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The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6–U2 and U6–U4 snRNA interactions during splicing

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ABSTRACT

The influenza virus NS1 protein is a unique posttranscriptional regulator that has two activities: inhibition of the nuclear export of poly A-containing mRNAs and inhibition of pre-mRNA splicing. Here we demonstrate that this protein binds to a specific region in one of the human spliceosomal snRNAs, U6 snRNA. Using U6 deletion mutations, we show that the binding of the NS1 protein requires both chains of a stem-bulge structure encompassing nucleotides 27–46 and nucleotides 83–101 of human U6 snRNA. A chemical modification/interference assay indicated that the primary binding site is centered around a purine-containing bulge in this stem-bulge structure. These results provide strong evidence that this postulated secondary structure in U6 snRNA actually exists. The NS1 protein also binds to a model U6–U4 snRNA complex, suggesting that the U6 stem-bulge comprising the NS1 protein binding site is also present in natural U6–U4 snRNA complexes. The U6 stem-bulge includes the U6 sequence that forms helix II with U2 snRNA during splicing, an interaction that is essential for mammalian splicing. We demonstrate that the NS1 protein blocks formation of the U6–U2 helix II both in a model system and during in vitro splicing. In addition, we show that the NS1 protein inhibits formation of U6–U4 snRNA complexes during in vitro splicing, presumably because the binding site of the NS1 protein includes the 3′-terminal region of U6 snRNA that has been shown to be important for the formation of U6–U4 complexes. We postulate that the inhibition of U6–U2 and U6–U4 snRNA complex formation is largely responsible for the inhibition of pre-mRNA splicing by the NS1 protein.

Keywords: influenza virus; NS1 protein; pre-mRNA splicing; U6–U2 and U6–U4 snRNA complexes

INTRODUCTION

Many proteins that regulate posttranscriptional processes of eukaryotic gene expression function by interacting with specific RNA sequences (Malim et al., 1989; Brown & Harland, 1990; Inoue et al., 1990; Tian & Maniatis, 1992). The influenza virus NS1 protein is a unique posttranscriptional regulator that has two activities: inhibition of the nuclear export of poly A-containing mRNAs and inhibition of pre-mRNA splicing (Alonso-Caplen et al., 1992; Fortes et al., 1994; Lu et al., 1994; Qian et al., 1994; Qiu & Krug, 1994). This protein has two functional domains: an RNA-binding domain and an effector domain (Qian et al., 1994). The RNA-binding domain is required for both activities (Lu et al., 1994; Qian et al., 1994). For the inhibition of nuclear export of mRNA, the NS1 protein recognizes the 3′ end poly A sequence (Qiu & Krug, 1994). However, the NS1 protein also inhibits pre-mRNA splicing both in vivo and in vitro, even in the absence of a poly A sequence (Fortes et al., 1994; Lu et al., 1994). This indicates that this protein probably interacts with RNA sequence(s) other than poly A to inhibit splicing.

Potential candidates for such RNA sequences are the small nuclear RNAs (snRNAs)—the U1, U2, U4, U5, and U6 snRNAs—that are essential components of the machinery that splices pre-mRNAs (see reviews by Nilsen, 1994; Sharp, 1994). A large body of evidence has indicated that these snRNAs function in splice site recognition, spliceosome assembly, and catalysis. The 5′ splice site and branchpoint sequences in the pre-

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mRNAs are recognized by base-pairing interactions with the U1 and U2 snRNAs, respectively. U5 snRNA has been shown to interact with exon sequences at both the 5′ and 3′ splice sites (Newman & Norman, 1992; Sontheimer & Steitz, 1993; Nilsen, 1994; Sharp, 1994). Of the spliceosomal snRNAs, U6 snRNA is the most highly conserved in sequence and secondary structure (Brow & Guthrie, 1988). U6 snRNA undergoes multiple interactions: association with U4 snRNA to form the U6–U4 snRNP; dissociation of the U6–U4 base pairing in the spliceosome, followed by the formation of a U6–U2 structure; and, after splicing, reformation of the U6–U4 snRNP (Nilsen, 1994; Sharp, 1994). The U6–U2 snRNA structure is thought to be closely associated with the catalytic center of the spliceosome, and this structure contains two regions of base pairing between U6 and U2 sequences. Recent evidence indicates that the U6–U2 complex functions at least in part to align the 5′ splice site and branchpoint of pre-mRNAs (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993).

Our previous results suggested that the NS1 protein interacts with one of the human spliceosomal snRNAs, U6 snRNA (Lu et al., 1994). In the present study, we demonstrate that the influenza virus NS1 protein interacts specifically with a stem-bulge region in human U6 snRNA. This binding inhibits the formation of U6–U2 and U6–U4 complexes during in vitro splicing, thereby allowing us to postulate a likely mechanism for the inhibition of splicing. Further, the characteristics of the NS1 protein binding site on U6 snRNA provide important new experimental evidence concerning the structure of U6 snRNA and of U6 snRNA-containing complexes.

RESULTS

Identification of the NS1 protein binding site in U6 snRNA

To demonstrate directly that the influenza virus NS1 protein binds to U6 snRNA, we carried out a gel shift assay with purified NS1 protein and U6 snRNA (Fig. 1). Indeed, full-length U6 snRNA (106 nucleotides long) efficiently bound to the NS1 protein: 80–90% of U6 snRNA (1.0 nM) was bound in the presence of 800 nM NS1 protein. When the 23 3′-terminal nucleotides of U6 snRNA were removed, binding to the NS1 protein was eliminated, indicating that the binding site for the NS1 protein included this region of U6 snRNA. The other spliceosomal snRNAs—U1, U2, U4, and U5 snRNAs—did not bind to the NS1 protein (data not shown).

To delineate the exact binding site in the 3′-terminal region of U6 snRNA, a series of U6 snRNAs containing different 3- or 4-nucleotide deletions from nucleotides 80 to 101 was tested for binding to the NS1 protein (Fig. 2A). Deletions Δ83–85 and Δ86–89 drastically reduced binding activity, and deletions Δ90–92, Δ93–95, Δ96–98, and Δ99–101 eliminated all detectable binding activity. In contrast, deletion Δ80–82 and a deletion of the five 3′-terminal nucleotides (Δ102–106) did not reduce binding activity. These results clearly indicate that nucleotides 83–101 of U6 snRNA are required for the binding of the NS1 protein.

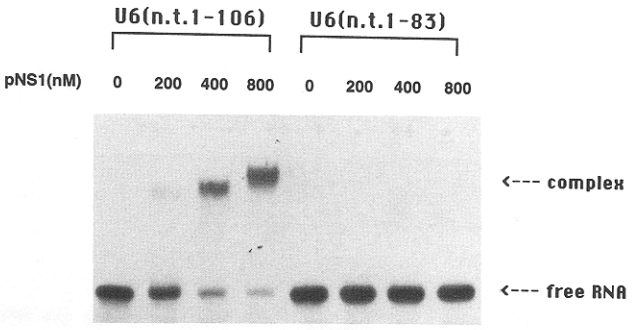

FIGURE 1. Full-length human U6 snRNA binds to the NS1 protein. The full-length U6 snRNA sequence (nucleotides 1–106) or the truncated U6 snRNA sequence (nucleotides 1–83) (each at 10,000 cpm, 1.0 nM) was incubated with the indicated concentration of the NS1 protein in the binding assays described in the Materials and methods. NS1 protein–RNA complexes were separated from free RNA by nondenaturing gel electrophoresis. Full-length and 3′ truncated U6 snRNAs had similar mobilities on these native gels.

To determine whether there was a stringent requirement for specific nucleotides at particular positions of this U6 snRNA sequence, the nucleotides at positions 93 and 94 were each separately mutated to each of the other three nucleotides (Fig. 2B). Changing the C at position 94 to A, G, or U abolished binding activity, indicating that there was a stringent requirement for C at this position. In contrast, at position 93, A or G, i.e., a purine residue, afforded strong binding, whereas a pyrimidine residue resulted in very weak binding (see Discussion).

