

U6atac snRNA, the highly divergent counterpart of U6 snRNA, is the specific target that mediates inhibition of AT-AC splicing by the influenza virus NS1 protein

WEIRONG WANG and ROBERT M. KRUG

Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, New Jersey 08855-1179, USA

ABSTRACT

The influenza virus NS1 protein inhibits the splicing of the major class of mammalian pre-mRNAs (GU-AG introns) by binding to a specific stem-bulge in U6 snRNA, thereby blocking the formation of U4/U6 and U2/U6 complexes. The splicing of the minor class of AT-AC introns takes place on spliceosomes that do not contain U6 snRNA, but rather U6atac snRNA—a highly divergent U6 snRNA counterpart. Nonetheless, we demonstrate that the NS1 protein inhibits AT-AC splicing *in vitro*, and specifically binds to only U6atac snRNA among the five minor class snRNAs. Chemical modification/interference assays show that the NS1 protein binds to the stem-bulge near the 3' end of U6atac snRNA, encompassing nt 82–95 and nt 105–114. Although the sequence of this stem-bulge differs significantly from the sequence of the stem-bulge to which the NS1 protein binds in U6 snRNA, RNA competition experiments indicate that U6 and U6atac snRNAs likely share the same binding site on the NS1 protein. Previously, the region of U6atac snRNA containing this 3' stem-bulge had not been implicated in any interactions of this snRNA with either U4atac or U12 snRNA. However, as assayed by psoralen crosslinking, we show that the NS1 protein inhibits the formation of U12/U6atac complexes, but not the formation of U4atac/U6atac complexes. We can conclude that the inhibition of AT-AC splicing results largely from the inhibition of formation of U12/U6atac complexes caused by the binding of the NS1 protein to the 3' stem-bulge of U6atac snRNA.

Keywords: AT-AC splicing; influenza virus NS1 protein; RNA-binding protein; U6atac snRNA; U6 snRNA

INTRODUCTION

Most eukaryotic pre-mRNAs contain 5' and 3' splice sites that conform to the GU-AG consensus (reviewed in Sharp, 1994). Splicing of these pre-mRNAs occurs on spliceosomes containing the U1, U2, U4, U6, and U5 snRNAs. During splicing, these snRNAs undergo interactions with the pre-mRNA and with each other (reviewed in Nilsen, 1994). U6 snRNA plays a central role. Initially, it is in the form of a hydrogen bonded complex with U4 snRNA. Subsequently, after dissociation from U4 snRNA, U6 snRNA interacts with the 5' splice site of the pre-mRNA and also with three different sequences in U2 snRNA. It has been postulated that this U6-U2 snRNA complex plays a direct catalytic role in splicing (Madhani & Guthrie, 1992). The ability of U6 snRNA to undergo these interactions is blocked by the influenza virus nonstructural protein 1 (NS1 protein), which binds to a specific stem-bulge in

U6 snRNA and inhibits the formation of U4/U6 and U2/U6 complexes (Lu et al., 1994; Qiu et al., 1995).

A small number of pre-mRNAs possess AU (or AT at the genomic level) and AC at the 5' and 3' ends of their introns, respectively (Jackson, 1991; Hall & Padgett, 1994). The splicing of AT-AC introns proceeds via a two-step reaction analogous to the splicing pathway for the major class of GU-AG introns (Tarn & Steitz, 1996a). However, other than U5 snRNA, a different set of snRNAs participates in the splicing reaction: U11, U12, U4atac, and U6atac instead of U1, U2, U4, and U6 (Hall & Padgett, 1996; Nilsen, 1996; Tarn & Steitz, 1996a, 1996b). The sequence conservation between U6atac snRNA and human U6 snRNA is less than that between human and yeast U6 snRNAs (Brow & Guthrie, 1988; Tarn & Steitz, 1996a). Based on computer-generated RNA structures, it is likely that U6atac snRNA lacks a stem-bulge structure similar to the one in U6 snRNA to which the influenza virus NS1 protein binds (Qiu et al., 1995; Tarn & Steitz, 1996b).

