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Two Functional Domains of the Influenza Virus NS1 Protein Are Required for Regulation of Nuclear Export of mRNA

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The influenza virus NS1 protein is the only known example of a protein that inhibits the nuclear export of mRNA. To identify the functional domains of this protein, we introduced 18 2- or 3-amino-acid substitutions at approximately equally spaced locations along the entire length of the protein. Two functional domains were identified. The domain near the amino end (amino acids 19 through 38) was shown to be the RNA-binding domain, by using a gel shift assay with purified NS1 protein and spliced viral NS2 mRNA as the RNA target. The second domain, which is in the carboxy half of the molecule, was presumed to be the effector domain that interacts with host nuclear proteins to carry out the nuclear RNA export function, by analogy with the effector domain of the Rev proteins of human immunodeficiency virus (HIV) and other lentiviruses which facilitate rather than inhibit nuclear RNA export. The NS1 protein has a 10-amino-acid sequence that is similar to the consensus sequence in the effector domains of lentivirus Rev proteins, specifically including two crucial leucines at positions 7 and 9 of this sequence. However, the effector domains of the NS1 and Rev (HIV type 1 [HIV-1]) proteins differed in several significant ways including the following: (i) unlike the HIV-1 Rev protein, NS1 effector domain mutants were negative recessive rather than negative dominant, (ii) the NS1 effector domain is about three times larger than the effector domain of the HIV-1 Rev protein, and (iii) unlike the HIV-1 protein, NS1 effector domain mutants exhibited a surprising property, a changed intracellular/intranuclear distribution, compared with the wild-type protein. These differences strongly suggest that the effector domains of the NS1 and Rev proteins interact with different nuclear protein targets, which likely explains the opposite effects of these two proteins on nuclear mRNA export.

The mechanism and regulation of the nuclear export of RNA are not well understood. Insight into these processes can be expected to be obtained from studies of the mechanism of action of proteins that regulate nuclear export of mRNA. This export has been shown to be regulated in opposite ways by proteins encoded by two different viral genomes. The Rev (and Rev-like) proteins encoded by lentiviruses, including human immunodeficiency virus type 1 (HIV-1), facilitate the nucleocytoplasmic transport of unspliced and partially spliced viral mRNAs (14, 16, 26). In contrast, the NS1 protein encoded by influenza A virus inhibits the nucleocytoplasmic transport of poly(A)-containing viral spliced mRNAs (2, 33).

The Rev protein of HIV-1 contains essentially two functional domains (24). Towards the amino end is an RNA-binding domain, which is responsible for the binding of the protein to a specific RNA sequence (Rev responsive region) in the viral pre-mRNA target. This RNA-binding domain is coincident with the nuclear localization signal (NLS) of the Rev protein and also overlaps with the multimerization domain of the protein. The Rev protein multimerizes prior to or after binding to the Rev responsive region on the target mRNA (25, 30, 32, 39). The second domain, located towards the carboxyl end, has been termed the activation or effector domain (24). Mutations in this domain result in a dominant negative phenotype (18, 24). A key feature of this effector domain is a 10-amino-acid sequence that is conserved among the Rev-like proteins of lentiviruses (17, 27). The working hypothesis is that the effector domain interacts with one or more cellular proteins that facilitate the nuclear export of the HIV pre-mRNA bound to the RNA-binding domain of the Rev protein (24). These cellular proteins might be components of the splicing machinery and/or of the nuclear machinery that exports mRNA (6, 11, 15, 20, 23, 24, 26).

In the present study, we carried out an extensive mutational analysis of the influenza virus NS1 protein which inhibits rather than facilitates the nuclear export of mRNA. We show that the NS1 protein has two functional domains. We demonstrate that the domain near the amino end is the RNA-binding domain, which binds to the poly(A) sequence in mRNAs (33). Our working hypothesis is that the second domain, which is in the carboxy-terminal half of the molecule, is the effector domain of the NS1 protein that interacts with cellular nuclear proteins in order to inhibit the nuclear export of mRNA. We determine several properties of this putative effector domain and demonstrate that this domain has an apparent sequence similarity to Rev effector domains but differs in several significant ways from the effector domain of the most extensively characterized Rev protein, that of HIV-1.

MATERIALS AND METHODS

Construction of molecular clones. The NS1 protein was expressed by using an NS1 gene containing a 3′ splice site mutation (NS3ss), which was ligated into the pBC12 vector via *Bam*HI linkers as described previously (2). Oligonucleotide-directed mutagenesis using either a bacteriophage M13 mutagenesis system (Boehringer Mannheim Biochemicals) or PCR (Perkin-Elmer Cetus) was used to introduce point mutations into this NS1 gene. All mutations were confirmed by dideoxynucleotide sequencing. The NS1 mRNA target was transcribed from the NS3ss(dm) gene, an NS1 gene containing both a 3′ splice site mutation and two amber mutations in the

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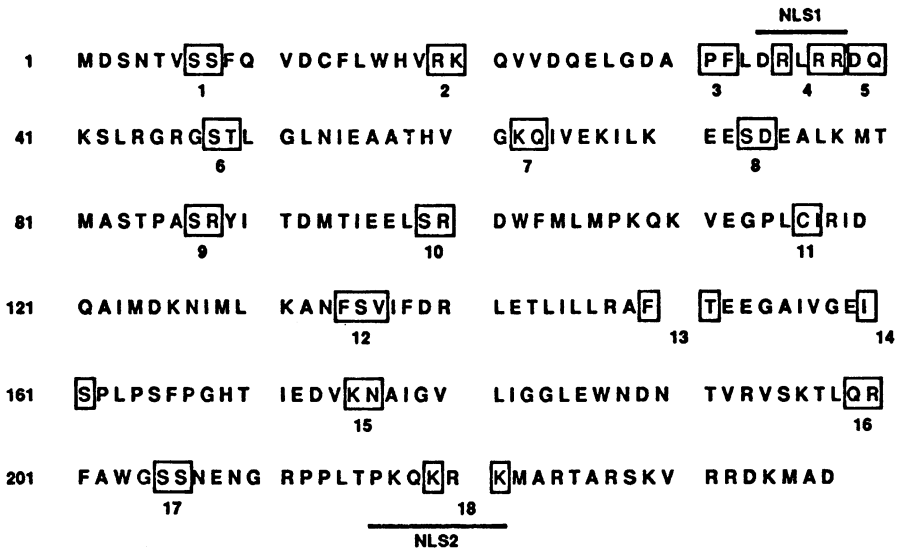

FIG. 1. Location of mutations introduced into the NS1 protein. The influenza virus A/Udorn NS1 gene encodes a 237-amino-acid protein with two NLSs (NLS1 and NLS2), as shown. The positions at which 2- or 3-amino-acid changes were made are boxed. These mutations were numbered according to their position within the NS1 protein, with 1 being the most N terminal and 18 being the most C terminal.