To ascertain whether other regions of the U6 snRNA molecule were required for binding, deletions near the 5′ end of the molecule were carried out (Fig. 3A). Deletion of the 5′-terminal 26 nucleotides of U6 snRNA had little or no effect on the binding activity. In contrast, the small deletions Δ27–30, Δ31–33, Δ34–36, Δ37–40, and Δ41–43 each eliminated detectable binding activity. Deletion Δ44–46 caused a loss of almost all binding activity, whereas deletion Δ47–49 had no effect on binding activity. These results indicate that nucleotides 27–46 of U6 snRNA are required for the binding of the NS1 protein. The deletion of even a single nucleotide, A39, in this region was sufficient to eliminate binding activity (Fig. 3B). When this nucleotide was mutated rather than deleted, about 30% of wild-type binding activity was retained with a G at this position, whereas much lower (C residue) or no (U residue) binding activity occurred with a pyrimidine at this position (see Discussion).

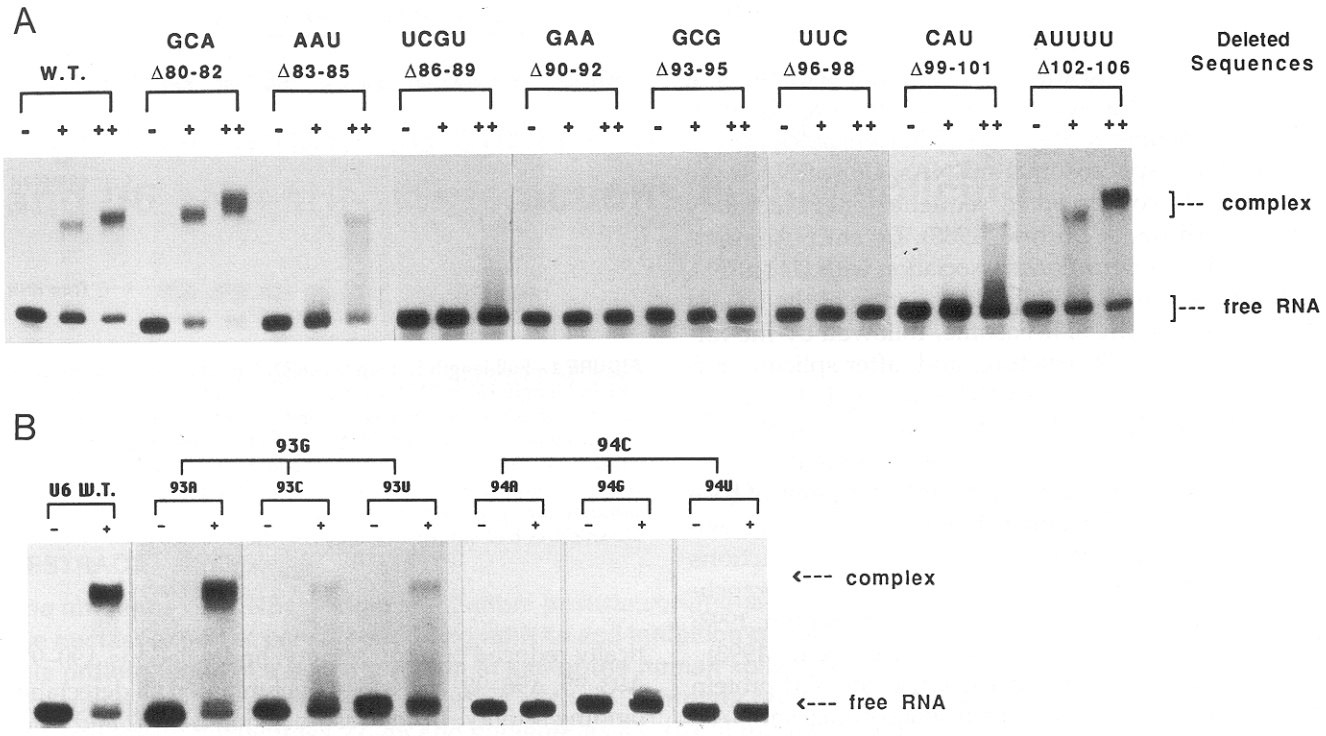

FIGURE 2. Effect of small deletions and point mutations in the 80–106 nucleotide region of U6 snRNA on its binding to the NS1 protein. Binding assays were performed using the indicated mutant or wild-type U6 snRNA. **A:** −, No NS1 protein; +, 400 nM of NS1 protein; ++, 800 nM of NS1 protein. **B:** −, No NS1 protein; +, 800 nM of NS1 protein.

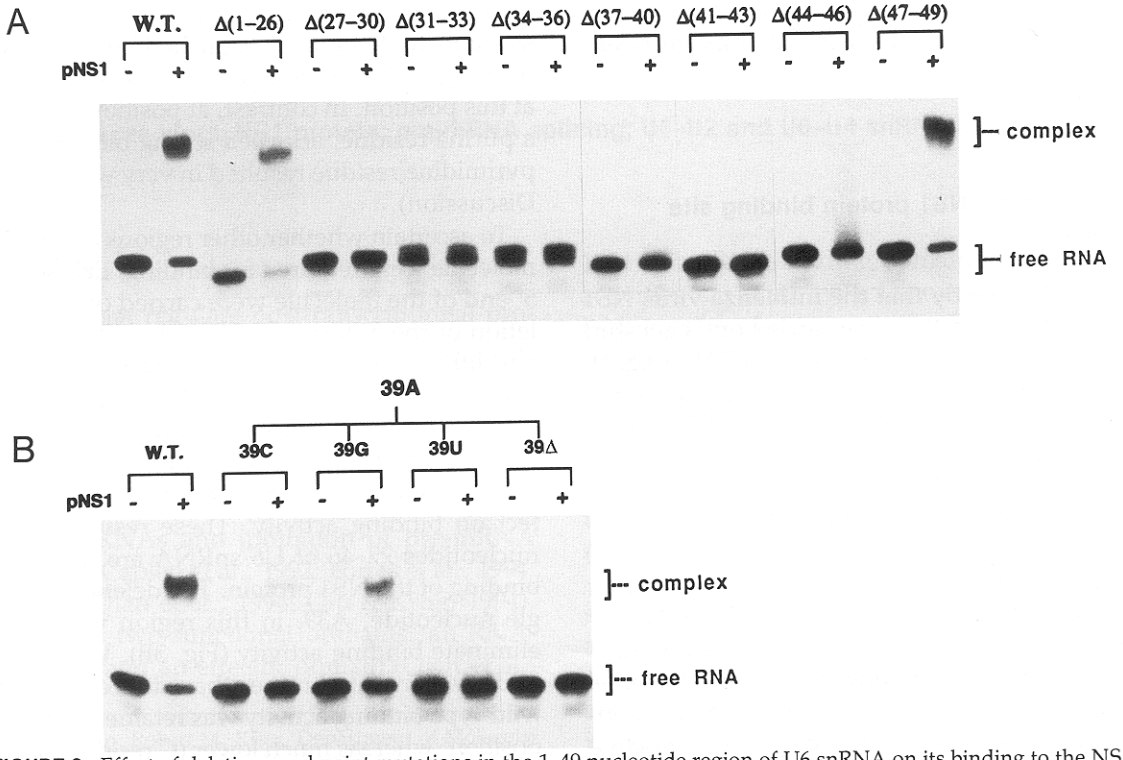

FIGURE 3. Effect of deletions and point mutations in the 1–49 nucleotide region of U6 snRNA on its binding to the NS1 protein. Binding assays were performed using the indicated U6 snRNA and either: −, no NS1 protein; or +, 800 nM NS1 protein.