We were therefore surprised to find that the NS1 protein inhibits AT-AC splicing *in vitro* by specifically

Reprint requests to: Robert M. Krug, Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, New Jersey 08855-1179, USA; e-mail: krug@mbcl.rutgers.edu.

binding to only U6atac snRNA among the five minor class snRNAs. The NS1 protein binds to the stem-bulge near the 3' end of U6atac snRNA, a region that had not been implicated in its interactions with either U4atac snRNA or U12 snRNA (Tarn & Steitz, 1996b). However, we show that the binding of the NS1 protein to this region of U6atac snRNA inhibits the formation of U12/U6atac complexes, whereas this binding has little or no effect on the formation of U4atac/U6atac complexes.

RESULTS

Influenza virus NS1 protein inhibits AT-AC splicing in vitro

One of the AT-AC pre-mRNAs is specified by the human P120 gene, which codes for a proliferation-associated nucleolar protein. As shown previously (Tarn & Steitz, 1996a), HeLa cell nuclear extracts catalyze the splicing of P120 pre-mRNA in vitro (Fig. 1A). This splicing is apparent only when the splicing of the major class of GU-AG pre-mRNAs is suppressed by the addition of a 2'-O-methyl oligoribonucleotide complementary to nt 27-49 of U2 snRNA (Fig. 1A, lane 2).

Verification that the minor, and not the major, class of spliceosomes was catalyzing this splicing was shown by supplementing the reactions with a second 2'-O-methyl oligoribonucleotide complementary to either the major U6 snRNA (Fig. 1A, lane 3) or the minor U12 snRNA (Fig. 1A, lane 4). The latter, but not the former, oligoribonucleotide inhibited splicing.

The addition of increasing amounts of a fusion protein containing GST linked to the NS1 protein of influenza A virus effectively inhibited P120 splicing (Fig. 1B, lanes 2-4). In the presence of 0.5 μ M GST-NS1 protein, the spliced mRNA product was reduced by approximately 90% and, when the GST-NS1 protein concentration was increased to 1.5 μ M, the spliced mRNA product was not detectable. This inhibition requires the RNA-binding domain of the NS1 protein, because P120 splicing was not inhibited in the presence of 1.5 μ M of a GST-NS1 fusion protein in which the NS1 protein (NS1 RM) contains a point mutation in its RNA-binding domain (Qian et al., 1994) (Fig. 1B, lane 5). In fact, the RNA-binding domain of the NS1 protein alone was sufficient for the inhibition of P120 splicing: 1.0 μ M of the RNA-binding domain consisting of the N-terminal 73 amino acids of the NS1 pro-

