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An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA-binding activity and largely helical backbone structure

XIAO-YAN QIAN,*[1,3] CHEN-YA CHIEN,*[1,2] YUAN LU,[1,4] GAETANO T. MONTELIONE,[1,2] and ROBERT M. KRUG[1]

[1] Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA
[2] Center for Advanced Biotechnology and Medicine, Piscataway, New Jersey 08854, USA

ABSTRACT

The NS1 protein of influenza A virus has the unique property of binding to three apparently different RNAs: poly A; a stem-bulge in U6 small nuclear RNA; and double-stranded RNA. One of our major goals is to determine how the NS1 protein recognizes and binds to its several RNA targets. As the first step for conducting structural studies, we have succeeded in identifying a fragment of the NS1 protein that possesses all the RNA-binding activities of the full-length protein. The RNA-binding fragment consists of the 73 amino-terminal amino acids of the protein. We have developed procedures for obtaining large amounts of the polypeptide in pure form. This has enabled us to establish the RNA-binding properties of this polypeptide and to demonstrate that it retains the ability to dimerize exhibited by the full-length protein. In addition, far-UV CD spectroscopy indicates that this RNA-binding polypeptide is largely (approximately 80%) helical, suggesting that the mode of dimerization of the NS1 protein and of its interaction with RNA is mediated, at least in part, by helices.

Keywords: circular dichroism; double-stranded RNA; helical protein; influenza virus NS1 protein; RNA-binding protein; U6 snRNA

INTRODUCTION

RNA-binding proteins play important roles in gene expression. However, relatively little is known about the structure of these proteins and of the ways they recognize their RNA targets. Based on sequence homologies, several RNA-binding motifs in proteins have been identified (Burd & Dreyfuss, 1994), but the amount of structural information remains limited. A few crystal and/or NMR structures have been solved (Banner et al., 1987; Nagai et al., 1990; Eberle et al., 1991; Hoffman et al., 1991; Wittekind et al., 1992; Schindelin et al., 1993, 1994; Schnuchel et al., 1993; Newkirk et al., 1994; Oubridge et al., 1994; Bycroft et al., 1995; Kharrat et al., 1995), but many more structural studies are needed to provide an understanding of the ways in which different protein motifs recognize RNA.

The NS1 protein encoded by influenza A virus is an RNA-binding protein that has unique properties. This protein, which functions in several posttranscriptional steps (Alonso-Caplen et al., 1992; Fortes et al., 1994; Lu et al., 1994, 1995; Qian et al., 1994; Qiu & Krug, 1994; Qiu et al., 1995), has been shown to bind to three apparently different RNAs: poly A; a stem-bulge region in U6 small nuclear RNA (snRNA); and double-stranded RNA (dsRNA) (Hatada & Fukuda, 1992; Lu et al., 1994, 1995; Qiu & Krug, 1994; Qiu et al., 1995). Mutational analysis of the protein and RNA competition experiments indicates that all three RNAs most likely share the same binding site on the protein (Lu et al., 1994, 1995; Qian et al., 1994). This RNA-binding region does not share any sequence homology with

Reprint requests to: Robert M. Krug, Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA; e-mail: krug@mbcl.rutgers.edu.

* The first two authors contributed equally to the work.

[3] Present address: Department of Microbiology, Duke University Medical Center, Durham, North Carolina 27710, USA.
[4] Present address: Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710, USA.

those of other known RNA-binding proteins (Qian et al., 1994). One of our major goals is to determine how the NS1 protein recognizes and binds to its several RNA targets. To achieve this goal, we decided to initiate structural studies of this protein and of its complex with at least one target RNA. As the crucial first step, we determined whether it was possible to identify a fragment of the NS1 protein that possesses the RNA-binding activities of the full-length protein. Such a fragment would greatly facilitate the structural analysis by NMR spectroscopy. Here we report the successful identification and purification of such an RNA-binding fragment, which our results indicate is highly ordered and largely (approximately 80%) helical.

RESULTS

Identification of a fragment of the NS1 protein that is sufficient for specific RNA binding

Previous mutational analysis of the influenza virus NS1 protein indicated that amino acids 19–38 are required for RNA binding (Qian et al., 1994). This was the experimental basis for defining this amino acid sequence as the RNA-binding region of the protein. Based on these results, we reasoned that an amino-terminal fragment that included the RNA-binding region might possess specific RNA-binding activity. Several amino-terminal fragments of the NS1 protein were expressed as GST fusion proteins (Fig. 1A). After purification, the amino-terminal fragments were cleaved from GST using protease factor Xa. These fragments were tested for their ability to bind to U6 snRNA in gel shift assays (Fig. 1B). Neither a 48- (NS 48) nor a 62- (NS 62) amino acid long, amino-terminal fragment was active in binding to U6 snRNA (lanes 3, 4). In contrast, the amino-terminal fragment containing 73 amino acids (NS 73) efficiently bound to U6 snRNA (lane 2). Thus, the addition of only 11 amino acids converted an inactive fragment (NS 62) into an active fragment (NS 73).

To verify that the RNA-binding region that is included in the 73-amino acid fragment was required for its RNA-binding activity, amino acids within this RNA-binding region were mutated. Amino acids at positions 19 and 20, or at positions 31 and 32 were mutated to alanines (Fig. 2). Either of these pairs of mutations rendered the 73-amino acid fragment inactive in RNA binding. Thus, the 73-amino acid fragment behaved like the full-length NS1 protein (Qian et al., 1994) with respect to the requirement for specific amino acids in the RNA-binding region.