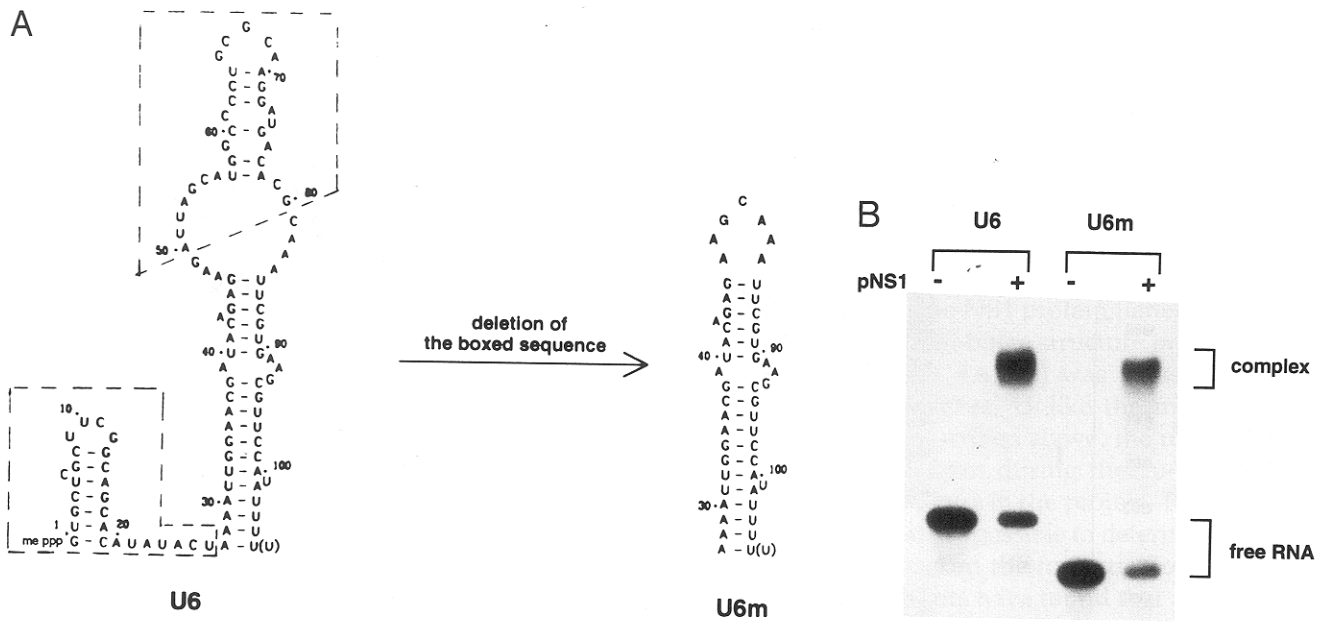

FIGURE 4. Fragment of human U6 snRNA that is sufficient for efficient binding to the NS1 protein. **A:** Based on the results shown in Figures 2 and 3, and on the proposed secondary structure of human U6 snRNA, two large deletions (nucleotides 1–26 and nucleotides 50–80) were made within U6 snRNA. **B:** Binding assay was carried out as described in Figure 2; −, no NS1 protein; +, 800 nM NS1 protein.

The two U6 snRNA sequences required for NS1 protein binding, nucleotides 85–106 and nucleotides 27–46, have been predicted to form a stem containing a few bulged out nucleotides (Rinke et al., 1985; Wolff & Bindereif, 1993) (Fig. 4A). To determine whether this stem-bulge structure was sufficient for efficient binding of the NS1 protein, we deleted the first 26 nucleotides of U6 snRNA as well as nucleotides 50–80 between the two sides of the stem. The resulting fragment (49 nucleotides long) bound to the NS1 protein almost as efficiently as the intact U6 snRNA molecule (Fig. 4B), establishing that this U6 snRNA stem-bulge was indeed sufficient for efficient binding.

To identify the actual binding site of the NS1 protein on this U6 snRNA stem-bulge, we used a chemical modification/interference assay (Peattie, 1979; Wolff & Bindereif, 1993). U6 snRNA, labeled with ^{32}P at its 3′ end, was treated with diethylpyrocarbonate (DEPC), which modifies purines (A and G). The modified U6 snRNA was incubated with the NS1 protein, and the bound and free U6 snRNAs were separated by native gel electrophoresis. These two U6 snRNA samples were cleaved with aniline, and the cleavage products were analyzed by denaturing gel electrophoresis (Fig. 5A). By comparing the cleavage patterns of the free and bound U6 snRNAs, strong interference was identified in two regions of the U6 snRNA bound to the NS1 protein: nucleotides 38–43 and nucleotides 88–95. As summarized in the proposed U6 snRNA structure (Fig. 5B), the purines at which strong interference occurred were G38, A39, A41, A43, G88, G90, A92, G93, and G95. These nucleotides are clustered around the major bulge in this stem-bulge structure of U6 snRNA. It can be concluded that this is the region of the U6 snRNA molecule to which the NS1 protein binds. Further, these results, along with the deletion data, provide strong experimental support for the existence of the postulated stem-bulge structure in this part of the U6 snRNA molecule.

Effect of the NS1 protein on the interactions of U6 snRNA with U2 and U4 snRNAs

During splicing, U6 snRNA forms complexes with both the U2 and U4 snRNAs (Nilsen, 1994; Sharp, 1994). One of the two regions of U6–U2 base-pairing, called helix II, which is essential for mammalian splicing, is formed between nucleotides 86 and 95 of U6 snRNA and a sequence at the 5′ end of U2 snRNA (Datta & Weiner, 1991; Wu & Manley, 1991). These U6 nucleotides are included in the NS1 binding site, suggesting that the NS1 protein could inhibit the formation of helix II. As one approach for determining the effect of the NS1 protein on the formation of U6–U2 snRNA helix II, we used a 15-mer oligoribonucleotide that is complementary to U6 nucleotides 85–99 and hence would form a model helix II with U6 snRNA. It should be noted that this model helix II is actually slightly longer than the natural U6–U2 helix II. When the complementary oligoribonucleotide and the NS1 protein were added at the same time, more than 50% of the U6 snRNA formed a helix II-like complex that had a slower

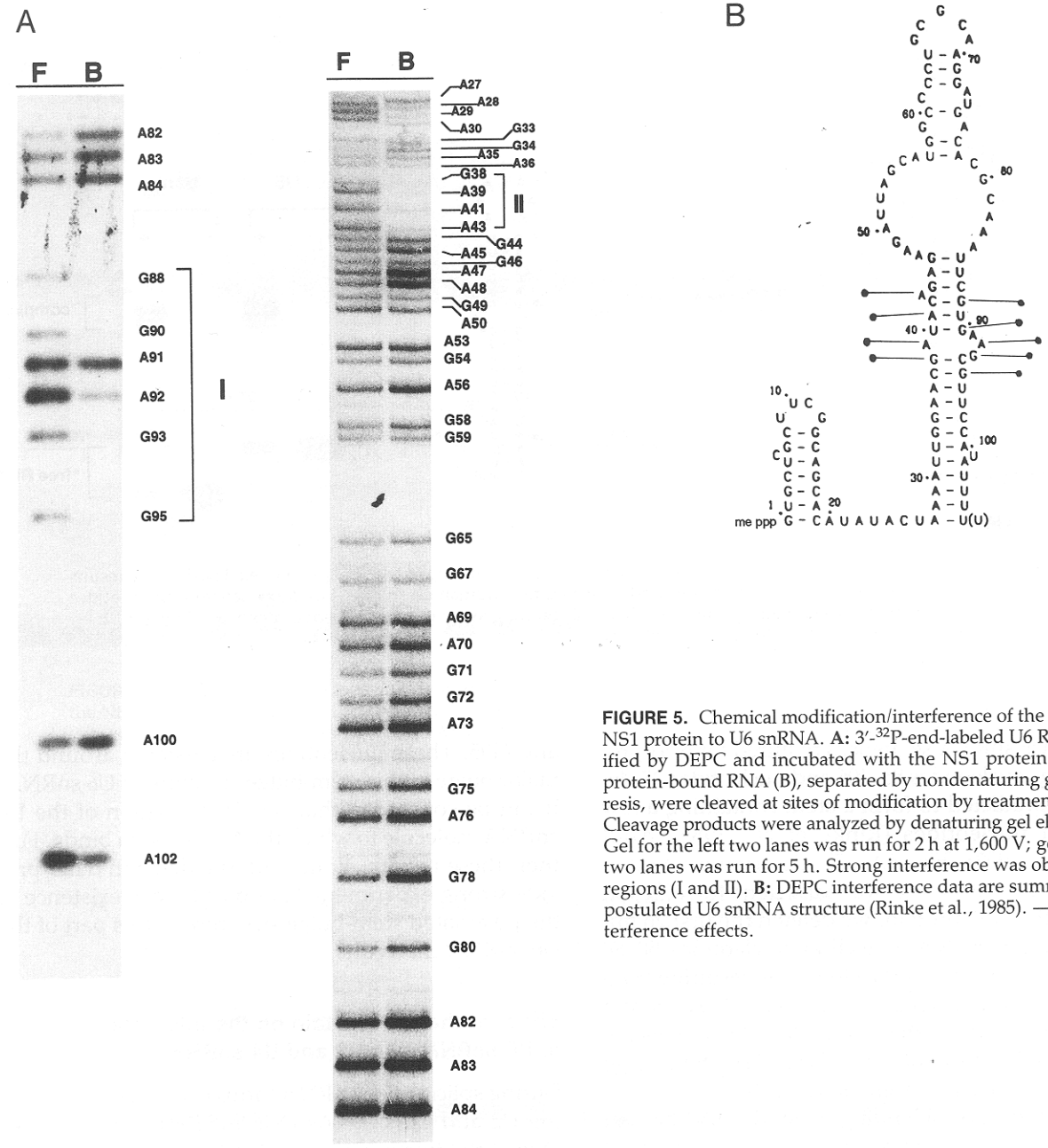

FIGURE 5. Chemical modification/interference of the binding of the NS1 protein to U6 snRNA. **A:** 3′-^{32}P-end-labeled U6 RNA was modified by DEPC and incubated with the NS1 protein. Free (F) and protein-bound RNA (B), separated by nondenaturing gel electrophoresis, were cleaved at sites of modification by treatment with aniline. Cleavage products were analyzed by denaturing gel electrophoresis. Gel for the left two lanes was run for 2 h at 1,600 V; gel for the right two lanes was run for 5 h. Strong interference was observed in two regions (I and II). **B:** DEPC interference data are summarized in the postulated U6 snRNA structure (Rinke et al., 1985). —●, Sites of interference effects.