NS1 protein reading frame. This gene was inserted into the PBC12 vector by blunt-end ligation. Wild-type NS1 and some mutant NS1 coding sequences were excised from the PBC12 vector at the *Bam*HI site and ligated into the unique *Bam*HI site of the *Escherichia coli* expression vector pGEX-3X (Pharmacia LKB Biotechnology) for the preparation of glutathione *S*-transferase (GST)-NS1 fusion proteins.

Cell culture and transfection. 293 cells were maintained in Dulbecco's modified eagle medium containing 10% fetal calf serum and were transfected by the calcium phosphate method (9). The level of plasmid DNA used in each transfection experiment is indicated in the corresponding figure legend.

RNA extraction and analysis. Transfected 293 cells were harvested 40 h posttransfection. The cells were fractionated into nuclei and cytoplasm, as described previously (2). The fractionation was monitored by agarose gel analysis to determine the presence of rRNA markers characteristic of the nucleus (45S) and of the cytoplasm (18S). RNA was extracted by the guanidinium isothiocyanate method (7). The amounts of RNA in the cytoplasm and nucleus were determined both by A_{260} and by quantitating the ethidium-stained agarose gel analysis of the cytoplasmic and nuclear RNAs. Cell-equivalent amounts of nuclear and cytoplasmic RNAs were assayed by S1 nuclease protection using 5′-end-labeled single-stranded DNA probes (9). The probe was obtained by ligating an *Nco*I-*Eco*RI fragment derived from the PBC12 plasmid containing NS3ss(dm) to the *Sma*I-*Eco*RI large fragment of M13mp19. A 24-mer oligonucleotide complementary to PBC12 sequences was phosphorylated at the 5′ end with [γ-^{32}P]ATP and was annealed to the single-stranded M13 DNA template. After primer extension using the Klenow fragment followed by *Ava*I digestion, the probe was purified on an alkaline-denaturing agarose gel. This probe detects only the NS1 3ss(dm) mRNA target and not the NS1 3ss mRNA that directs the synthesis of the NS1 protein, because the 3′ cloning sites of the pair of NS1 genes in PBC12 differ. After S1 nuclease digestion, the protected fragment(s) was resolved by gel electrophoresis (5% acrylamide). The relative amounts of nuclear and cytoplasmic NS1 mRNA were determined with either a densitometer or a phosphorimager.

RNA-binding analysis. The synthesis of [α-^{32}P]UTP-labeled RNA used in the RNA binding assays was performed by using standard protocols. Full-length NS2 cDNA was subcloned into the PGEM1 vector at a *Bam*HI site so that its synthesis was under the control of T7 RNA polymerase. The transcript was purified on a denaturing polyacrylamide gel. The GST-NS1 fusion protein (wild-type or mutant NS1 protein) was purified as described in Qiu and Krug (33). An RNA-binding experiment was performed by incubating a particular purified GST-NS1 protein with labeled NS2 mRNA, and the protein-RNA complexes were resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel (33). The gels were then dried and visualized by autoradiography.

Immunofluorescence. 293 cells, which were grown on glass coverslips in 35-mm culture dishes, were transfected with 5 μg of one of the plasmids specified in Fig. 7. Forty hours posttransfection the cells were washed with phosphate-buffered saline, fixed with methanol for 20 min at -20°C, and treated with rabbit polyclonal anti-NS1 antiserum (1:50) and then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (1:100). The anti-NS1 antiserum was obtained from M. Krystal and P. Palese (12, 38).

RESULTS

Identification of the functional domains of the influenza virus NS1 protein by site-directed mutagenesis. The influenza A virus (Udorn strain) NS1 protein consists of 237 amino acids (Fig. 1). To identify the functional domains of this protein, we introduced 18 point mutations into the protein. These mutations were approximately evenly spaced throughout the protein. The locations of these mutations are shown in Fig. 1. The NS1 protein contains two NLSs (13), and mutations were introduced into each of these NLSs (mutations 4 and 18). The majority of the mutations involved the replacement of a pair of amino acids by aspartic acid (D) and leucine (L). This replacement introduced *Bgl*II sites, so that deletion mutants could be readily made. If the DL mutation resulted in a mutant phenotype, a different, less drastic mutation—namely, the substitution of two alanines (AA) for the normal pair of amino