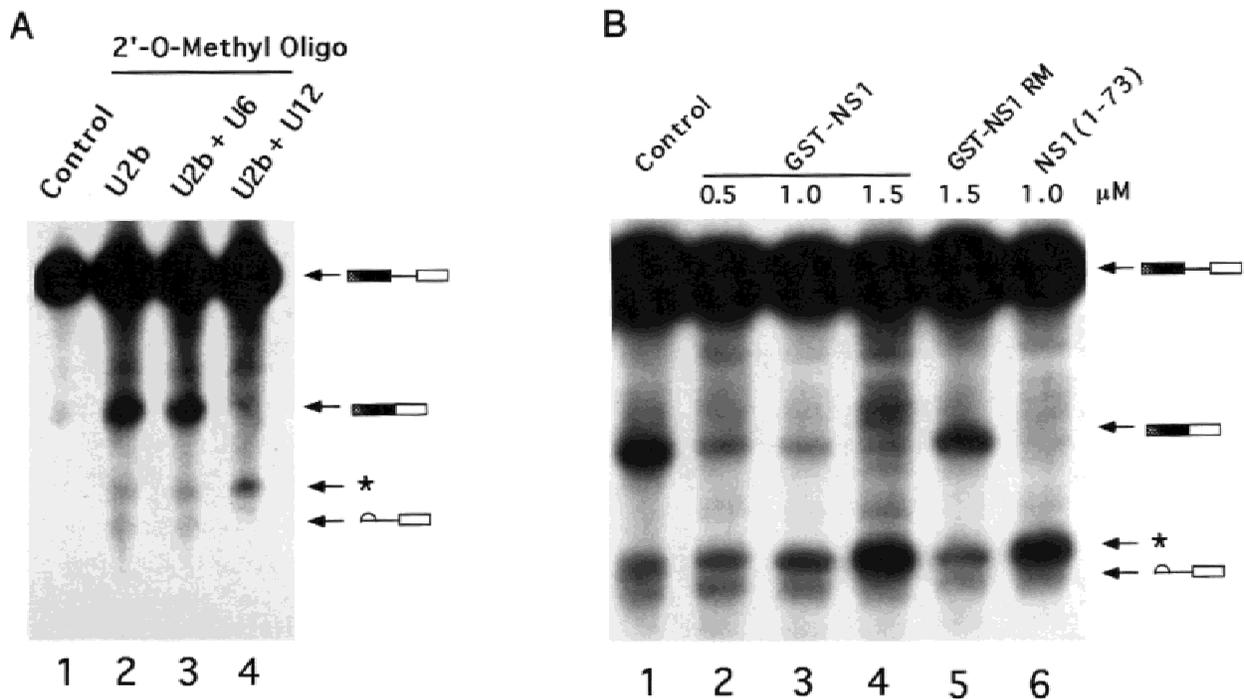


FIGURE 1. The NS1 protein of influenza A virus inhibits the splicing of P120 pre-mRNA in vitro. **A:** 32 P-labeled P120 pre-mRNA was incubated for 2 h at 30 °C with HeLa cell nuclear extract under splicing conditions as described in Materials and Methods in the absence (lane 1) or the presence (lanes 2-4) of the U2b 2'-O-methyl oligoribonucleotide. Where indicated, the reactions were supplemented with a 2'-O-methyl oligoribonucleotide complementary to either a U6 snRNA (lane 3) or a U12 snRNA sequence (lane 4) (Tarn & Steitz, 1996a). RNA products were analyzed by denaturing gel electrophoresis. Positions of the pre-mRNA and spliced products, which had been established previously (Tarn & Steitz, 1996a), are indicated. **B:** 32 P-labeled P120 pre-mRNA was incubated for 2 h at 30 °C with HeLa cell nuclear extract under splicing conditions in the absence (lane 1) or the presence of the indicated concentration of the GST-NS1 fusion protein (lanes 2-4), GST-NS1RM (lane 5), or the NS1(1-73) polypeptide (lane 6). U2b oligoribonucleotide was present in all reactions.

tein [NS1(1-73)] (Qian et al., 1995) effectively inhibited splicing (Fig. 1B, lane 6).

NS1 protein specifically binds to the highly divergent U6 snRNA counterpart, U6atac snRNA, among the five minor class snRNAs

As an initial approach to determining whether the NS1 protein binds to specific RNAs in the minor class spliceosomes, a splicing reaction containing the GST-NS1 protein and labeled P120 pre-mRNA was incubated for 2 h at 30 °C, and the reaction mixture was then subjected to selection on protein A agarose beads containing anti-NS1 antibody. If the GST-NS1 protein had associated with spliceosomes or spliceosome precursors, then this antibody selection should yield the pre-mRNA and the snRNAs that participate in splicing. As shown in Figure 2A (lane 4), the P120 pre-mRNA was indeed selected by the NS1 antibody. In addition, as detected using Northern blot analysis, the five snRNAs that participate in AT-AC splicing were also selected by the NS1 antibody (Fig. 2B, lane 4). In contrast, the P120 pre-mRNA was not selected when the GST-NS1 protein was not added to the splicing reaction (Fig. 2A, lane 2), nor when the NS1 protein containing a mutation in its RNA-binding domain (GST-NS1RM protein) was added to the splicing reaction (Fig. 2A, lane 3). Under these conditions, no snRNAs were selected by the NS1 antibody (Fig. 2B, lanes 2 and 3). These results indicate that the RNA-binding domain of the NS1 protein binds to one or more RNAs that are associated with spliceosomes or spliceosome precursors.

To verify this conclusion, a control reaction was performed in which spliceosome formation was eliminated by omitting ATP from the splicing reaction (Fig. 2A,B, lanes 5). Under these conditions, selection by the NS1 antibody did not yield either the P120 pre-mRNA or snRNAs. Thus, the binding of the NS1 protein to one or more RNAs requires the formation of spliceosomes or spliceosome precursors. A similar result was obtained for the binding of the NS1 protein to the major class U6 snRNA: this binding also requires the formation of spliceosomes (Lu et al., 1994).