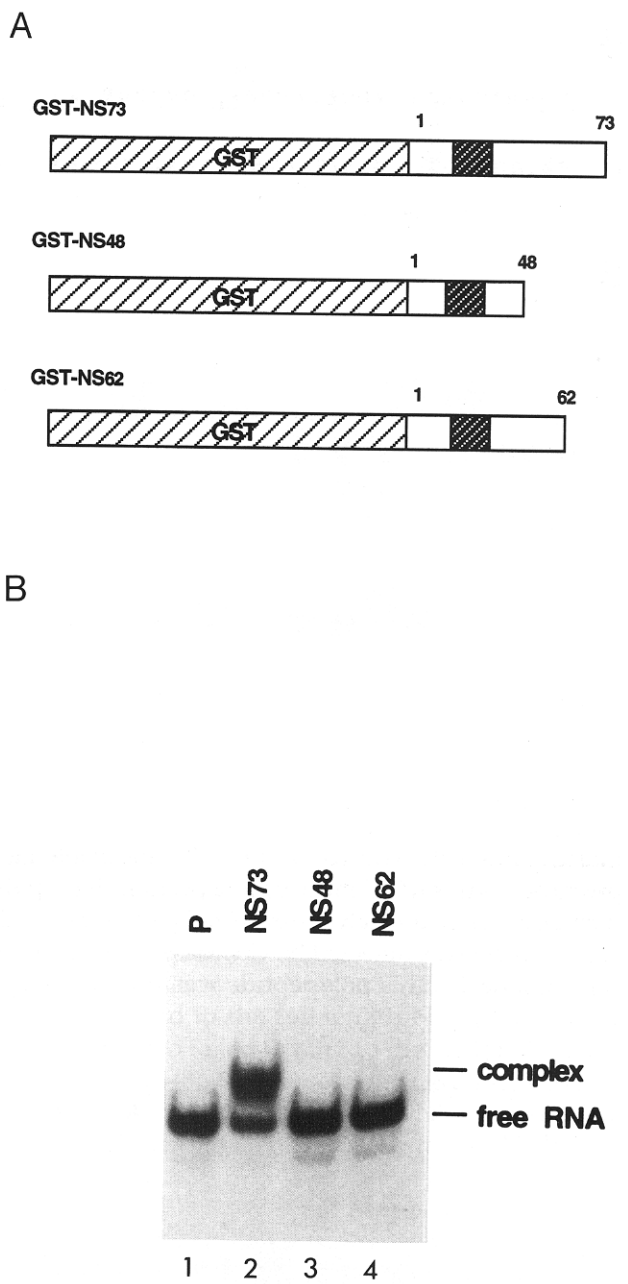

FIGURE 1. RNA-binding activity of amino-terminal fragments of the NS1 protein. **A:** Diagram of the GST fusion proteins containing various lengths (73, 48, and 62 amino acids) of the amino terminus of the NS1 protein. The speckled box in the NS1 sequence corresponds to amino acids 19–38, which have been shown to be required for RNA binding. **B:** Gel shift assay. ^{32}P-labeled U6 snRNA (1.0 nM) was mixed with 0.4 μM of the indicated amino-terminal fragment of the NS1 protein (cleaved from the GST fusion protein using factor Xa) under standard RNA-binding conditions (see the Materials and methods), and the mixtures were subjected to nondenaturing gel electrophoresis. Lane 1, P, U6 snRNA alone.

Purification of a nonfusion 73-amino acid NS1 fragment

The above results prompted us to express and purify a nonfusion 73-amino acid polypeptide [NS1(1–73)]. The DNA sequence encoding the NS1 polypeptide was cloned into the pET-11a plasmid under the control of the T7 RNA polymerase promoter (Studier & Moffatt, 1986). This plasmid was transformed into *Escherichia coli* BL21(DE3) containing the gene for the T7 RNA poly-