mobility than free U6 snRNA during nondenaturing gel electrophoresis (Fig. 6A, lane 3). The NS1 protein blocked the formation of this helix II-like complex, and all the U6 snRNA formed a complex that had the same mobility as that formed between the NS1 protein and U6 snRNA alone (lane 4). These results were verified by experiments that used labeled oligoribonucleotide and unlabeled U6 snRNA (Fig. 6B). When the labeled oligoribonucleotide and the unlabeled U6 snRNA were added at the same time, the resulting helix II-like complex contained labeled oligoribonucleotide (lane 1) and, in the presence of the NS1 protein, labeled oligoribonucleotide could not be detected at the position of either the helix II-like complex or the NS1 protein–U6 complex (lane 2). Under the latter conditions the NS1 protein–U6 complex was efficiently formed as demonstrated in a parallel reaction containing labeled U6 snRNA and unlabeled oligoribonucleotide (lanes 3, 4). These results strongly suggested that the NS1 protein could inhibit helix II formation during splicing.

To form a model U6–U4 complex containing stem I and stem II base-paired regions like those in native U6–U4 snRNA complexes (Brow & Guthrie, 1988; Guthrie & Patterson, 1988), a 25-mer oligoribonucleotide containing the U4 stem I and stem II sequences was base-paired to labeled U6 snRNA. The resulting complex had a slower mobility than free U6 snRNA during nondenaturing gel electrophoresis (Fig. 7A, compare lanes 1

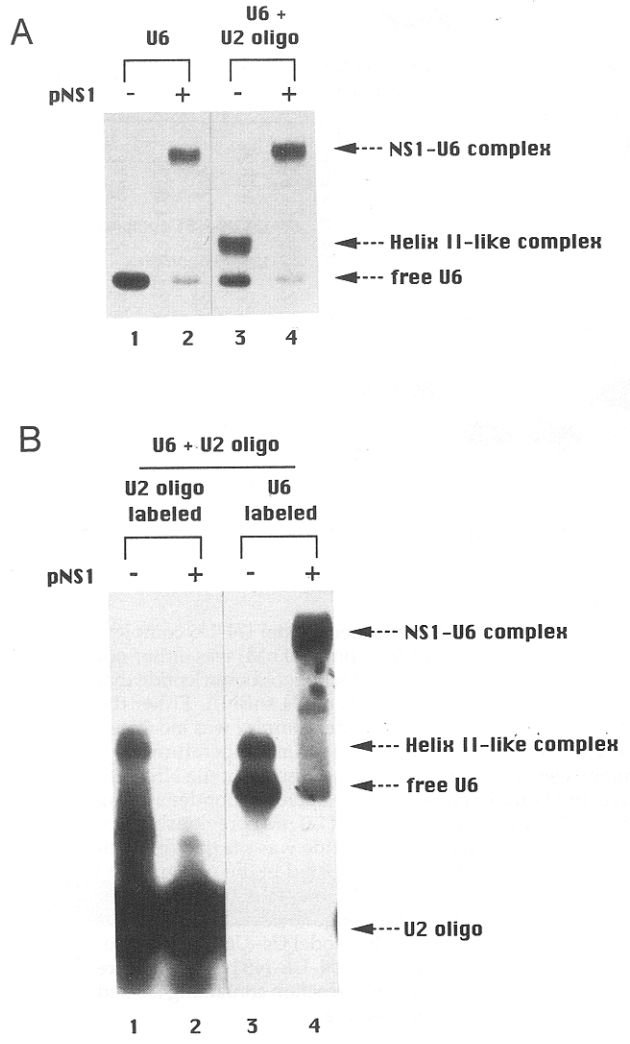

FIGURE 6. NS1 protein inhibits the formation of a model U6–U2 helix II. **A:** Full-length labeled U6 snRNA (10,000 cpm, 1.0 nM) (lanes 1, 2) or the U6 snRNA plus a 1,000-fold excess of the 15-mer oligoribonucleotide complementary to U6 nucleotides 85–99 (U2 oligo) (lanes 3, 4) was incubated with 800 nM NS1 protein (+) for 20 min at room temperature. U2 oligo and the NS1 protein were added at the same time. As a control, incubations were also carried out in the absence of the NS1 protein (−). The mixture was subjected to nondenaturing gel electrophoresis. **B:** Unlabeled U6 snRNA (1.0 nM) plus a 1,000-fold excess of the labeled U2 oligo was incubated with 800 nM NS1 protein (+) for 20 min at room temperature. Again, the U2 oligo and NS1 protein were added at the same time. A parallel reaction containing labeled U6 snRNA plus unlabeled U2 oligo was also carried out. Control incubations were carried out in the absence of NS1 protein (−). Mixtures were subjected to nondenaturing gel electrophoresis.

and 3). Surprisingly, the NS1 protein efficiently bound to this model U6–U4 complex, and the resulting protein–RNA complex had a slower mobility than the complex formed between the NS1 protein and U6 snRNA alone (lane 4). To verify that the U4 oligoribonucleotide was present in the complex shown in lane 4, we repeated this experiment using a labeled U4 oligoribonucleotide and unlabeled U6 snRNA (Fig. 7B): the labeled U4 oligoribonucleotide was indeed present in the complex containing the NS1 protein. To establish that the NS1 protein was in fact binding to the same U6 site in this model U6–U4 complex as in free U6 snRNA, we employed two small deletion mutants of U6 snRNA (Δ37–40 and Δ90–92) (Fig. 7A). These experiments employed labeled U6 snRNA and unlabeled U4 oligoribonucleotide. As was the case for the free mutant U6 snRNAs, model U6–U4 complexes containing either of these two mutant U6 snRNAs did not support the binding of the NS1 protein (lanes 5–12). This indicated that the stem-bulge structure present in free U6 snRNA (see Figs. 4A, 5B) was preserved in the model U6–U4 complexes. Unlike the model U6–U2 helix II complexes described above, the model U6–U4 complexes did not form during the 20-min incubation at room temperature in the protein–RNA binding buffer, so that it was not possible to determine whether the NS1 protein blocked the formation of this model U6–U4 complex. Others have found that under conditions compatible with splicing one or more protein factors are required for the formation of U6–U4 complexes (Wolff & Bindereif, 1993).

To examine the effect of the NS1 protein on the formation of U6–U2 and U6–U4 complexes during in vitro splicing, we used a psoralen crosslinking assay (Hausner et al., 1990; Wassarman & Steitz, 1993). It has been documented that psoralen crosslinking detects the formation of U6–U2 helix II (but not helix I) and the formation of U6–U4 complexes. In vitro splicing reactions employing HeLa cell nuclear extracts and the MINX pre-mRNA (Zillman et al., 1988) were incubated for 30 min at 30 °C. In the presence of 0.5 μM GST–NS1 fusion protein, both U6–U2 and U6–U4 crosslinking were reduced approximately 50% compared to the control reaction (nuclear extract alone) (Fig. 8A, lanes 2, 3). When the concentration of the GST–NS1 protein was increased to 1.0 and 1.5 μM, U6–U2 and U6–U4 crosslinking were reduced to almost undetectable levels (lanes 4, 5). The same levels of a GST–NS1 fusion protein containing a deletion (Δ1) of the RNA-binding domain did not inhibit U6–U2 and U6–U4 crosslinking (lanes 6–8). In contrast, a GST–NS1 fusion protein containing a deletion of the effector domain was as active as the fusion protein containing the wild-type NS1 protein (data not shown). These results indicate that the NS1 protein, which, as shown above, binds to a specific region of U6 snRNA, inhibits the formation of U6–U2 helix II and U6–U4 complexes during splicing.