The most likely RNA target of the NS1 protein in the minor class spliceosomes is a minor class snRNA that is associated with the spliceosomes. As a first approach, we employed the strategy that initially identified U6 snRNA as the target of the NS1 protein on the major spliceosomes: when nuclear extracts were heat inactivated (65 °C for 5 min) to inactivate splicing factors prior to the splicing reaction, U6 snRNA was the only major class snRNA that was rendered accessible to the NS1 protein in the absence of spliceosome formation (Lu et al., 1994). In heat-inactivated extracts, selection by the NS1 antibody (Fig. 2C) yielded a single minor snRNA, U6atac snRNA, in a reaction containing the NS1 protein (Fig. 2c, lane 2), whereas no

detectable snRNAs were selected in a reaction containing the NS1 protein with a mutation in its RNA-binding domain (Fig. 2C, lane 1). No P120 pre-mRNA was selected under either condition (data not shown), verifying that spliceosome formation did not occur. Thus, the NS1 protein binds specifically to U6atac snRNA in heat-inactivated nuclear extracts in the absence of spliceosome formation.

To verify that U6atac snRNA is the RNA target, we determined whether the NS1 protein bound to each of the minor class snRNAs *in vitro*. Purified NS1 protein (cleaved from the GST-NS1 fusion protein) was incubated at 4 °C with each of the labeled minor class snRNAs in the presence of a large excess of unlabeled tRNA, and the formation of labeled RNA-protein complexes was assayed by native gel electrophoresis (Fig. 3A). The NS1 protein did not form complexes with U11 (Fig. 3A, lanes 1 and 2), U12 (Fig. 3A, lanes 3 and 4), or U4atac snRNA (Fig. 3A, lanes 5 and 6). Previous experiments showed that U5 snRNA, which functions in both the major and minor spliceosomes, does not bind to the NS1 protein (Qiu et al., 1995). In contrast, the NS1 protein did form a lower-mobility complex with U6atac snRNA (Fig. 3A, lanes 7 and 8). The only other snRNA that has been found to bind to the NS1 protein is the major class human U6 snRNA (Fig. 3A, lanes 9 and 10) (Qiu et al., 1995). Not even yeast U6 snRNA binds to the NS1 protein (Fig. 3A, lanes 11 and 12).

The binding of U6atac snRNA to the NS1 protein increased as the concentration of the protein was increased (Fig. 3B, lanes 2-5). However, it was necessary to use about a 5-10-fold higher concentration of the NS1 protein to obtain U6atac snRNA binding comparable to that obtained with U6 snRNA (Qiu et al., 1995), suggesting that the affinity of the NS1 protein for U6 snRNA is about 5-10-fold higher than that for U6atac snRNA. Nonetheless, U6 and U6atac snRNAs most likely share the same binding site on the NS1 protein. Thus, as found previously for U6 snRNA (Qiu et al., 1995), an NS1 protein containing a point mutation in its RNA-binding domain (NS1 RM protein) did not bind to U6atac snRNA (Fig. 3B, lane 6). In addition, U6 and U6atac snRNAs competed with each other for binding to the NS1 protein (Fig. 3C). However, the concentration of U6 snRNA that displaced U6atac snRNA was 5-10-fold lower than the concentration of U6atac needed to displace U6 snRNA (Fig. 3C, compare lanes 8-10 to lanes 3-5). This also suggests that the affinity of the NS1 protein for U6 snRNA is about 5-10-fold higher than that for U6atac snRNA.

Identification of the NS1 protein binding site in U6atac snRNA

We used chemical modification/interference assays to identify the NS1 protein binding site in U6atac snRNA