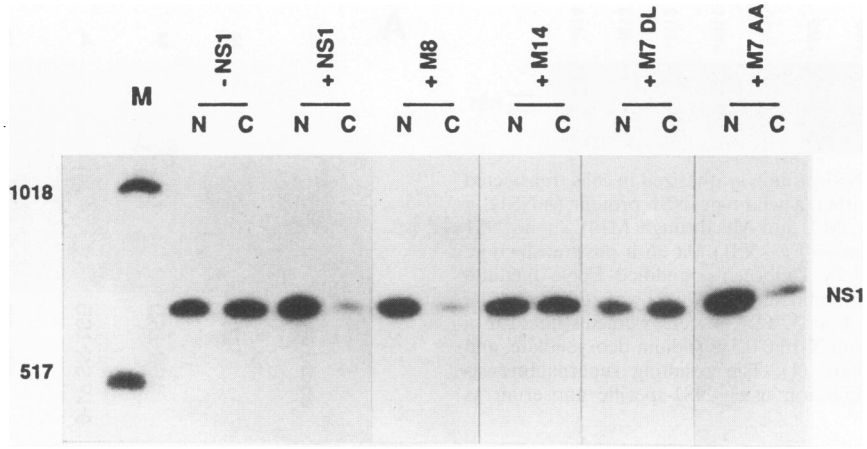

FIG. 2. Effects of several representative mutated NS1 proteins on the nuclear export of NS1 mRNA. 293 cells were cotransfected with 5 μg of the PBC12 plasmid encoding the target NS1 mRNA and 10 μg of the indicated PBC12 plasmid encoding the NS1 coding sequence with two amber mutations [NS3ss(dm)] (−NS1), wild-type protein (NS3ss) (+NS1), mutant 8 (DL), mutant 14 (DL), or mutant 7 (DL or AA). At 40 h posttransfection, the cells were fractionated into nuclei and cytoplasm, and the nuclear (N) and cytoplasmic (C) RNAs were extracted and subjected to S1 analysis as described in Materials and Methods. The protected fragments were resolved by gel electrophoresis, followed by autoradiography. Lane M, markers.

acids—was made at the same position. Only if the AA as well as the DL replacement resulted in a mutant phenotype did we consider the site of the amino acid replacement as being part of a functional domain. In some cases, three amino acids were replaced (mutants 4 and 12), and in some cases only the AA (or AAA) and not the DL mutation was made (mutants 3 to 5 and 15 to 18).

We have previously documented the function of the NS1 protein in regulating the nuclear export of mRNA. Initially, it was shown that this protein inhibits the nuclear export of two viral mRNAs: spliced NS2 mRNA and unspliced NS1 mRNA containing a 3′ splice site mutation (2). Subsequently, we showed that the NS1 protein inhibits the nuclear export of all mRNAs containing poly(A) (33). In the present study, we determined by transfection assays whether the mutated NS1 proteins retained the ability to inhibit the nuclear export of NS1 mRNA containing a 3′ splice site mutation. The target NS1 mRNA also contained two amber mutations and hence did not encode a functional NS1 protein. The vector specifying this target NS1 mRNA was mixed with a vector specifying wild-type or mutated NS1 protein, and the mixture was transfected into 293 cells. At 40 h after transfection, the cells were fractionated into nuclear and cytoplasmic fractions. The purity of the nuclear and cytoplasmic fractions was monitored as described in Materials and Methods. The amounts of the target NS1 mRNA in the nucleus and cytoplasm were determined by S1 analysis of cell-equivalent amounts of nuclear and cytoplasmic RNAs.

The effects of several representative mutated NS1 proteins on the nuclear/cytoplasmic distribution ratio of the target NS1 mRNA are shown in Fig. 2. In the absence of the NS1 protein, about 60% of the target NS1 mRNA was in the cytoplasm; this varied from 50 to 80% in different experiments. In contrast, in the presence of the NS1 protein, only about 5% of the NS1 mRNA was in the cytoplasm. The shift in the nuclear/cytoplasmic distribution ratio of the NS1 mRNA caused by the NS1 protein was approximately 25-fold. As previously documented (2), this shift is due to the inhibition of nuclear export of NS1 mRNA by the NS1 protein. Some of the mutated NS1 proteins (e.g., mutant 8, containing a DL substitution) retained the ability to inhibit the nuclear export of NS1 mRNA, as the nuclear/cytoplasmic distribution ratio of NS1 mRNA with these mutated NS1 proteins was the same as that with wild-type NS1 protein. Some of the mutated NS1 proteins clearly lost the ability to inhibit the nuclear export of NS1 mRNA. For example, mutant 14 NS1 protein with either a DL or an AA substitution did not affect the nuclear/cytoplasmic distribution ratio of NS1 mRNA. Consequently, this mutation was considered to be in a functional domain of the NS1 protein. In contrast, mutation 7 was considered not to be in a functional domain, because only the DL substitution and not the AA substitution caused a loss of NS1 protein function in the inhibition of nuclear NS1 mRNA export. Presumably the KQ-to-DL change at this position in the protein was drastic enough to cause an overall conformational change in the protein that led to a loss of function.

Table 1 summarizes the results obtained with the 18 mutants. Only six of these mutants lost function in nuclear mRNA

TABLE 1. Phenotypic analysis of influenza virus NS1 gene mutants

Mutant	Position of mutation	Mutation(s)[a]
1	7, 8	SS→DL (−), SS→AA (+)
2	19, 20	RK→DL (−), RK→AA (−)
3	31, 32	PF→AA (−)
4	35, 37, 38	RRR→AAA (+/−)
5	39, 40	DQ→AA (+)
6	48, 49	ST→DL (+)
7	62, 63	KQ→DL (−), KQ→AA (+)
8	73, 74	SD→DL (+)
9	87, 88	SR→DL (+)
10	99, 100	SR→DL (+)
11	116, 117	CI→DL (+)
12	134–136	FSV→LDL (−), FSV→AAA (−)
13	150, 151	FT→DL (−), FT→AA (−)
14	160, 161	IS→DL (−), IS→AA (−)
15	175, 176	KN→AA (+)
16	199, 200	QR→AA (+)
17	205, 206	SS→AA (+)
18	219, 221	KK→AA (+)

[a] Phenotypes: +, wild type; −, mutant; +/−, partial mutant.