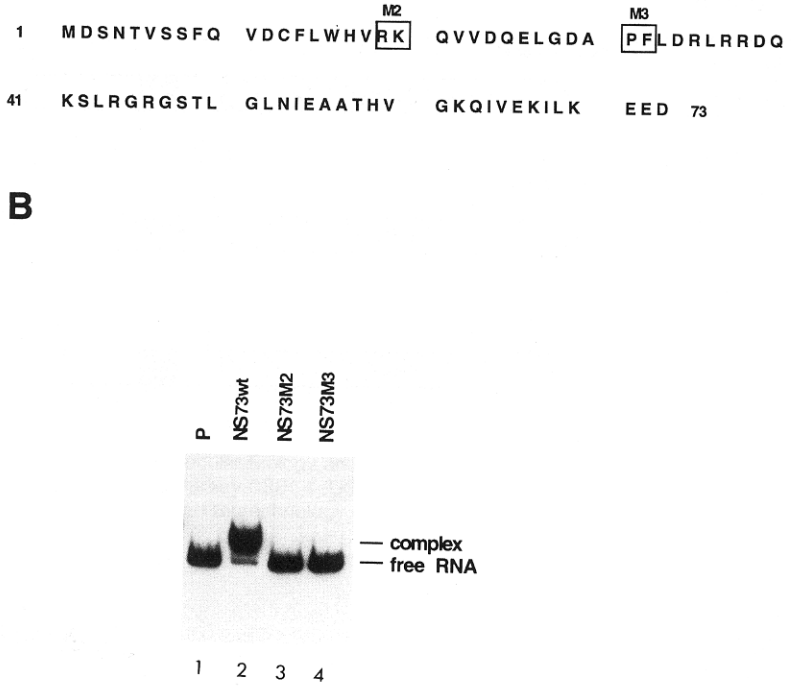

FIGURE 2. RNA-binding region is required for RNA-binding activity of the 73-amino acid amino-terminal fragments of the NS1 protein. **A:** Amino acid sequence of the 73 amino-terminal amino acids, and the positions of the M2 and M3 mutations (RK → AA; PF → AA). **B:** Gel shift assay. ^{32}P-labeled U6 snRNA (1.0 nM) was mixed with 0.4 μM of the wild-type (wt) (lane 2), M2 mutant (lane 3), or M3 mutant (lane 4) 73-amino acid amino-terminal fragment of the NS1 protein (cleaved from the GST fusion protein using factor Xa). After incubation under standard RNA-binding conditions, the mixtures were subjected to nondenaturing gel electrophoresis. Lane 1, P, U6 snRNA alone.

merase under the control of the IPTG-inducible lac z promoter. After IPTG induction, a predominant polypeptide of approximately the expected molecular weight of NS1(1–73) was detected by Coomassie Blue staining (Fig. 3, lane 1). This polypeptide was not detected in the absence of IPTG induction (data not shown). The NS1(1–73) polypeptide was purified, as described in the Materials and methods, by differential centrifugation (lane 2), ammonium sulfate fractionation (lane 3), Q-Sepharose anion exchange chromatography (lane 4), Mono S cation exchange chromatography (lane 5), and gel filtration on Superdex 75 (lane 6). The final purified protein contained only a single band as detected by either Coomassie Blue (lane 6) or silver staining (data not shown).

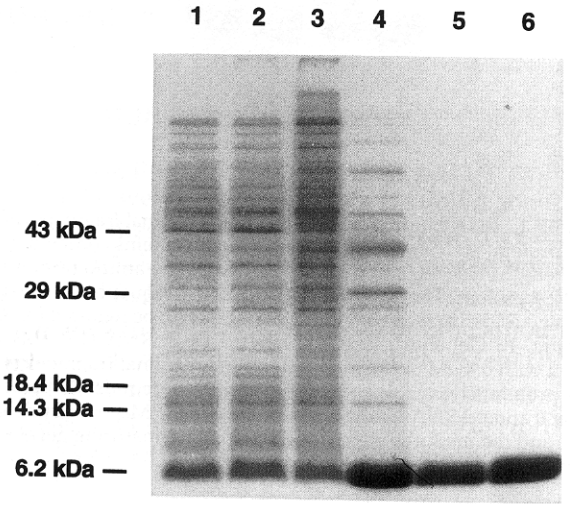

FIGURE 3. Purification of the NS1(1–73) polypeptide. Equivalent aliquots of each sample were subjected to SDS electrophoresis on 17.5% gels. Lane 1, total cell extract; lane 2, 100,000 × *g* supernatant; lane 3, 70% ammonium sulfate precipitate; lane 4, after Q-Sepharose anion exchange chromatography; lane 5, after Mono-S cation exchange chromatography; lane 6, after Superdex 75 gel filtration. The gel was stained with Coomassie Blue. Positions of marker proteins are labeled with their corresponding molecular weights on the left.

Characterization of purified NS1(1–73)

Oligomerization state

The full-length NS1 protein exists as a dimer in vitro even in the absence of its RNA target, and mutational analysis indicated that the RNA-binding and dimerization regions of the protein are coincident (Nemeroff et al., 1995). To determine whether the NS1(1–73) polypeptide retained the ability to dimerize, we used gel filtration on Superdex 75 to estimate its molecular weight at pH 6.0. The NS1(1–73) polypeptide had an elution time of 24.5 min from this column (Fig. 4A). Based on the elution times of several protein standards, this result indicated that the NS1(1–73) polypeptide possessed an apparent molecular weight of 18 kDa (Fig. 4B). This is the molecular weight expected for a dimer of the polypeptide. Thus, the dimerization exhibited by the full-length NS1 protein is retained by the NS1(1–73) polypeptide.