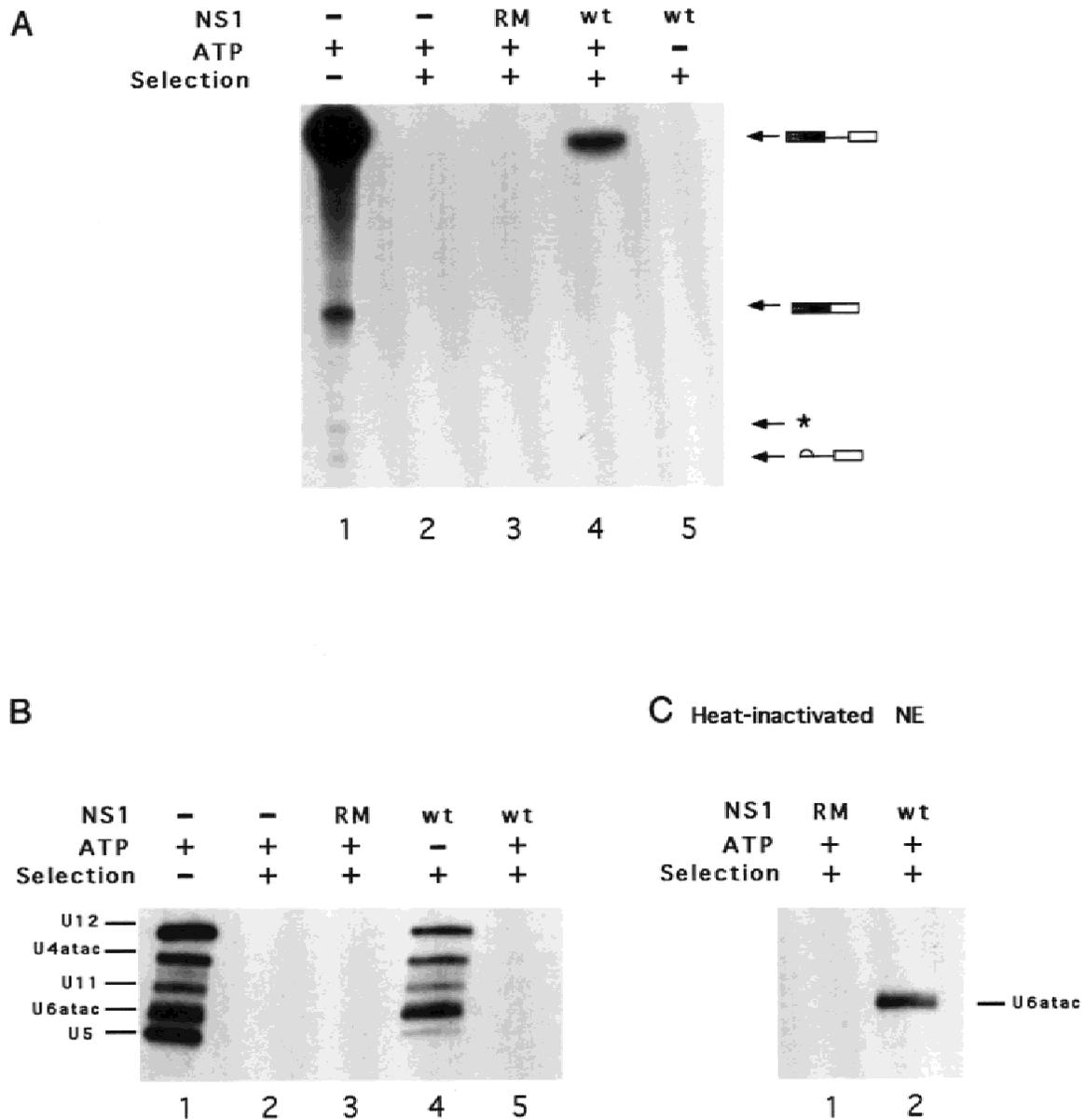


FIGURE 2. Binding of the NS1 protein to RNA(s) in the minor class spliceosomes. **A,B:** ^{32}P -labeled P120 pre-mRNA was incubated for 2 h at 30°C with the HeLa cell nuclear extract under splicing conditions (U2b oligoribonucleotide present) in the absence of the NS1 protein (lane 2), in the presence of 1.5 μM GST-NS1RM protein (lane 3), or in the presence of 1.5 μM GST-NS1 protein (lanes 4 and 5). ATP and the ATP-generating system were omitted from the splicing reaction of lane 5. After incubation, the mixtures were subject to antibody selection. Lane 1, same splicing reaction as lane 2 (no NS1 protein) prior to antibody selection. In A, the RNA was extracted from the selected material and electrophoresed on a 5% denaturing gel. In B, the RNAs were separated on a 7% denaturing gel and electrotransferred onto a nylon membrane. The membrane was probed with digoxigenin-labeled probes specific to U11, U12, U4atac, U5, and U6atac snRNAs. The identities of the individual snRNA bands were verified by using individual riboprobe in separate assays. **C:** The HeLa cell nuclear extract was inactivated by preheating at 65°C for 5 min before its addition to the splicing reactions containing either GST-NS1RM or GST-NS1 protein. Antibody selection and Northern blot assays were performed as described in B.