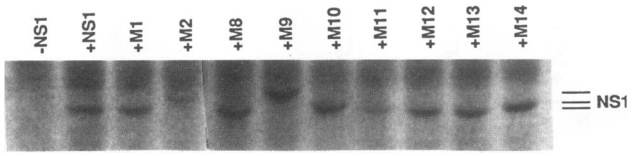

FIG. 3. Amount of the NS1 protein synthesized in cells transfected by a plasmid expressing either a wild-type NS1 protein (+NS1), a mutated NS1 protein (M1, M2, and M8 through M14), or no NS1 protein [the NS3ss(dm) plasmid] (−NS1). At 36 h posttransfection, the medium was replaced by Dulbecco's modified Eagle medium lacking methionine. ^{35}S-methionine at 100 μCi/ml was added, and the cells were incubated for 6 h at 37°C. The cells were disrupted in a buffer containing 0.5% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (1). The resulting supernatant was subjected to immunoprecipitation using NS1-specific antiserum as previously described (2).

export. To determine whether all the mutants synthesized the NS1 protein, we measured the amount of the NS1 protein synthesized in cells transfected with a plasmid expressing either wild-type NS1 protein or a mutated NS1 protein. After a 6-h labeling period with ^{35}S-methionine, cell extracts were immunoprecipitated with NS1 antiserum, and the precipitated proteins were analyzed by gel electrophoresis (Fig. 3). The amount of the NS1 protein synthesized varied to a certain extent. Some of the mutated NS1 proteins were synthesized at about the same level as the wild-type NS1 protein (mutants 1 and 10 [wild-type phenotype] and mutants 12 and 13 [loss-of-function phenotype]). Some were synthesized at higher levels (mutants 8 and 9 [wild-type phenotype] and mutant 14 [loss-of-function phenotype]). Some were synthesized at lower levels (mutant 11 [wild-type phenotype] and mutant 2 [loss-of-function phenotype]). Clearly, there was no correlation between the amount of the NS1 protein synthesized and the phenotype of the mutant. In fact, the particular small differences in the amount of synthesis of the various mutated NS1 proteins shown in Fig. 3 were not reproducible, suggesting that there was little or no significant difference between the amount of the NS1 protein synthesized by the wild type and the amounts synthesized the various mutants (also see Fig. 7). It should also be noted that some of the mutations caused a change in the mobility of the NS1 protein (mutants 2, 8, and 9).

The six loss-of-function mutants clearly defined two functional domains (Table 1). One domain was defined by mutants 2 through 4 and encompassed amino acids 19 through 38. It should be noted that mutant 4, in which the three arginines at positions 35, 37, and 38 were all changed to alanines, did not lose all function. The assays for regulation of NS1 mRNA nuclear export indicated that mutant 4 retained about 25% of wild-type function (data not shown). These three arginine residues were apparently at the carboxy-terminal border of this functional domain, because mutation of amino acids 39 and 40 (mutant 5) did not lead to the loss of function. The second domain was defined by mutants 12 through 14 and was in the carboxy-terminal half of the NS1 protein, encompassing amino acids 134 to 161. As expected, deletions of either protein domain (Δ1-6 or Δ11-14) caused a loss of function.

The amino-proximal functional domain is the RNA-binding domain. To determine which domain functioned in RNA binding, we employed the specific in vitro RNA binding assay that identified poly(A) as the binding site of the NS1 protein on NS2 mRNA and other mRNAs (33). In this assay ^{32}P-labeled NS2 mRNA was mixed with a purified GST-NS1 fusion protein in the presence of an excess of tRNA, and binding of

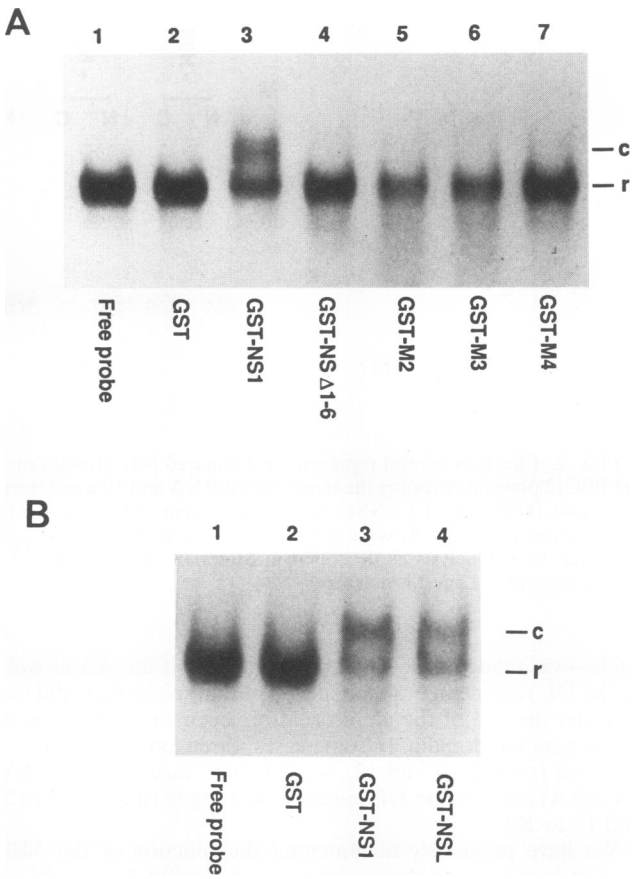

FIG. 4. The amino-proximal functional domain is the RNA-binding domain. Equal amounts (250 ng) of GST or of each of the indicated GST-NS1 fusion proteins were incubated with ^{32}P-labeled NS2 mRNA (10,000 cpm). The protein-RNA complex (c) was resolved from free RNA (r) by native gel electrophoresis. (A) Mutants in the amino-proximal functional domain. (B) Mutant (NSL) in the second functional domain.

the protein to the labeled RNA was assayed by gel shift, i.e., the shift of some of the NS2 mRNA to a more slowly migrating species during gel electrophoresis. The wild-type NS1 fusion protein bound to NS2 mRNA (Fig. 4A) as described previously (33). In contrast, the Δ1-6 deletion mutant lacking the first domain, as well as three of the domain 1 mutant NS1 proteins (mutants 2 to 4), failed to bind to and gel shift NS2 mRNA. Mutants in the second domain did not share this property, indicating that the second domain in the NS1 protein was not involved in RNA binding. For the experiment in Fig. 4B we used an NS1 protein (termed NSL) containing three L-to-A mutations in the second domain (see below). The GST-NSL fusion protein bound to and gel shifted NS2 mRNA like the wild-type NS1 fusion protein. Other mutants in this domain behaved the same way. The same results were obtained with labeled poly(A) itself as the target RNA (data not shown). These results indicated that domain 1 is the RNA-binding domain of the NS1 protein and that RNA binding by the NS1 protein is required for the inhibition of the nuclear export of mRNA.