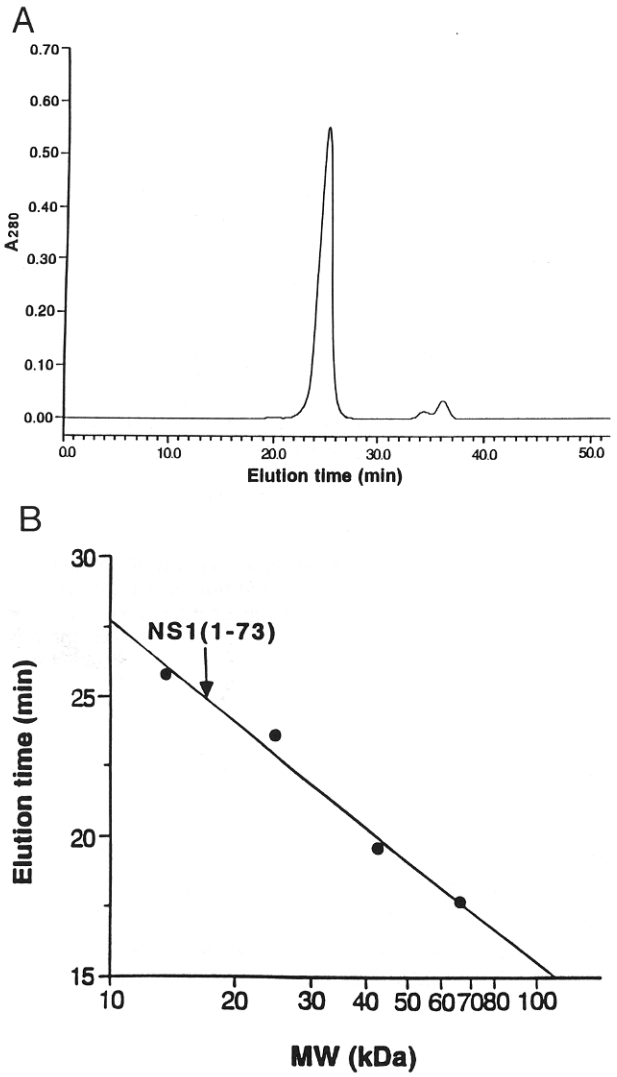

FIGURE 4. NS1(1–73) polypeptide behaves as a dimer during gel filtration. **A:** Chromatographic profile of the NS1(1–73) polypeptide. The major peak at 24.5 min corresponds to the NS1(1–73) polypeptide. The minor peaks between 33 and 38 min correspond to small molecules in the solution used in the previous Mono S ion exchange step. **B:** Molecular weight calibration curve for gel filtration chromatography. The gel filtration column was calibrated using albumin (56 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The relative molecular weight of the NS1(1–73) polypeptide calculated by this method, 18 kDa, is about twice the molecular weight of the NS1(1–73) monomer (8.3 kDa). The buffer for analytical gel filtration at 20 °C was 0.3 M ammonium acetate, pH 6.0.

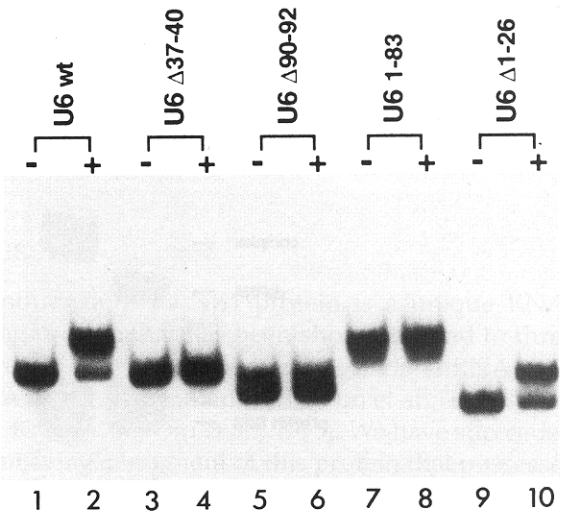

FIGURE 5. Effect of various deletions of U6 snRNA on its ability to bind to the NS1(1–73) polypeptide. Binding assays were performed under standard conditions using the indicated ^{32}P-labeled wild-type or deleted U6 snRNA (1.0 nM) and either: (−), no NS1(1–73) polypeptide; or (+), 0.4 μM NS1(1–73) polypeptide.

RNA-binding activity

The purified NS1(1–73) polypeptide was next tested for its specific RNA-binding activity using both full-length U6 snRNA and various deletion mutants of U6 snRNA as targets (Fig. 5). Using 0.40 μM of the NS1(1–73) polypeptide, 80–90% of full-length U6 snRNA (1.0 nM) was bound to the polypeptide (lanes 1, 2). A molar excess of this magnitude was also needed for the full-length NS1 protein to quantitatively gel shift U6 snRNA (Qiu et al., 1995). As discussed previously (Qiu et al., 1995), this may indicate that gel shift assays underestimate the RNA-binding activity of protein preparations, particularly as compared to filter-binding assays. Several U6 snRNA deletion mutants, Δ37–40, Δ90–92, 1–83, that do not bind to the full-length NS1 protein (Qiu et al., 1995), also failed to bind to the NS1(1–73) polypeptide (lanes 3–8). Conversely, the U6 Δ1–26 deletion mutant that binds to the full-length NS1 protein (Qiu et al., 1995) also efficiently bound to the NS1(1–73) polypeptide (lanes 9, 10). These results indicate that the binding of the NS1(1–73) polypeptide and of the full-length NS1 protein to U6 snRNA require the same region of this RNA, namely the stem-bulge encompassing nt 27–46 and nt 83–101 (Qiu et al., 1995). A chemical modification/interference assay carried out as described previously (Qiu et al., 1995) verified that the full-length NS1 protein and the NS1(1–73) polypeptide bound to the same region of U6 snRNA (data not shown).