(Peattie, 1979; Qiu et al., 1995; Wang & Krug, 1996). U6atac snRNA was either labeled with ^{32}P at its 5' end and treated with diethylpyrocarbonate (DEPC) to modify predominantly A residues, or was labeled with ^{32}P at its 3' end and treated with 50% hydrazine/50% H_2O to modify predominantly U residues (and to a lesser extent C residues). The modified U6atac snRNA was

incubated with the NS1 protein under standard binding conditions, and the bound and free U6atac snRNAs were separated by native gel electrophoresis. The two RNA samples were recovered from the gel and cleaved with aniline. The cleaved products were analyzed by denaturing gel electrophoresis (Fig. 4A). By comparing the cleavage patterns of the free and bound U6atac

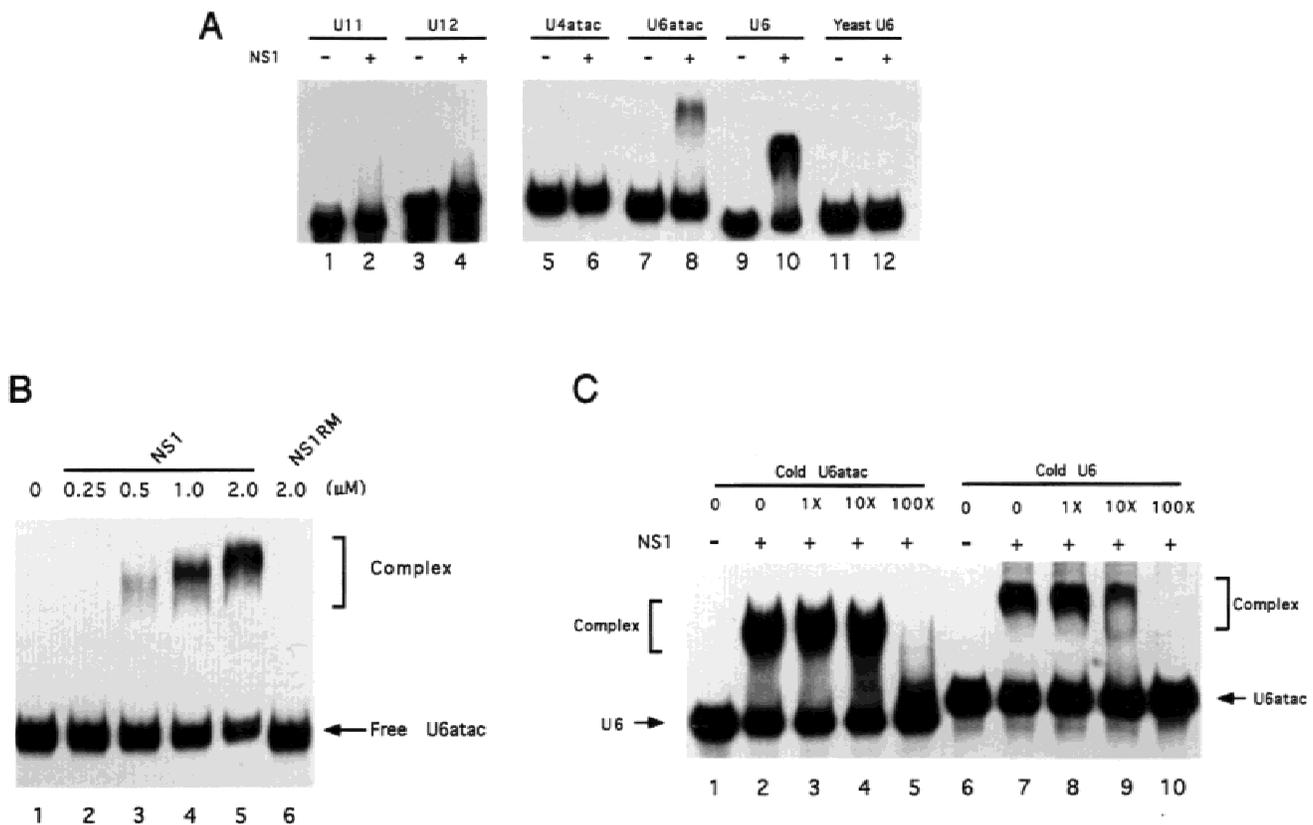


FIGURE 3. The NS1 protein specifically binds to the highly divergent U6 snRNA counterpart, U6atac snRNA, among the five minor class snRNAs, but with a lower affinity than its binding to U6 snRNA. **A:** RNA-binding assays with different U snRNAs. The NS1 protein (0.4 μM) was incubated with ³²P-labeled U11 (lane 2), U12 (lane 4), U4atac (lane 6), U6atac (lane 8), human U6 (lane 10), or yeast U6 (lane 12) snRNA (10,000 cpm, 1 pmol each). The RNA-protein complexes were separated from the free RNAs by nondenaturing gel electrophoresis. The odd-numbered lanes show the U snRNAs that were incubated in the absence of the NS1 protein. **B:** Binding of U6atac snRNA to increasing concentrations of the NS1 protein. ³²P-labeled U6atac snRNA (10,000 cpm, 1 pmol) was incubated with the indicated concentrations of the NS1 protein (lanes 2–5), or the NS1RM protein (lane 6), and the RNA-protein complexes were separated from free RNA by nondenaturing gel electrophoresis. Lane 1, U6atac alone. **C:** U6 and U6atac snRNAs compete for binding to the NS1 protein. ³²P-labeled U6 snRNA (1 pmol) (lanes 3–5) or U6atac snRNA (1 pmol) (lanes 8–10) was mixed with the indicated increasing amounts of unlabeled U6atac snRNA or U6 snRNA, respectively, followed