Characterization of second-domain mutants. By analogy with the HIV Rev protein, our working hypothesis is that the second functional domain of the NS1 protein is the effector

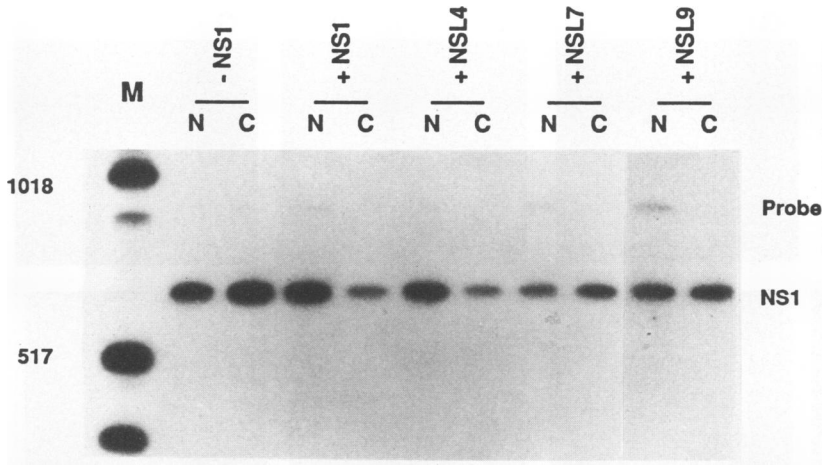

FIG. 5. The leucines at positions 7 and 9 of the 10-amino-acid effector sequence are required for NS1 protein function. 293 cells were cotransfected with 5 μg of the PBC12 plasmid encoding the target NS1 mRNA and 10 μg of a PBC12 plasmid encoding either no NS1 protein [the NS3ss(dm) gene] (−NS1), wild-type NS1 protein (+NS1), or NS1 protein with an L-to-A mutation at position 4 (NSL4), position 7 (NSL7), or position 9 (NSL9) of the 10-amino-acid sequence in the effector domain. At 40 h posttransfection, the cells were fractionated into nuclei and cytoplasm, and the nuclear (N) and cytoplasmic (C) RNAs were subjected to S1 analysis. The protected fragments were resolved by gel electrophoresis, followed by autoradiography. Lane M, markers.

domain, i.e., the domain that interacts with host nuclear targets to carry out the inhibition of mRNA transport. The effector domains of lentivirus Rev and Rev-like proteins contain a consensus sequence, Z- - -LJJLTLJ (Z is a hydrophobic amino acid and J is a hydrophilic amino acid) (17, 27). The putative effector domain of the NS1 protein contains a 10-amino-acid sequence, FDRLETLILL (amino acids 138 through 147), that is similar to this consensus sequence. In particular, an L residue is found at positions 7 and 9 of both the NS1 and most of the Rev sequences; a few Rev-like proteins contain isoleucine rather than L at position 9. These two L residues, as well as the L at position 4, have been shown to be required for the function of the Rev protein of HIV-1. When A was substituted for any of these three Ls, then the function of this Rev protein was lost (27). To determine whether the NS1 protein behaved similarly, we individually changed each L at positions 4, 7, and 9 in the NS1 sequence to an A (Fig. 5). When L at position 4 was changed to A, the mutated NS1 protein behaved like wild-type NS 1 protein: NS1 mRNA was retained in the nucleus. In contrast, when either the L at position 7 or the L at position 9 was changed to A, the mutated NS1 protein lost function: NS1 mRNA was transported to the cytoplasm essentially as efficiently as in the absence of the NS1 protein. Hence, analogously to the situation with the HIV-1 Rev protein, these two Ls in this 10-amino-acid sequence are required for the activity of the NS1 protein in regulating the nuclear export of NS1 mRNA. The NSL mutant used in the RNA-binding experiment described above contained L-to-A mutations at positions 4, 7, and 9 of this 10-amino-acid sequence.

The HIV-1 Rev proteins that contain a loss-of-function mutation in the effector domain exhibit dominant negative phenotypes, i.e., they inhibit the function of the wild-type Rev protein in *trans* (18, 24). Efficient inhibition occurred only when the mutant Rev proteins were present in excess. To determine whether the NS1 loss-of-function mutants in the second domain exhibited a dominant negative phenotype, increasing amounts of a plasmid expressing the NS1 protein with an L-to-A mutation at position 7 of the 10-amino-acid effector domain sequence (NSL7) were cotransfected with the plasmid expressing wild-type NS1 protein, and the nuclear/cytoplasmic distribution ratio of target NS1 mRNA was determined (Fig. 6). Even when the ratio of NSL7 to wild-type NS1 plasmid was 20 to 1, wild-type function in regulating NS1 mRNA transport was not inhibited. In other experiments it was found that even a slightly higher ratio, 25 to 1, of NSL7 to wild-type NS1 plasmid did not inhibit wild-type function. The same result was obtained with the effector domain deletion mutant Δ11-14. This indicates that NS1 effector domain mutants are negative recessive rather than negative dominant mutants, in contrast to the HIV-1 Rev effector domain mutants

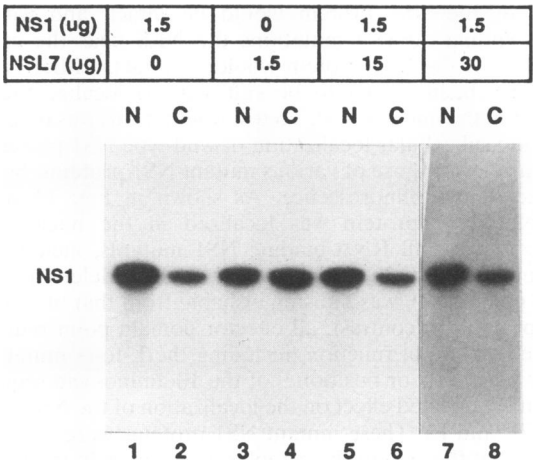

FIG. 6. NS1 effector domain mutants are negative recessive mutants. 293 cells were cotransfected with 3 μg of the PBC12 plasmid encoding the target NS1 mRNA and the indicated amounts of the PBC12 plasmids encoding wild-type NS1 protein and/or the PBC12 plasmid encoding the NSL7 effector domain mutant (see the legend to Fig. 5). The amount of total plasmid added to each transfection was normalized by the addition of an appropriate amount of the PBC12 plasmid lacking an NS1 protein coding insert. The amounts of the target NS1 mRNAs in the nucleus (N) and cytoplasm (C) were determined by S1 analysis.