The experiment with the model U6–U4 complexes indicated that the NS1 protein did not dissociate these preformed complexes (Fig. 7). To determine the effect of the NS1 protein on preformed, authentic U6–U2 and U6–U4 complexes present in nuclear extracts, these extracts were incubated in the absence and presence of 1.5 μM GST–NS1 protein for 30 min at 30 °C in the absence of ATP and an ATP-generating system, conditions under which splicing is abrogated (Fig. 8B). The

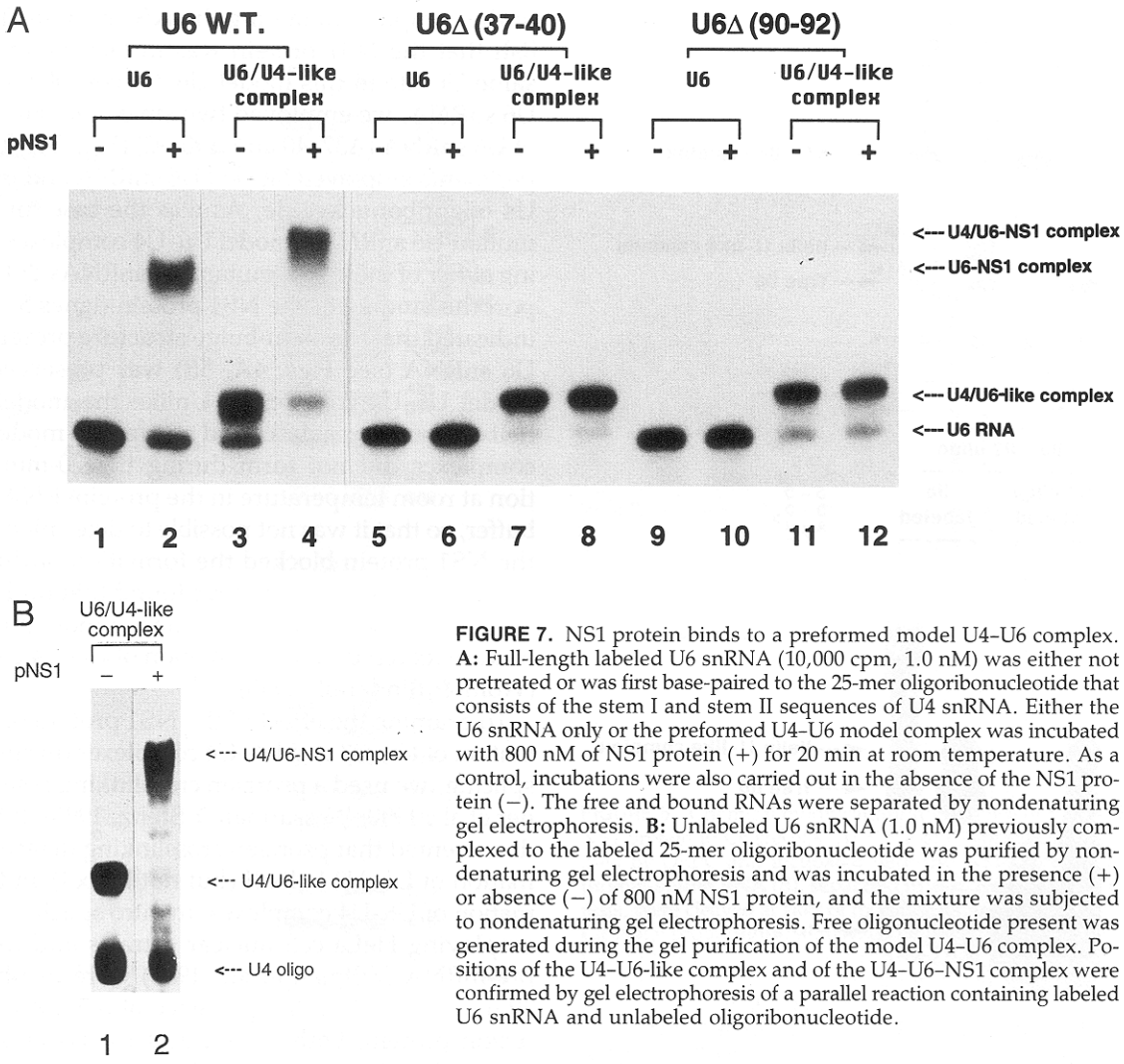

FIGURE 7. NS1 protein binds to a preformed model U4–U6 complex. **A:** Full-length labeled U6 snRNA (10,000 cpm, 1.0 nM) was either not pretreated or was first base-paired to the 25-mer oligoribonucleotide that consists of the stem I and stem II sequences of U4 snRNA. Either the U6 snRNA only or the preformed U4–U6 model complex was incubated with 800 nM of NS1 protein (+) for 20 min at room temperature. As a control, incubations were also carried out in the absence of the NS1 protein (−). The free and bound RNAs were separated by nondenaturing gel electrophoresis. **B:** Unlabeled U6 snRNA (1.0 nM) previously complexed to the labeled 25-mer oligoribonucleotide was purified by nondenaturing gel electrophoresis and was incubated in the presence (+) or absence (−) of 800 nM NS1 protein, and the mixture was subjected to nondenaturing gel electrophoresis. Free oligonucleotide present was generated during the gel purification of the model U4–U6 complex. Positions of the U4–U6-like complex and of the U4–U6–NS1 complex were confirmed by gel electrophoresis of a parallel reaction containing labeled U6 snRNA and unlabeled oligoribonucleotide.

GST–NS1 protein had no effect on the amount of U6–U2 and U6–U4 crosslinking, indicating that the NS1 protein did not cause the dissociation of preformed U6–U2 and U6–U4 complexes present in nuclear extracts.

Splicing activity and spliceosome formation were assayed in the presence of ATP at the three levels of the wild-type GST–NS1 fusion protein used in the psoralen crosslinking assays. In the presence of 0.5 μM of this protein, the production of spliced mRNA was inhibited 90%, whereas there was actually an increase in the amount of the lariat-3′ exon intermediate (Fig. 9A). The amount of spliceosomes (B complexes) assayed by native gel electrophoresis was similar to that in the absence of the GST–NS1 protein (Fig. 9B). However, a small amount of a species migrating between the B complexes and A complexes (prespliceosomes) appeared. This species, which was barely detectable in the absence of the wild-type GST–NS1 fusion protein, contained only U1 and U2 snRNAs (data not shown) and may be the A′ complexes previously described by others (Zillman et al., 1988; Krainer et al., 1990). Thus, splicing, predominantly the second step of splicing, was inhibited, whereas spliceosome formation was largely unaffected at this level of the GST–NS1 protein (see the Discussion). These data are similar to our previous results (Lu et al., 1994).

Increasing the amount of the GST–NS1 fusion protein to 1.0 μM resulted in 90% inhibition in spliceosome (B complex) formation and an increase in the putative A′ complexes (Fig. 9B). Splicing was almost completely inhibited, though a very small amount of the lariat-3′ exon intermediate was still made (Fig. 9A). With the highest concentration of the GST–NS1 protein tested, 1.5 μM, spliceosome (B complex) formation and splicing were completely inhibited (Fig. 9A,B). Thus, inhibition of spliceosome formation occurred only at these higher levels of the GST–NS1 protein (see the Discussion). In contrast, the interaction of the pre-mRNA with U1 and U2 snRNAs persisted in the presence of 1.0 and 1.5 μM GST–NS1 protein. The amount of the putative A′ complexes containing U1 and U2 snRNA