Like the full-length NS1 protein (Hatada & Fukuda, 1992; Lu et al., 1995), the NS1(1–73) polypeptide also binds to poly A (data not shown) and dsRNA (Fig. 6A). The dsRNA used in this experiment was a small dsRNA of 29 base pairs generated by annealing the sense and antisense transcripts of the polylinker of the pGEM1 plasmid. The NS1(1–73) polypeptide also bound to other dsRNAs, including reovirus RNAs (data not shown). Competition experiments indicated that the binding site on the NS1(1–73) polypeptide for dsRNA and for U6 snRNA is most likely the same (Fig. 6B). Thus, unlabeled dsRNA, either poly (I):(C)

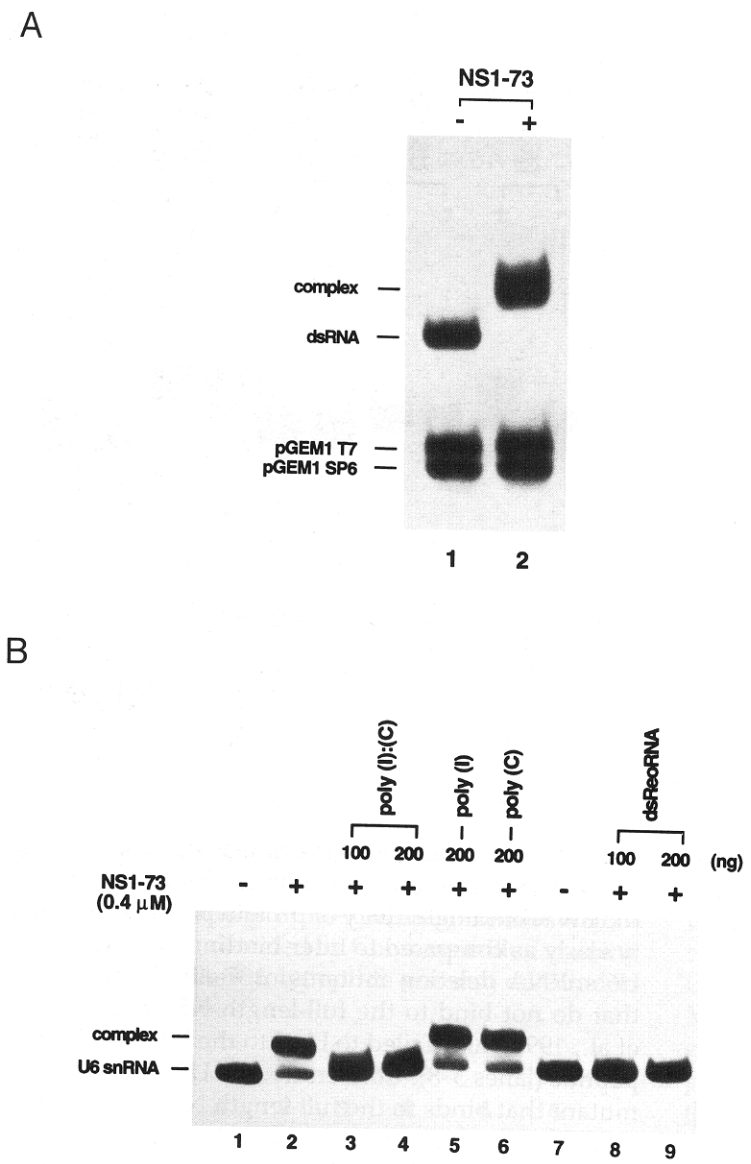

FIGURE 6. dsRNA binds to the NS1(1–73) polypeptide and competes with U6 snRNA for binding. **A:** ^{32}P-labeled 29-base pair pGEM1 dsRNA (1.0 nM) was incubated without (−) or with (+) 0.4 μM of the NS1(1–73) polypeptide, and the mixture was analyzed by nondenaturing gel electrophoresis. **B:** ^{32}P-labeled U6 snRNA (1.0 nM) was mixed with the indicated amount of double-stranded poly (I):(C) (lanes 3, 4), single-stranded poly (I) (lane 5), single-stranded poly (C) (lane 6), or double-stranded reovirus RNA (lanes 8, 9), followed by the addition of 0.4 μM NS1(1–73) polypeptide. After incubation, the mixtures were subjected to nondenaturing gel electrophoresis. Lanes 1 and 7, U6 snRNA in the absence of the NS1(1–73) polypeptide; lane 2, U6 snRNA incubated with the NS1(1–73) polypeptide in the absence of a competitor RNA. pGEM1T7 and pGEM1Sp6, the unannealed sense and antisense ssRNAs.

(lanes 3, 4) or reovirus RNA (lanes 8, 9), competed with U6 snRNA for binding to the NS1(1–73) polypeptide. In contrast, unlabeled single-stranded poly (I) or poly (C) had no effect on the binding of U6 snRNA (lanes 5, 6).

The binding of the full-length NS1 protein to dsRNA has been shown to block this dsRNA from activating PKR (Lu et al., 1995), the kinase that phosphorylates the α subunit of the eukaryotic translation initiation factor eIF-2 (reviewed in Hershey, 1991; Merrick, 1992; Rhoads, 1993). This phosphorylation causes an inhibition of the rate of initiation of translation. To establish whether the NS1(1–73) polypeptide retained this activity of the full-length protein, we determined whether this polypeptide blocked the inhibition of translation caused by dsRNA (Fig. 7). Reovirus RNA (0.1 μg/mL) inhibited the translation of luciferase mRNA catalyzed by reticulocyte extracts (lane 2). Preincubation of this dsRNA with 0.1 or 0.2 μM NS1(1–73) polypeptide blocked the ability of the dsRNA to inhibit translation (lanes 4, 5). This concentration is approximately the same as that needed for the full-length NS1 protein to block the inhibition of translation caused by 0.1 μg/mL ds reovirus RNA (Lu et al., 1995).