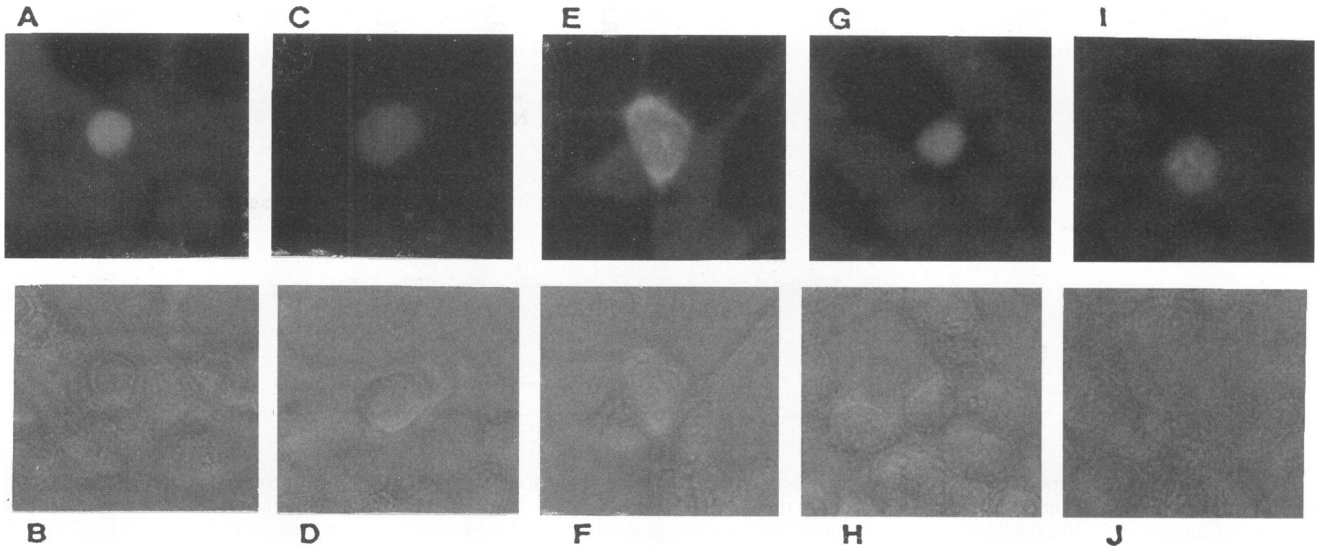

FIG. 7. Subcellular localization of wild-type and mutated NS1 proteins determined by indirect immunofluorescence. Immunofluorescence (upper panels) and corresponding phase-contrast photographs (lower panels) of transfected, fixed 293 cell cultures are presented. Cells were treated with rabbit polyclonal anti-NS1 antiserum and then with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G. Representative examples are shown. (A and B) Wild-type NS1; (C and D) M4 mutant; (E and F) M12 mutant; (G and H) NSL4 mutant (see the legend to Fig. 5); (I and J) Δ11-14 deletion mutant.

(18, 24). As is the case for HIV-1 Rev RNA-binding mutants (24), the NS1 mutant 2 RNA-binding mutant was also found to be a negative recessive mutant. At a 25-to-1 ratio of mutant 2 to wild-type plasmid, wild-type function in regulating NS1 mRNA transport was not inhibited (data not shown).

Rev proteins of HIV-1 that contain mutations in the effector domain are localized in the nucleus like the wild-type protein (24). It was anticipated that mutations in the NS1 protein second functional domain, as well as mutations in the RNA-binding domain would not alter the nuclear localization of the NS1 protein. With mutations in the second domain, both of the NLSs of the NS1 protein would be intact, and with the RNA-binding domain mutations the NLS near the carboxy terminus of the NS1 protein would be intact. Either NLS by itself has been shown to be sufficient to localize the NS1 protein in the nucleus (13). To test whether this was indeed the case, the subcellular localization of wild-type NS1 protein was compared with those of various mutant NS1 proteins, by using indirect immunofluorescence. As shown in Fig. 7A and B, wild-type NS1 protein was localized in the nucleus. The localization of all RNA-binding NS1 mutants, including the M4 mutant (Fig. 7C and D) and the Δ1-6 deletion mutant (data not shown), was indistinguishable from that of wild-type NS1 protein. In contrast, all effector domain point mutations that led to loss of function, including the L-to-A mutation at either position 7 or position 9 of the 10-amino-acid sequence, had an unexpected effect on the localization of the NS1 protein (Fig. 7E and F). These mutant NS1 proteins were spread out throughout the cell, in the cytoplasm as well as in the nucleus, even though the two NLSs were intact. In contrast, the NS1 protein with the L-to-A mutation at position 4 of the 10-amino-acid sequence in the effector domain was localized in the nucleus-like wild-type NS1 protein (Fig. 7G and H); unlike the other effector domain mutants, this NS1 protein retained wild-type function in regulating nuclear export of mRNA. These results suggested that the loss of effector domain function per se might cause some of the NS1 protein to appear in the cytoplasm. To determine whether this was the case, we examined the localization of the NS1 protein deletion mutant, Δ11-14, lacking the entirety of the effector domain (also a loss-of-function mutant). This NS1 protein was localized in the nucleus, in the form of speckles (Fig. 7I and J), indicating that the loss-of-effector domain function by itself was not sufficient to cause the localization of a substantial amount of the NS1 protein in the cytoplasm. Rather, this cytoplasmic localization occurred when the effector domain was present but contained point mutations that resulted in the loss of function in the regulation of nuclear export of mRNA. These immunofluorescence results also showed that (i) the cells expressing functional NS1 protein were considerably smaller than the cells expressing nonfunctional NS1 protein, presumably indicating that functional NS1 proteins inhibited cell growth, and (ii) mutant and wild-type NS1 proteins were expressed at comparable levels, confirming the immunoprecipitation assays (Fig. 3).