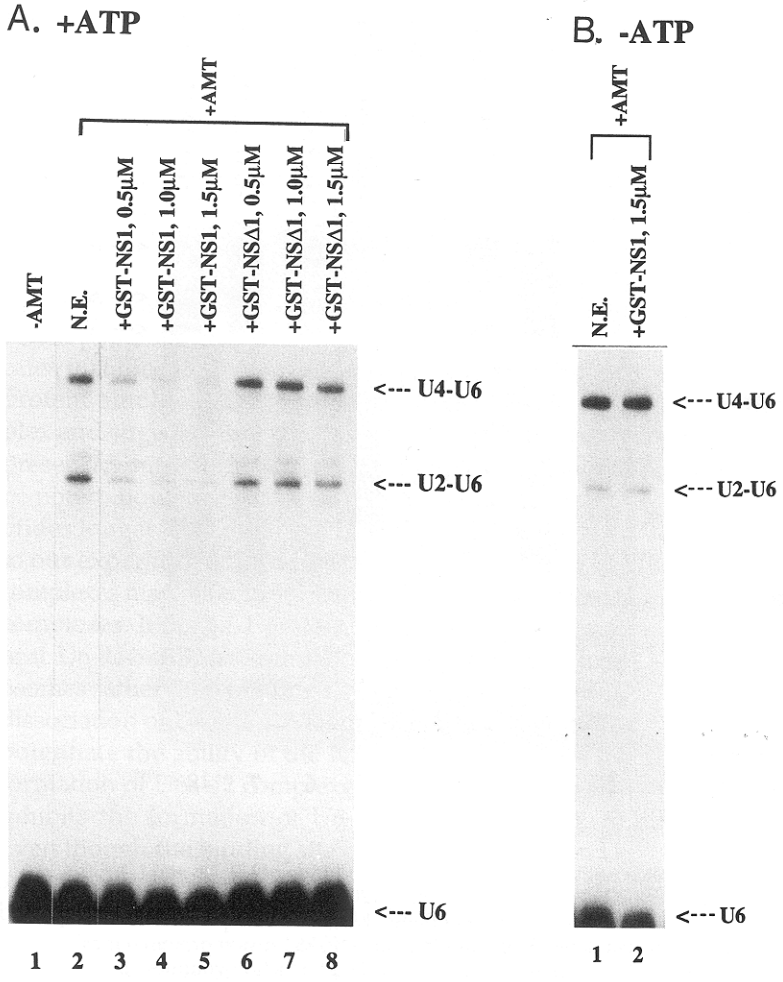

FIGURE 8. NS1 protein inhibits U2–U6 and U4–U6 complex formation during splicing, as assayed by psoralen crosslinking. **A:** Unlabeled MINX pre-mRNA (20 ng) was incubated for 30 min at 30 °C in a splicing reaction (HeLa cell nuclear extract) in the presence of ATP and an ATP-generating system under the conditions described previously (Lu et al., 1994), in the absence of a GST–NS1 fusion protein (lanes 1, 2), or in the presence of the indicated concentration of the wild-type GST–NS1 fusion protein (lanes 3–5), or of the GST–NS1Δ1 fusion protein (Δ1, deletion of NS1 RNA binding domain) (lanes 6–8). After incubation, all reactions except that in lane 1 were treated with AMT, UV-irradiated, and the RNA extracted as described (Hausner et al., 1990; Wassarman & Steitz, 1993). Reaction in lane 1 was not treated with AMT, but otherwise was processed like the other reactions. RNA products were resolved by electrophoresis on a denaturing gel, followed by Northern blotting using a ^{32}P-labeled U6 probe. **B:** Unlabeled MINX pre-mRNA (20 ng) was incubated with HeLa cell nuclear extract in the absence of ATP and an ATP-generating system either in the absence of a GST–NS1 fusion protein (lane 1) or in the presence of 1.5 μM GST–NS1 protein (lane 2). After AMT treatment and UV irradiation, RNA was subjected to gel electrophoresis, followed by Northern blotting using a ^{32}P-labeled U6 probe.

increased when the GST–NS1 concentration was increased from 0.5 to 1.0 μM, and the prespliceosomes (A complexes) continued to form in the presence of 1.0 and 1.5 μM GST–NS1, although in reduced amounts. It should be emphasized that the same levels of the GST–NS1Δ1 protein (Fig. 9A,B) and of other RNA-binding proteins (Lu et al., 1994) did not have any effect on splicing activity or spliceosome formation.

DISCUSSION

The influenza virus NS1 protein has the unique property of binding to a specific stem-bulge in U6 snRNA (Fig. 6B). Both the deletion and chemical modification/interference results established that the NS1 protein binds to both nucleotide chains of the stem-bulge of U6 snRNA. This provides strong evidence that this postulated secondary structure in U6 snRNA (Rinke et al., 1985) actually exists. Based on our chemical modification results, the primary binding site of the NS1 protein includes the U6 snRNA purine-containing bulge (nucleotides 39, 91, 92, and 93) as well as nucleotides in the stem structure adjacent to this bulge, specifically nucleotides 38–43 and nucleotides 88–95. However, U6 sequences that are at a distance from this primary binding site are also required for the binding of the NS1 protein. For example, deletion of nucleotides 27–30 or of nucleotides 99–101 eliminates most, if not all, of the binding activity. It is likely that these more distant nucleotides are needed to maintain secondary structure, i.e., the double-stranded stem is required for binding activity. In fact, our recent experiments indicate that the NS1 protein recognizes largely double-stranded regions in RNA (Y. Lu & R. Krug, manuscript in prep.). This raises the issue of why the NS1 protein bound at and near the bulge region of the stem-bulge. It may be significant that binding occurred only when purines but not pyrimidines were present at two positions of the bulge (nucleotides 39 and 93). The other residues in this bulge are also purines. This suggests that purines, but not pyrimidines, in this bulge maintain the continuity of the double-stranded character of the U6 stem-bulge.

Our mutational results establish that the NS1 protein is highly specific in its ability to bind to a particular region in U6 snRNA. As assayed by gel shift experiments, this specific interaction requires an excess of the NS1 protein, 400–800 nM to quantitatively gel shift