Backbone structure analysis by CD spectroscopy

Far-UV CD spectroscopy was used to analyze the regular backbone structure in the NS1(1–73) polypeptide. Samples of the polypeptide were prepared in 25 mM sodium phosphate buffer at pH 6.5. Nearly identical spectra were obtained at temperatures of 25 °C and ~0 °C (Fig. 8), suggesting that the NS1(1–73) polypeptide is fully folded at both of these temperatures. The

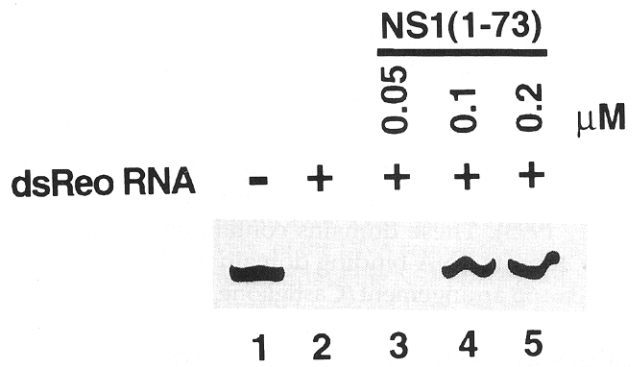

FIGURE 7. NS1(1–73) polypeptide blocks the inhibition of translation caused by dsRNA. Reovirus RNA (0.1 μg/mL) was incubated with the indicated concentration of the NS1(1–73) polypeptide (lanes 3–5) for 10 min at 30 °C. As a control, no protein was added to the reovirus RNA (lane 2). Lane 1: neither reovirus RNA or NS1(1–73) was added. Reticulocyte extract was added, and the mixtures were incubated at 30 °C for 10 min. The reaction mixtures were then supplemented with translation components as described in the Materials and methods. After incubation for 60 min at 30 °C, proteins were analyzed by SDS gel electrophoresis.

CD spectrum of the NS1(1–73) polypeptide exhibits minima at ~208 nm and ~222 nm, a property that is characteristic of highly α-helical backbone structure (Greenfield & Fasman, 1969). Using the self-consistent spectral decomposition method of Sreerama and Woody (1993) to analyze this CD spectrum, we estimate that the NS1(1–73) polypeptide is comprised of approximately 80% helical backbone structure. In addition, initial NMR results are also consistent with a highly helical backbone structure; for example, two-dimensional nuclear Overhauser enhancements spectra of the NS1(1–73) polypeptide recorded at pH 6.5 and 20 °C exhibit many well-defined amide proton/amide proton cross peaks, characteristic of helical backbone structure (data not shown).

DISCUSSION

The influenza virus NS1 protein is a unique RNA-binding protein that has been shown to bind to three apparently different RNAs: poly A, U6 snRNA, and dsRNA (Hatada & Fukuda, 1992; Lu et al., 1994, 1995; Qiu & Krug, 1994; Qiu et al., 1995). We have succeeded in identifying a fragment of this protein that possesses all the RNA-binding properties of the full-length protein. The RNA-binding fragment consists of the 73 amino-terminal amino acids of the protein. It is significant that a slightly shorter amino-terminal fragment containing 62 amino acids was inactive in RNA binding. This demonstrates that some, if not all, of the last 11 carboxy-terminal amino acids of the amino-terminal fragment are needed for it to acquire RNA-binding activity. However, mutagenesis indicates that only a subset of these 73 amino acids is absolutely required for binding activity. Specifically, mutation of two or three amino acid blocks between residues 19 and 38 causes a loss of RNA-binding activity (Qian et al., 1994). These mutations also cause a loss of the ability of the protein to dimerize (Nemeroff et al., 1995). In contrast, simi-

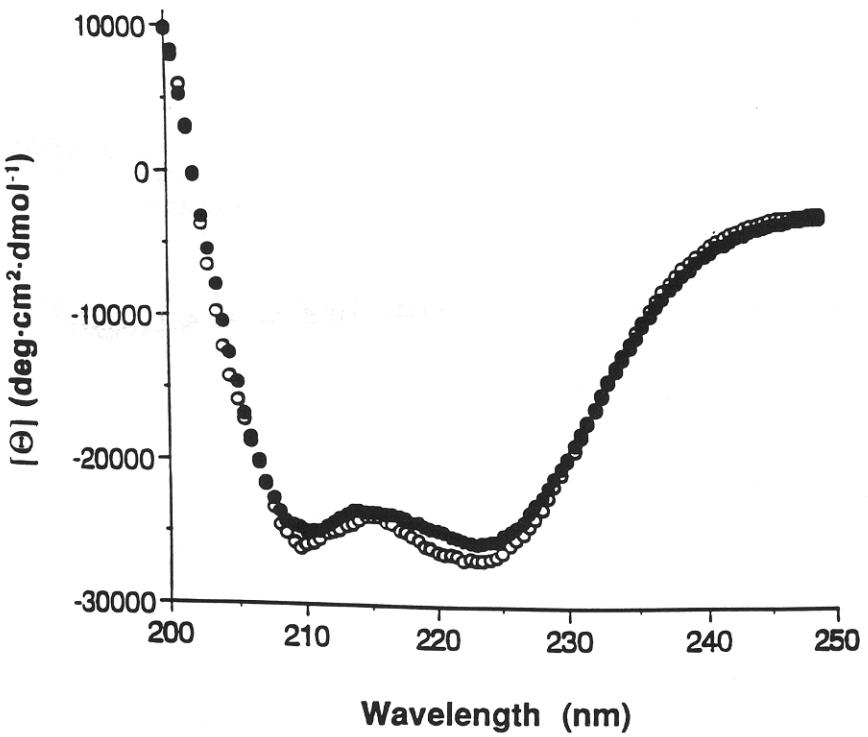

FIGURE 8. CD spectra of NS1(1–73) showing plot of mean residue molar ellipticity in the far-UV region. Samples were prepared at 24 μM in 25 mM sodium phosphate, 25 mM sodium chloride, and 0.5 mM sodium azide buffer, at pH 6.5. Spectra were recorded at 25 °C (closed circles) and ~0 °C (open circles). The double minimum at 208 nM and 222 nm is indicative of a highly helical backbone conformation.