by the addition of 0.8 μM NS1 protein. Lanes 1 and 6, U6 snRNA and U6atac snRNA, respectively, in the absence of the NS1 protein and the competitor RNA. Lanes 2 and 7, U6 snRNA and U6atac snRNA, respectively, incubated with the NS1 protein in the absence of the competitor RNA. After incubation, the mixtures were subject to nondenaturing gel electrophoresis.

snRNAs, interference was observed on both sides of the stem-bulge at the 3' end of the U6atac snRNA. At the 5' side of this stem-bulge, strong interference occurred at nt 82–85 (UUAA) and at nt 95 (A); at the 3' side of this stem-bulge, strong interference occurred at nt 113 and 114 (AA), and less strong interference occurred at nt 105 (U), 109 (A), 110 (U), and 112 (U) [as well as possible interference at nucleotide 111 (C)]. The interference at 101 (U) was not reproducible. No interference was observed in other regions of the molecule. The results are summarized in the proposed structure for U6atac snRNA (Tarn & Steitz, 1996b) (Fig. 4B). We conclude that the NS1 protein binds to the stem-bulge region near the 3' end of U6atac snRNA, although interference was observed at not all of the A and U nucleotides in this stem-bulge (see Discussion).

Binding of the NS1 protein to U6atac snRNA inhibits the formation of U12/U6atac snRNA complexes and of functional spliceosomes

It was unexpected that the NS1 protein, which inhibits splicing, specifically binds to the 3' stem-bulge of U6atac snRNA, because this region in U6atac snRNA had not been implicated previously in any interactions with other snRNAs during splicing (Tarn & Steitz, 1996b). We determined whether the binding of the NS1 protein to this region of U6atac snRNA affected the formation of U4atac/U6atac and U12/U6atac snRNA complexes during *in vitro* splicing, using the psoralen crosslinking assay previously described by others (Tarn & Steitz, 1996b). Unlabeled P120 pre-mRNA was incubated for 60 min under splicing conditions in either the absence or the presence of 1.5 μM GST-NS1 pro-

