointing out the similarity in the polysome distribution of NP mRNA in cells expressing NP alone (Fig. 6, upper panel) and in extracts of cells expressing NP and NS1 protein, after treatment with EDTA (Fig. 6, lower panel). As a whole, these results suggest that influenza virus NS1 protein stimulates translation by, at least, enhancing the rate of initiation.

To check the specificity of the effect of NS1 protein on the protein initiation rate, the size of *lacZ*-specific polysomes in cells transfected with plasmids pSVβgal or pSVβgalNS1 was determined. The experiments were carried out as described above for NP-specific polysomes, and the results are shown in Fig. 6, middle panel. The pattern of *lacZ*-specific polysomes did not change upon coexpression of NS1 protein (Fig. 6, middle panel). This is in accordance with the absence of NS1 protein effect on the efficiency of β-galactosidase mRNA translation in vivo (Fig. 3C) and indicates that NS1 protein acts as a virus-specific translation enhancer.

In addition to the NS1 protein association with the nucleus, earlier reports (4, 27) had shown that a fraction of the protein had sedimentation properties compatible with it being associated with polysomes in influenza virus-infected cells. We have confirmed these findings by Western analysis of the polysomal gradient fractions isolated from influenza virus-infected cells (data not shown).

DISCUSSION

The results presented in this report indicate that the influenza virus NS1 protein is a powerful translational enhancer that specifically induces the usage of viral mRNAs. Thus, the

quantitative determinations of NP- or M1-specific cytoplasmic mRNA and their translation products show that the efficiency of translation is increased 5- to 100-fold by the concomitant expression of NS1 protein (Fig. 1 and 2). Our results are in agreement and extend those obtained by a transfection/infection approach (12, 13) and from a recent report in which a reconstituted viral transcription system was used (8). The translational enhancement was not a consequence of protein stabilization, since pulse-chase experiments indicated that NP half-life is not altered by coexpression of NS1 protein. A mechanism involving either a transcriptional induction or a stabilization of the mRNAs is also excluded, since the accumulation of the cytoplasmic mRNAs is directly measured and the half-life of NP mRNA is not affected by NS1 protein coexpression (10a). Furthermore, contrary to the experimental system used by Enami et al. (8), our experiments imply the expression of the NS1 protein and its target genes from cloned DNA, independent of the viral transcription machinery, i.e., other possible control mechanisms that might operate in the infected cell cannot be operative under these circumstances. The NS1 protein blocks the transport of RNA polymerase II transcripts from the nucleus to the cytoplasm (11, 36), and indeed, the absolute cytoplasmic concentrations of the target mRNAs are lower in its presence than in its absence. Therefore, it could be argued that the observed effect is the consequence of saturation of the translation machinery when large amounts of target mRNAs are present in the cytoplasm. However, these concentrations are about 10 times lower than in influenza virus-infected cells, and yet, no saturation is detected in the latter case, as judged by the incorporation of the viral NP mRNA to the polysomal fractions (Fig. 6, upper panel).

The NS1-mediated induction is virus specific, i.e., none of the nonviral mRNAs tested (CAT or β-galactosidase) showed significant translational enhancement upon expression of NS1 protein (Fig. 3). In addition, constructs of the *cat* gene containing the 5′-extracistronic regions of several viral RNA segments showed a reconstitution of the enhancing effect of NS1 protein on viral mRNAs (Fig. 5), suggesting that some of the *cis* elements required for the specific action of NS1 protein on viral mRNAs are located at that position in the genes. The translational stimulation observed for NP mRNA was larger than those detected for any of the influenza virus-CAT chimeric mRNAs (compare Fig. 1 and 4). This difference suggests that, in addition to a general enhancement of viral mRNA translation, NS1 protein may induce to a larger extent the expression of some viral mRNAs.

NS1 protein enhances the rate of translation initiation. The increase in the size of NP-specific polysomes when the NP gene is coexpressed with NS1 protein (Fig. 6), together with the observed enhancement in NP translation (Fig. 1), strongly supports the notion that NS1 protein stimulates translation initiation. This is also in agreement with the previously reported and here confirmed presence of NS1 protein in the polysomes of influenza virus-infected cells (4, 27). The viral specificity of the change in polysomal size (Fig. 6) correlates with the specificity of translational enhancement (Fig. 3) and is in line with the concept of the NS1 protein as a virus-specific translational enhancer.

During infection of susceptible cells with influenza virus, two subunits of the eIF-4F translation initiation factor are altered: the cap-binding protein component (eIF-4E; p24) is partially dephosphorylated, and hence inactivated, and the p220 component becomes hyperphosporylated (9), a modification that appears to stimulate its binding to protein synthesis initiation complexes (3). It is tempting to speculate that NS1 protein might interact with some component of the translation initiation machinery and thereby help the viral mRNAs to overcome the translational block induced by influenza virus infection.

The properties of NS1 protein as a translational enhancer are not without precedent. Thus, the human immunodeficiency virus Rev protein has been described to increase the initiation rate of polysome formation on mRNAs containing the Rev-responsive element (5). Likewise, the adenovirus L4 100-kDa protein, a nonspecific RNA-binding protein (37), shows similar activity on adenovirus late mRNAs, mainly on those containing the tripartite leader sequence (19). However, no information is available for any of these examples in regard to the way their biological effects are brought about. In these experimental systems, as well as for the action of NS1 protein (18), a gene gating model (2) to explain their biological activities has been proposed. We had proposed a similar model to explain the fast transport of the influenza virus mRNAs in infected cells (44), but the results presented here do not support such a hypothesis enough to justify the NS1-mediated translational enhancement. Thus, both the NS1 gene and the influenza virus genes used as targets are expressed from cloned DNA in the absence of any viral replication or transcription processes, their mRNAs are retained in the nucleus, and yet the fraction of these mRNAs that reaches the cytoplasm is preferentially translated.

In conclusion, the NS1 protein, a small nonstructural protein induced in influenza virus-infected cells, shows a plethora of biological effects on RNA metabolism: retention of poly(A)$^+$ RNA in the nucleus (11, 36), modulation of the splicing of pre-mRNAs (11), and, as shown here, specific stimulation of viral mRNA translation. This pleiotropy might not be a rare property of the NS1 protein but might simply reflect the identity among protein factors that play roles in several distinct processes relative to RNA modification and expression, as described for the polypyrimidine binding protein (p57) that is required for internal initiation of translation in poliovirus RNA (20) and the La antigen, a nuclear protein that enhances translation of poliovirus RNA (29, 43).

ACKNOWLEDGMENTS

We thank F. Gebauer, J. A. Melero, A. Nieto, A. Portela, and J. Valcárcel for critically reading the manuscript. We are indebted to W. Doerfler, M. Krystal, and P. Palese for generously providing biological materials. The technical assistance of J. Fernández, M. Pastor, and A. Sanz is gratefully acknowledged.

P.F. was a fellow from the Programa Nacional de Formación de Personal Investigador. This work was supported by the Comisión Interministerial de Ciencia y Tecnología (grant BIO92-1044), the Comunidad Autónoma de Madrid (grant A0063), and the EC SCIENCE Program (grant SC1*CT91-0688).

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