Several approaches were taken to try to transform the NS1 protein from an inhibitor of nuclear RNA export into a facilitator of RNA export like the Rev protein. One possibility was that the NS1 protein was an inhibitor rather than a facilitator because of the presence of a second NLS in the NS1 protein. Because the first NLS and the RNA-binding domain overlap, the first NLS might become nonfunctional after this domain of the NS1 protein binds to its target RNA sequence. However, the second NLS in the NS1 protein might then be responsible for causing the nuclear retention of the target RNA. Because the Rev protein does not have a second NLS, nuclear retention would not be expected to occur. Two experiments eliminated this possibility. When the second NLS of the NS1 protein was eliminated by a 2-amino-acid substitution (mutant 18) (Table 1) or by deletion (removal of the 23 carboxy-terminal amino acids) (data not shown), the mutated NS1 protein fully retained wild-type activity in inhibiting NS1 mRNA transport. Thus, the 23 carboxy-terminal amino acids are not required for the function of the NS1 protein in regulating nuclear mRNA export. The second approach was to change the 10-amino-acid sequence in the NS1 protein second

domain so that it was almost identical, rather than simply similar, to the 10-amino-acid effector domain in a specific Rev protein that facilitates nuclear RNA export. By changing only three amino acids, at positions 6, 8, and 10 of the NS1 second-domain 10-amino-acid sequence, the last seven amino acids of this sequence were transformed from LETLILL to LERLTLD, which is identical to the sequence in the HIV-1 Rev protein. However, even this small change in the NS1 protein sequence resulted in the loss of all function in regulating nuclear mRNA transport (data not shown).

DISCUSSION

The influenza virus NS1 protein is the only known example of a protein that inhibits the nuclear export of mRNA. In the present study we establish that this protein has two functional domains: an RNA-binding domain and a second domain which is most likely the effector domain. The RNA-binding domain, which binds to the poly(A) in mRNAs (33), was identified by using a specific gel shift assay with purified NS1 protein and spliced viral NS2 mRNA as the RNA target (33). Mutations that caused a loss of binding in the gel shift assay also caused a loss of function in nuclear mRNA export regulation, indicating that the same domain is required for both the in vitro and the in vivo functions. The mutational analysis identified a 20-amino-acid sequence (amino acids 19 through 38) in the NS1 protein of the Udorn A type virus as the RNA-binding domain. This sequence is well conserved among some type A influenza virus strains (94 to 100% identity), is partially conserved among other type A strains (71 to 89% identity), and is only weakly conserved among a few type A strains (57% identity) (4). This sequence in the Udorn virus NS1 protein is not homologous with the binding domains of other poly(A)-containing proteins. These domains consist of one or more RNA recognition motifs about 80 nucleotides in length, each of which contains both an 8-amino-acid and a 6-amino-acid conserved sequences (RNP-1 and RNP-2, respectively) (3, 5, 19, 28, 31, 37). Consequently, our results indicate that an amino acid sequence that differs from this previously identified consensus sequence can also bind specifically to poly(A). The RNA-binding domain of the NS1 protein also does not have any evident homology with other known RNA-binding domains. In addition, this sequence is not arginine rich, as in some RNA-binding domains (22, 24, 34). In fact, the NS1 RNA-binding domain does not even have a high net positive charge. Of the 20 amino acids, 6 are basic and 4 are acidic. It will be of great interest to determine the mode by which this amino acid sequence binds to poly(A).

Our working hypothesis is that the second functional domain is the effector domain, the domain that interacts with cellular nuclear targets to carry out the function of inhibiting the nuclear export of mRNA. The HIV-1 Rev protein, which also regulates the nuclear export of RNA, has a functional domain separate from its RNA-binding domain, and this second domain, termed the effector domain, is presumed to interact with host nuclear proteins to accomplish the regulation of the nuclear export of viral pre-mRNAs (24). These putative cellular proteins have not yet been identified, and consequently the mechanism of action of the Rev effector domain has not yet been established. In the present study we have determined several characteristics of the presumed effector domain of the NS1 protein and have compared these characteristics with those of the effector domain of Rev proteins.

The NS1 effector domain contains a 10-amino-acid sequence that is similar to a consensus sequence found in the effector domains of Rev proteins. In particular, the two Ls which are located at positions 7 and 9 of this sequence are crucial for the function of both the NS1 and Rev proteins (27; also this study). However, this similarity may be more apparent than real. The actual identity of the amino acids in this effector sequence differs between the NS1 protein and individual Rev proteins, including the Rev protein of HIV-1, and changing only three amino acids in the NS1 effector domain to increase the similarity to the HIV-1 10-amino-acid effector domain sequence inactivated the function of the NS1 protein. In addition, the NS1 effector domain is significantly larger than the effector domain of the HIV-1 Rev protein, which, on the basis of mutational analysis, consists of at most only a 10-amino-acid consensus sequence (27). Our mutational analysis indicates that the NS1 effector domain is at least 28 amino acids in length, extending from amino acid 134 to 161. In fact, this analysis does not eliminate the possibility that the NS1 effector domain is even larger, i.e., starting at some amino acid between mutant 11 (amino acids 116 and 117) and mutant 12 (amino acids 134 to 136) and ending at some amino acid between mutant 14 (amino acids 160 and 161) and mutant 15 (amino acids 175 and 176). Figure 8 shows the comparison of the functional domains of the HIV-1 Rev and influenza virus NS1 proteins. There are also several other significant differences between the NS1 and Rev effector domains.