lar mutations in other regions of the amino-terminal 73-amino acid sequence do not lead to a loss of either RNA-binding or dimerization activity. These results can be interpreted as indicating that: (1) the amino acid sequence from residues 19–38 is required for dimerization, which may be necessary for creating the RNA-binding site on the protein (Nemeroff et al., 1995); and/or (2) this sequence constitutes the region of the protein that is in direct contact with the RNA target. In contrast, the other amino acids in the 73-amino acid sequence might be needed primarily to maintain the proper fold of the polypeptide. This function might be less susceptible to disruption by mutation.

Our results establish that the NS1 protein is at least partially modular. The amino-terminal 73-amino acid sequence functions as an RNA-binding and dimerization domain independently of the rest of the protein. The remaining 164 amino acid sequence of the NS1 protein contains a second functional region that is required for the in vivo activity of the protein (Qian et al., 1994). This region, which has been designated the effector domain, is presumed to interact with host cell nuclear proteins. It has not yet been determined whether an amino acid sequence from this region of the protein can act as an independent module, i.e., carry out the effector function of the NS1 protein independently of the rest of the protein.

Three RNAs have been shown to bind to both the full-length NS1 protein and the NS1(1–73) polypeptide: poly A, a stem-bulge region in U6 snRNA, and dsRNA (Hatada & Fukuda, 1992; Lu et al., 1994, 1995; Qiu & Krug, 1994; Qiu et al., 1995). The differences between these three RNAs may be more apparent than real, in that all three RNAs probably share structural similarities. dsRNA is clearly a highly ordered structure, and the stem-bulge in U6 snRNA likely also possesses an ordered structure (Qiu et al., 1995). Poly A can also be highly ordered, consisting of two parallel polynucleotide chains that are hydrogen bonded to each other (Rich et al., 1961). It is not known what features of these ordered structures the RNA-binding domain of the NS1 protein recognizes. Nor is it known how this recognition and binding by the NS1 protein occurs. The primary sequence of the RNA-binding domain [i.e., the NS1(1–73) polypeptide] does not provide any insight. This sequence does not share any significant homology with the RNA-binding domains of other known proteins, including other poly A-binding and dsRNA-binding proteins (Burd & Dreyfuss, 1994; Qian et al., 1994). This suggests that the RNA-binding domain of the NS1 protein could possess unique structural features.

Aside from the ribonucleases and virus coat proteins, the structures of only a few RNA-binding proteins have been determined by NMR or X-ray crystallography to date. The N-terminal RNA-binding domain of U1 snRNP A (Nagai et al., 1990; Hoffman et al., 1991) and the single-stranded (ss) RNA-binding domain of hnRNP C (Wittekind et al., 1992) have a $\beta\alpha\beta\beta\alpha\beta$ secondary structure, whereas the ssRNA-binding "cold-shock" domain is a β-barrel structure (Schindelin et al., 1993, 1994; Schnuchel et al., 1993; Newkirk et al., 1994). The three-dimensional structures of two dsRNA-binding domains have also been solved recently (Bycroft et al., 1995; Kharrat et al., 1995). These domains contain an $\alpha\beta\beta\beta\alpha$ motif. Also, the KH RNA-binding domain has been shown to be a $\beta\alpha\alpha\beta\beta$ arrangement (Castiglione Morelli et al., 1995). Thus, all of these RNA-binding proteins are composed either exclusively of β-sheets or of mixtures of α and β structures in which the β structures predominate. The RNP domains of U1 snRNP A (Oubridge et al., 1994), hnRNP C (Wittekind et al., 1992), and *E. coli* cold-shock protein A (Newkirk et al., 1994) interact with RNA using the amino acids located primarily in β-sheet backbone conformations. One RNA-binding protein has been shown to be α-helical: the Rop protein encoded by the *E. coli* ColE1 plasmid, which forms a four-helical bundle structure (Banner et al., 1987; Eberle et al., 1991; Predki et al., 1995). In addition, the RNA-binding proteins with arginine-rich motifs may use α-helices to interact with their RNA targets. For example, an α-helical Rev peptide binds to the same specific RNA target as the full-length protein (Kjems et al., 1992; Tan et al., 1993), but the structure of the entire RNA-binding domain of the Rev protein has not yet been determined. Our results indicate that the NS1(1–73) N-terminal fragment of NS1, which contains the dimerization and RNA-binding activities of the full-length protein, is largely (~80%) helical. These results suggest that the mode of dimerization of the NS1 protein and of its interaction with RNA is mediated, at least in part, by α-helices. However, a clear demonstration of the role of the helices of the NS1(1–73) polypeptide in dimerization and/or RNA-binding awaits further structural studies. These studies will be greatly facilitated by the results of the present paper: the availability of a high-level production system for the NS1(1–73) polypeptide, the development of a purification procedure of this polypeptide, and the characterization of its RNA-binding properties.