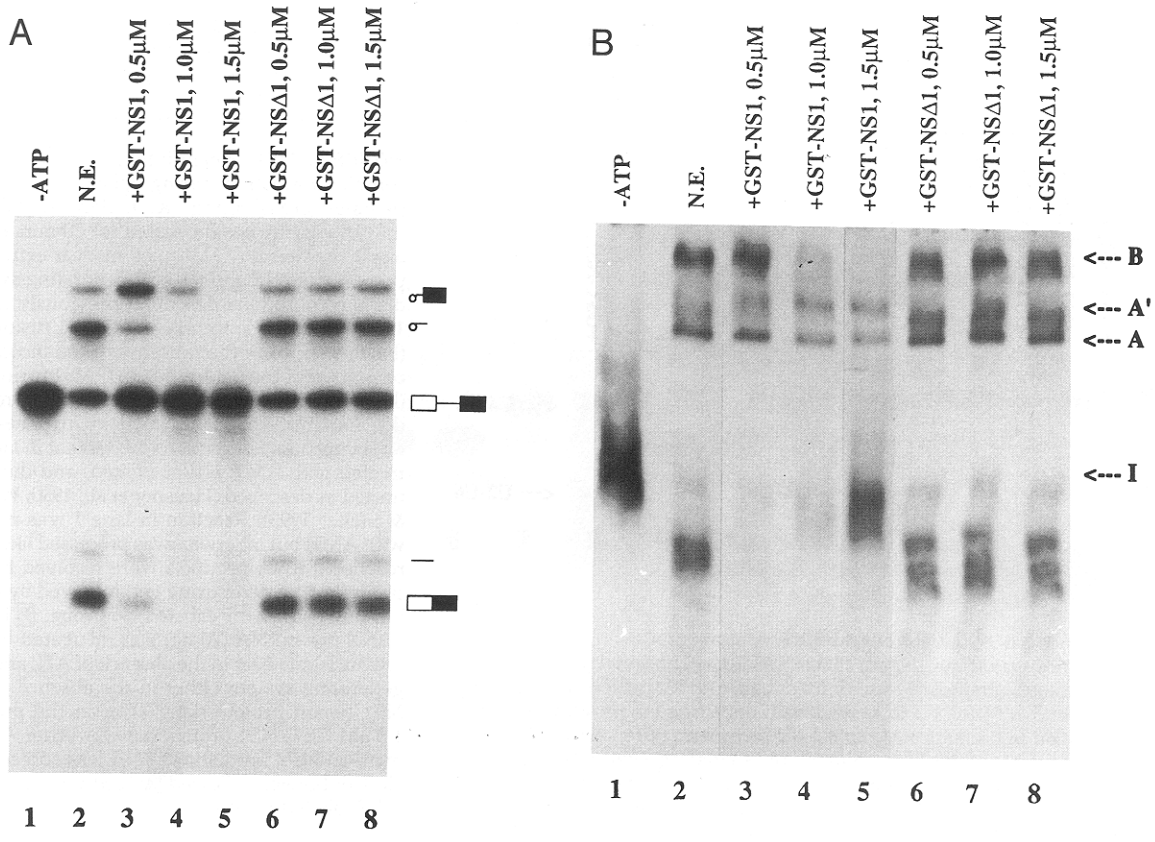

FIGURE 9. Correlation of the inhibition of splicing and of spliceosome formation with the inhibition of U2–U6 and U4–U6 complex formation by the NS1 protein. **A:** Splicing assays. ^{32}P-labeled MINX pre-mRNA (20,000 cpm) was incubated for 30 min at 30 °C in a splicing reaction in the absence of a GST–NS1 fusion protein (lanes 1, 2), or in the presence of the indicated concentration of the wild-type GST–NS1 fusion protein (lanes 3–5), or of the GST–NS1Δ1 fusion protein (lanes 6–8). ATP and the ATP-generating system were omitted from the reaction in lane 1. Of the 20-μL reaction volumes, 15 μL were extracted with pronase and phenol–chloroform, and the RNA products were analyzed by gel electrophoresis (13% gel containing 7 M urea). **B:** Spliceosome assays. To 5 μL of the reactions described in A, heparin was added to a final concentration of 1 mg/mL. This mixture was incubated at 30 °C for 10 min and then subjected to electrophoresis on a nondenaturing 3.5% acrylamide–0.5% agarose gel for 4 h at 175 V. B, A, A′ are described in the text; I is the initial complex made in the absence of ATP (lane 1) (Zillman et al., 1988; Krainer et al., 1990).

1 nM U6 snRNA. A similar requirement for an excess of protein has been found for the specific interaction of the human immunodeficiency virus (HIV) 1 Rev protein with its RNA target: 67–200 nM Rev protein was needed to gel shift 50% of 0.1–0.2 nM target RNA (Heaphy et al., 1990; Malim et al., 1990). More than one protein molecule apparently binds to the cognate RNA target, four in the case of Rev (Olsen et al., 1990; Zapp et al., 1991) and two in the case of the NS1 protein (M. Nemeroff & R. Krug, manuscript in prep.), but this does not account for the magnitude of the excess of protein needed for efficient binding as assayed by gel shift analysis. One possibility is that a substantial fraction of the purified, bacterially expressed Rev and NS1 proteins is not active in binding to the specific RNA target. It should be noted, however, that the gel shift assay likely underestimates the RNA-binding activity of these protein preparations. The concentration of the Rev protein needed in a filter-binding assay was about 50-fold lower than that needed in a gel shift assay (Heaphy et al., 1990). Regardless of the percentage of the bacterially expressed Rev and NS1 proteins that are active, it is clear that the active molecules exhibit great specificity in their binding to the cognate RNA target (Heaphy et al., 1990; Malim et al., 1990; present study).

U6 snRNA interacts with U2 and U4 snRNAs during splicing (Nilsen, 1994; Sharp, 1994). The U6–U2 interaction involves two regions of base-pairing between U6 and U2 sequences: helix I, involving U6 nucleotides 48–56, and helix II, involving U6 nucleotides 86–95 (Nilsen, 1994; Sharp, 1994). The latter, but not the former, U6 sequence is part of the NS1 protein binding site, and we demonstrated that the NS1 protein blocks the formation of helix II both in a model system and in nuclear splicing extracts. Helix II has been shown to be essential for mammalian splicing (Datta & Weiner, 1991; Wu & Manley, 1991). In addition, the primary binding site of the NS1 protein on U6 snRNA includes at least part of the invariant ACAGAG sequence (nucleotides 41–46) that has been shown to play a critical

role in 5′ splice site selection (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993). The formation of U6–U2 complexes during splicing requires the dissociation of U6 snRNA from U6–U4 complexes (Nilsen, 1994; Sharp, 1994).

Our results suggest that the NS1 protein can bind not only to free U6 snRNA but also to the U6 snRNA in the U6–U4 complex. Thus, the NS1 protein binds to a model U6–U4 complex consisting of U6 snRNA and a 25-mer oligoribonucleotide containing the complementary stem I and stem II U4 sequences. This indicates that the U6 stem-bulge structure required for NS1 protein binding is present in this model U6–U4 complex and suggests that this U6 stem-bulge may also be present in natural U6–U4 snRNA complexes. It should be noted, however, that U4 snRNA is about 120 nucleotides longer than the model U4 oligonucleotide used in our experiments, and hence natural U6–U4 snRNA complexes may differ in structure from our model U6–U4 complexes. If the NS1 protein were able to bind to natural U6–U4 snRNA complexes that enter the spliceosomes rather than binding to U6 snRNA only after the dissociation of U4 snRNA from U6 snRNA, this would potentiate the ability of the NS1 protein to inhibit the formation of U6–U2 complexes. The NS1 protein also inhibits the formation of U6–U4 complexes (Fig. 8). Even though the binding site of the NS1 protein on U6 snRNA does not include the region of U6 snRNA that base pairs with U4 snRNA, this binding site does include the 3′-terminal region of U6 snRNA that has been shown to be important for the transitions that lead to the formation of U6–U4 complexes (Wolff & Bindereif, 1993).

In contrast to the ability of the NS1 protein to inhibit the formation of U6–U2 and U6–U4 complexes, this protein did not cause the dissociation of preformed U6–U2 and U6–U4 complexes. This was demonstrated both in the U6–U4 model system described above and in nuclear splicing extracts. In the nuclear extracts, preformed U6–U4 and U6–U2 complexes have been shown to exist (Wassarman, 1993; Wassarman & Steitz, 1993; present study), and these complexes were not dissociated when the NS1 protein was incubated with the nuclear extracts in the absence of ATP, conditions under which splicing does not occur. Consequently, we can conclude that the observed inhibition of the amount of U6–U4 and U6–U2 complexes caused by the NS1 protein in the presence of ATP is due to the inhibition of the association and reassociation of U6 snRNA with U4 and U2 snRNAs that occurs during splicing.

Based on current models of splicing (Nilsen, 1994; Sharp, 1994), our working hypothesis is that the inhibition of U6–U2 complex formation largely accounts for the inhibition of splicing, whereas the inhibition of U6–U4 complex formation largely accounts for the inhibition of spliceosome formation. At the lowest level of the GST–NS1 protein added to the splicing reaction (0.5 μM), the amount of the U6–U4 complexes that remained was apparently sufficient for almost normal levels of spliceosome formation. Under the same conditions, the reduction in the amount of the U6–U2 helix II complexes resulted in a 90% inhibition of the second step of splicing. The first step in splicing, yielding the lariat-3′ exon, continued to a certain extent, as found previously (Lu et al., 1994). It will be of interest to determine how the inhibition of U6–U2 helix II formation by the NS1 protein inhibits the second step of splicing more than the first step, and to determine whether U6–U2 helix I formation is inhibited in the same way as helix II formation. When the concentration of the GST–NS1 protein in the splicing reaction was increased to 1.0 and 1.5 μM, spliceosome formation was inhibited, presumably as a result of the almost complete absence of U6–U4 complexes. Basically, we propose that the binding of the NS1 protein to U6 snRNA serves to sequester U6 snRNA, predominantly in the singlet form, so that it would be unable to undergo the interactions with U2 and U4 snRNAs that are required for splicing and spliceosome formation.

This hypothesis predicts that essentially all of the U6 snRNA in the nuclear extracts would be bound to the NS1 protein when this protein was present at its inhibitory levels of 0.5–1.5 μM. Based on previous calculations of the amount of U6 snRNA in nuclear extracts (Wolff & Bindereif, 1993), the concentration of U6 snRNA in our in vitro splicing assays should be approximately 16 nM. Our gel shift assays indicate that the concentration of the NS1 protein required to complex all of this U6 snRNA would be approximately 10 μM. If, as is the case for Rev binding (Heaphy et al., 1990), the gel shift assays underestimate the binding activity of the NS1 protein by about 50-fold, then the concentration of the NS1 protein required to bind all the U6 snRNA in the nuclear extract would be comparable to the concentration that was experimentally found to be needed for the inhibition of splicing.