The NS1 effector domain mutants exhibited a surprising property, which is not shared by Rev effector domain mutants. All of the NS1 proteins containing a loss-of-function point mutation in this domain were not localized predominantly in the nucleus like wild-type NS1 protein but, instead, were spread throughout the cell, in the cytoplasm as well as in the nucleus. Even a single amino acid change, L to A at position 9 of the consensus effector sequence, caused this change in localization. This is particularly surprising because these mutant NS1 proteins, like the wild-type protein, contain two NLSs (13). In contrast, HIV-1 Rev effector domain mutants retain their nuclear localization (24). It was not loss of effector function per se that resulted in the cytoplasmic accumulation of some of the NS1 protein, because an NS1 protein containing a deletion of the entirety of the effector domain (also a loss-of-function mutation) localized predominately in the nucleus. This result also made it unlikely that the incomplete nuclear localization of the NS1 proteins containing a single L-to-A mutation in the effector domain was due to protein misfolding, as the effector domain deletion is a much more drastic alteration of the protein. Also, both of these types of effector domain mutants retained their abilities to bind to NS2 mRNA and poly(A). Hence, substantial cytoplasmic as well as nuclear localization of the NS1 protein occurred only when the effector domain was present and was inactivated by a point mutation. A possible explanation stems from the observation that even wild-type NS1 protein has some affinity for one or more cytoplasmic targets. Specifically, the small amount of the wild-type NS1 protein that is present in the cytoplasm has been shown to be associated with ribosomes and polyribosomes (8, 21). It has not been established whether this ribosome-associated NS1 protein has any function. Perhaps the loss of function point mutations in the effector domain of the NS1 protein lowered its affinity for nuclear targets but did not affect its already existing affinity for polyribosomes, so that the cytoplasmic affinity became predominant even over the two NLSs.

Unlike the effector domain mutants of the HIV-1 Rev protein (18, 24), the effector domain mutants of the NS1 protein, both point mutations and deletions, were not dominant negative versus the wild-type NS1 protein. With at least one Rev protein, the visna virus Rev protein, effector domain mutants exhibited only a weakly dominant negative phenotype

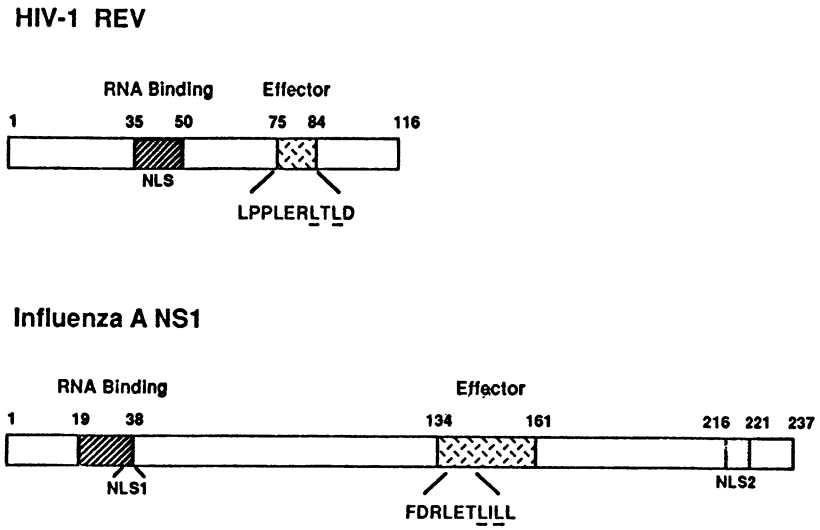

FIG. 8. Comparison of the functional domains of the influenza virus A NS1 protein with those in the HIV-1 Rev protein. The approximate locations of RNA-binding and effector domains are indicated. The 10-amino-acid effector domain sequence is shown with the two critical leucine residues underlined. The NLS2 in the NS1 protein is dispensable for the function of the NS1 protein in regulating the nuclear export of mRNA.

(35). Consequently, a dominant negative phenotype may not be a common property of effector domain mutants of proteins that regulate nuclear mRNA export. Two general mechanisms for the dominant negative activities of effector domain mutants have been proposed. In one mechanism, these mutant proteins bind to target mRNA molecules, thereby sequestering these mRNA molecules away from the wild-type protein (24). The NS1 protein effector mutants might be expected to be capable of participating in this type of mechanism. These mutant NS1 proteins retained the ability to bind to poly(A)-containing mRNAs. Though some of the NS1 protein molecules containing loss-of-function point mutations in the effector domain were found in the cytoplasm, a substantial amount remained in the nucleus, and the vast majority of the NS1 protein molecules lacking the entirety of the effector domain were located in the nucleus. However, because the NS1 protein binds to the poly(A) sequence that is common to almost all mRNAs (33), NS1 protein effector domain mutants would probably not be able to sequester all the target mRNA molecules away from the wild-type protein and, therefore, might not be dominant negative. This possibility can be tested by changing the RNA-binding specificity of the NS1 protein (see below). In the second mechanism, if multimerization of the transport regulatory protein were required for its activity, the effector domain mutants might inhibit activity by forming mixed multimers with wild-type protein (18). Preliminary experiments indicate that the NS1 protein forms multimers (30a), but it has not yet been established whether this multimerization is required for activity.

In summary, our results indicate that two types of proteins that regulate the nuclear export of mRNA, lentivirus Rev (and Rev-like) proteins and the influenza virus NS1 protein, have the common property of containing two functional domains, an RNA-binding domain and an effector domain. One type (the Rev proteins) facilitates nuclear export, whereas the other type (the NS1 protein) inhibits nuclear export. This is comparable to the situation with transcriptional activators and repressors, both of which usually contain two major domains: a DNA-binding domain and an effector domain which either activates or represses transcription (10). Analogously to transcriptional activators and repressors, the effector domain of the Rev proteins (29, 36) and of the NS1 protein (32a) can function with a heterologous RNA-binding domain. However, as we have shown in the present study, there are several significant differences between the effector domains of the NS1 and Rev proteins. These differences most likely indicate that the cellular nuclear target(s) and the function of the effector domains of the NS1 and Rev proteins are quite different. We can presume that one consequence of such a difference is that the NS1 and Rev proteins have opposite effects on nuclear mRNA transport. It is very likely that the interactions between proteins that regulate nuclear mRNA export and their nuclear targets will be varied and complex, possibly as varied and complex as the interaction of transcription factors with their protein targets. We are currently endeavoring to identify the nuclear protein target(s) of the effector domain of the NS1 protein.

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