MATERIALS AND METHODS

Preparation and cleavage of NS1-GST fusion proteins

The GST fusion protein containing the full-length NS1 protein was prepared as described previously (Qiu & Krug, 1994). To prepare the GST fusion proteins containing the 48, 62, or 73 amino-terminal amino acids of the NS1 protein, *Bam*H I sites were introduced into the NS1 coding sequence at the appropriate positions using PCR, and the *Bam*H I fragments were then excised and cloned into the *Bam*H I site of the pGEX-3X vector. For the preparation of the GST fusion proteins containing the M2 or M3 mutation in the 73-amino acid amino-terminal sequence, a *Bam*H I site was introduced

into the appropriate position of the full-length NS1 protein containing the indicated mutation. These GST fusion proteins were purified and cleaved using factor Xa as described previously (Qiu & Krug, 1994).

Expression and purification of NS1(1–73)

PCR was used to introduce *Nde* I and *Bam*H I sites at the 5′ and 3′ ends, respectively, of the DNA sequence encoding the 73 amino-terminal amino acids of the NS1 protein. After PCR amplification, this fragment was cloned into the pET-11a plasmid (Studier & Moffatt, 1986). The resulting plasmid was transformed into *E. coli* BL21(DE3) for expression of the protein. The transformed cells were grown at 37 °C in LB medium. One-liter fermentations were conducted in 2-L shaker flasks. When the cells reached an absorbance of 0.6 (at 600 nm), the production of the NS1(1–73) polypeptide was induced for 4 h in the presence of 1 mM IPTG. Cells were harvested by centrifugation at 4,500 × *g* for 30 min at 4 °C and resuspended in 30 mL of PBS buffer, pH 7.0. Cells were broken by sonication, followed by centrifugation at 100,000 × *g* at 4 °C for 1 h. Ammonium sulfate was then added to the supernatant to 70% saturation. The pellet obtained by centrifugation at 50,000 × *g* for 30 min was solubilized in 20 mL of 50 mM Tris, 0.1 mM EDTA, and 0.1 mM DTT, pH 8.0 (TED), and dialyzed twice against 2 L of the same buffer. The dialyzed solution was loaded onto a Q-Sepharose (Pharmacia) fast flow column (2.5 × 15 cm). The protein was eluted in TED using a gradient of 0–300 mM NaCl. The fractions corresponding to the NS1(1–73) peak were pooled and dialyzed three times against 1 L of 50 mM MES, 0.1 mM EDTA, and 0.1 mM DTT, pH 6.0 (MED). This protein solution was then loaded onto an FPLC Mono S HR 10/10 column (Pharmacia). The protein was eluted in MED using a NaCl gradient from 0 to 1.0 M. Fractions containing NS1(1–73) were pooled and subjected to gel filtration on a Superdex 75 gel column (Pharmacia) in 0.3 M ammonium acetate, pH 6.0, at a flow rate of 0.5 mL/min. In all chromatographic steps, the protein was detected by absorbance at 280 nm. NS1(1–73) fractions from gel filtration were pooled, lyophilized, and stored at −20 °C.

RNA-binding assays

The following ^{32}P-labeled RNA targets were prepared as previously described: U6 snRNA and its deletion mutants, and pGEM1 dsRNA (29 base pairs) (Qiu & Krug, 1994; Lu et al., 1995; Qiu et al., 1995). Binding assays were carried out as described previously (Qiu & Krug, 1994). Briefly, the indicated labeled RNA (at 1.0 nM) and the indicated concentration of the NS1(1–73) polypeptide were incubated in the standard RNA-binding buffer (43 mM Tris, pH 8.0, 50 mM KC1, 8% glycerol, 5 mM dithiothreitol, 50 ng/μL *E. coli* tRNA, 0.5 units/μL RNasin) in a final volume of 20 μL on ice for 20 min. The RNA–polypeptide complexes were resolved from unbound RNA by electrophoresis on 6% nondenaturing gels as described previously (Qiu & Krug, 1994).

In vitro translation assays

Nuclease-treated rabbit reticulocyte extracts were programmed with luciferase mRNA generated in situ by T7 RNA polymerase transcription of the luciferase gene (TNT-coupled reticulocyte system, Promega). Reaction mixtures contained ^{35}S-methionine, an amino acid mixture (minus methionine), T7 RNA polymerase, DNA-encoding luciferase mRNA. Translation was conducted for 60 min at 30 °C. The amount of luciferase synthesized was determined by gel electrophoresis on SDS-containing 12.5% polyacrylamide gels, followed by fluorography.

Analytical gel filtration chromatography

High-performance gel filtration chromatography was performed on a Superdex 75 HR 10/30 column (Pharmacia). This column was calibrated using four standard proteins: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). Chromatography was conducted in 0.3 M ammonium acetate, pH 6.0, at 20 °C using a flow rate of 0.5 mL/min. The sample was applied to the column at a protein concentration of 1 mg/mL. Column fractions were monitored for the presence of protein by absorbance at 280 nm.

CD spectroscopy

Far-UV CD spectra were obtained using an Aviv Model 62-DS spectropolarimeter. The NS1(1–73) polypeptide at a concentration of 24 μM was in a solution containing 25 mM sodium phosphate, 25 mM sodium chloride, and 0.5 mM sodium azide buffer at pH 6.5. Protein concentrations were determined by quantitative amino acid analysis and then used to calculate molar ellipticities.

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