In addition, the underlying premise of our hypothesis is that the inhibition of splicing and of spliceosome formation results primarily, if not entirely, from the interaction of the NS1 protein with U6 snRNA, and that NS1 protein interactions with other components of the splicing machinery are not involved. As one approach to proving this premise, we attempted to use the U6 reconstitution assay, i.e., deplete U6 snRNA from nuclear extracts and reconstitute splicing activity with wild-type or mutant U6 snRNA molecules (Wolff & Bindereif, 1992). If an added mutated U6 snRNA were unable to bind the NS1 protein but were still able to form U2–U6 helix II, then splicing should occur but should be refractory to inhibition by the NS1 protein. Unfortunately, efficient reconstitution of splicing activity required about 40-fold more U6 snRNA than is normally present in nuclear extracts (Wolff & Bindereif, 1992), so that it was not feasible to add sufficient con-

centrations of the NS1 protein to inhibit splicing (unpubl.). The results described in the present study do indicate that other RNA–RNA splicing interactions persisted under conditions in which the NS1 protein disrupted U6 snRNA interaction. Thus, the interaction of the pre-mRNA with U1 and U2 snRNAs persisted in the presence of 1.0 and 1.5 μM of the GST–NS1 protein: A′ complexes and prespliceosomes (A complexes), both of which contain U1 and U2 snRNAs, continued to form. In fact, the A′ complexes were actually generated by the addition of the GST–NS1 protein. Further investigation is required to establish definitively that the NS1 protein inhibits splicing solely via blocking U6 snRNA interactions.

In influenza virus-infected cells the NS1 protein also specifically interacts with U6 snRNA (Lu et al., 1994). This strongly suggests that the NS1 protein inhibits splicing in infected cells via the mechanism described in the present study. As previously discussed (Lu et al., 1994), it has not been possible to make a meaningful analysis of the splicing of host pre-mRNAs in influenza virus infected cells themselves, because all host polymerase II transcripts are degraded in the nucleus of infected cells due at least in part to the cleavage of their 5′ ends by the viral cap-dependent endonuclease (Katze & Krug, 1984; Krug et al., 1989). Our results indicate that the influenza virus NS1 protein regulates pre-mRNA splicing in a unique way. Other proteins that regulate pre-mRNA splicing bind to specific sites on particular pre-mRNAs and inhibit or enhance the formation of spliceosomes at specific splice site(s) on these pre-mRNAs (Inoue et al., 1990; Tian & Maniatis, 1992). Instead, the NS1 protein binds to a specific site on a key spliceosomal RNA, U6 snRNA, leading to the inhibition of splicing, followed by the inhibition of spliceosome formation at higher levels of the protein.

MATERIALS AND METHODS

RNA binding assays

The GST–NS1 fusion protein was purified, and the NS1 protein was cleaved from this fusion as previously described (Qiu & Krug, 1994). The purity of these proteins was established by gel electrophoresis followed by Coomassie blue dye staining. Binding assays were carried out essentially as previously described (Qiu & Krug, 1994). Briefly, the indicated labeled RNA (at 1.0 nM except where indicated) and the indicated concentration of the nonfusion NS1 protein were incubated in an RNA-binding buffer (43 mM Tris, pH 8.0, 50 mM KCl, 8% glycerol, 5 mM dithiothreitol, 50 ng/μL *Escherichia coli* tRNA, 0.5 units/μL RNasin) in a final volume of 20 μL on ice for 20 min. Where indicated, some incubations were carried out at room temperature. The protein–RNA complexes were separated from unbound RNA by electrophoresis on 4% nondenaturing polyacrylamide gels using 50 mM Tris, pH 8.0, 50 mM boric acid, 1 mM EDTA as running buffer. The gels were run for 3 h at 10 V/cm.

Mutagenesis of U6 snRNA

The full-length U6 snRNA sequence was produced by PCR from a human genomic U6 DNA (Konarska & Sharp, 1987). The resulting DNA was cloned into the *Hin*d III and *Bam*H I sites of pGEM1. Deletion and point mutations of U6 snRNA were made using oligonucleotide-directed PCR mutagenesis. The mutations were confirmed by sequencing. The small deletion mutants between U6 nucleotides 80 and 106 were kindly provided by Dr. A. Bindereif (Wolff & Bindereif, 1992). Each U6 RNA sequence was synthesized by SP6 RNA polymerase in the presence of [α-^{32}P]UTP.

DEPC modification/interference analysis

In vitro-transcribed U6 RNA was 3′-end-labeled using [^{32}P]pCp and T4 RNA ligase and purified by gel electrophoresis. The RNA was modified with DEPC as described (Peattie, 1979). Modified U6 RNA (5×10^5 cpm, 5 nM) was incubated with 2 μM NS1 protein in a 50-μL reaction for 20 min on ice, and the mixture was subjected to nondenaturing gel electrophoresis. Under these preparative conditions, approximately 50% of the labeled RNA was in a complex with the NS1 protein. The gel slices containing the free RNA and bound RNA were cut out, and the two RNAs were recovered by electroelution followed by phenol extraction and ethanol precipitation. The free and bound RNAs (5,000 cpm each) were subjected to aniline cleavage, and the cleavage products were analyzed by denaturing gel electrophoresis as described by Peattie (1979).

Formation of U6–U2 and U6–U4 model complexes

Two oligoribonucleotides were synthesized: 5′-GGAACG CUUCACGAA-3′ (U2 oligo) and 5′-GCUUUGCGCAGUGG CAUUGCUAAUU-3′ (U4 oligo). The U2 oligo oligoribonucleotide is complementary to nucleotides 85–99 of U6 snRNA; and the U4 oligo consists of the following U4 snRNA nucleotides: stem II (U4 nucleotides 2–17) and stem I (U4 nucleotides 55–63). Each of these two oligoribonucleotides has only one potential sequence in U6 snRNA to which it can base pair. Either the U6 snRNA was labeled (by SP6 RNA polymerase transcription in the presence of [α-^{32}P]UTP) or the oligoribonucleotide was labeled (using polynucleotide kinase and [γ-^{32}P]ATP). To form U6–U4 model complexes prior to RNA-binding assays, a 1,000 molar excess of the U4 oligo was mixed with U6 snRNA (1.0 nM). The mixture was heated in the RNA-binding buffer (modified in that it also contained 3 mM $MgCl_2$) for 2 min at 90 °C, followed by renaturation by slow cooling to room temperature. For the U6–U4 complex, the comparative ribonuclease T1 digestion patterns of free U6 snRNA and of the U6 snRNA–U4 oligo complex confirmed that the U4 oligo was base-paired with the appropriate U6 snRNA sequence.

In vitro splicing and spliceosome assays

In vitro splicing assays were performed as described previously (Lu et al., 1994). Briefly, ^{32}P-labeled MINX pre-mRNA (20,000 cpm) was incubated with HeLa nuclear extract in a

final volume of 20 μL under the conditions described previously for 30 min at 30 °C. Where indicated, the NS1 protein was added. The reaction was stopped by the addition of pronase followed by phenol extraction and ethanol precipitation. The RNAs were analyzed by electrophoresis on 13% polyacrylamide gels. Spliceosome assays were carried out as described previously (Nelson & Green, 1988). Heparin (1 mg/mL final concentration) was added to 5 μL of the splicing reaction described above. This mixture was incubated at 30 °C for 10 min and then subjected to electrophoresis on a nondenaturing 3.5% acrylamide–0.5% agarose gel for 4 h at 175 V.

Psoralen crosslinking assays

Unlabeled MINX pre-mRNA (20 ng) was incubated with the HeLa cell nuclear extract in a splicing reaction as described above. Where indicated, the NS1 protein was added and/or ATP and the ATP-generating system were omitted. After 30 min at 30 °C, 20 μg/mL AMT (4′-aminomethyl-4,5′,8-trimethyl psoralen) was added, followed by UV irradiation at 365 mM for 20 min on ice (Hausner et al., 1990; Wassarman & Steitz, 1993). The RNAs were isolated by pronase treatment, phenol extraction, and ethanol precipitation. The RNAs were then subjected to electrophoresis on a 6% denaturing polyacrylamide gel, and the separated RNAs were transferred by electroblotting onto a Nylon membrane. The membranes were hybridized with [α-^{32}P]UTP-labeled RNA complementary to U6, U2, or U4 snRNA. The blots were washed and then autoradiographed. The blot was reprobed with labeled RNA complementary to U2 or U4 snRNA to confirm the identity of the crosslinked complexes.

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