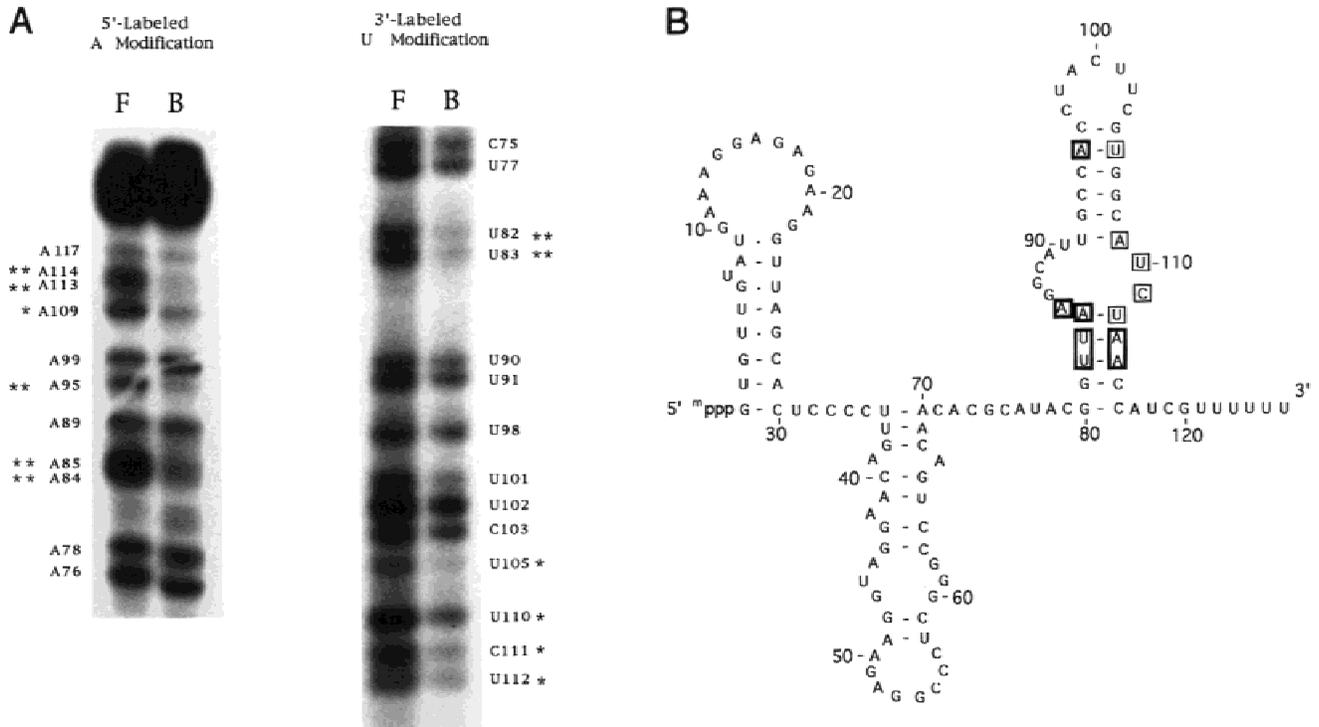


FIGURE 4. Chemical modification/interference assays map the NS1 protein binding site to the stem-bulge near the 3' end of U6atac snRNA. **A:** U6atac snRNA was ^{32}P -end-labeled as described in Materials and Methods, and its A residues were modified by DEPC, or its U residues were modified by 50% hydrazine/50% H_2O . The modified RNAs were incubated with the NS1 protein. The free (F) and protein-bound (B) RNA, separated by nondenaturing gel electrophoresis, were cleaved at modification sites by treatment with aniline. Cleavage products were analyzed by denaturing gel electrophoresis. **, Strong interference. *, Weak interference. **B:** Modification/interference data are summarized in the postulated U6atac snRNA structure (Tarn & Steitz, 1996b).

tein, and the reaction mixtures were irradiated on ice in the presence of AMT psoralen. The RNAs extracted from these mixtures were resolved by electrophoresis on a denaturing gel. Northern blot analysis using a digoxigenin-labeled RNA probe complementary to U6atac snRNA detected two slowly migrating species containing U6atac (Fig. 5A,B, lanes 1). The more slowly migrating of these RNA species, which was present in the absence of either ATP or the P120 pre-mRNA (Fig. 5B, lanes 2 and 3), was shown to be the complex of U4atac/U6atac: RNase H digestion in the presence of a U4atac-specific oligodeoxynucleotide selectively removed this RNA species (Fig. 5B, lane 4). The other RNA species, which is not present in the absence of either ATP or the P120 pre-mRNA (Fig. 5B, lane 4), is the U12/U6atac complex: RNase H digestion in the presence of a U12-specific oligodeoxynucleotide selectively removed this species (Fig. 5B, lane 5). The NS1 protein inhibited the formation of only one of these two U6 snRNA-containing complexes. Thus, in the presence of $1.5 \mu\text{M}$ GST-NS1 protein, the formation of U12/U6atac complexes was reduced approximately 90%, whereas no effect on the formation of U4atac/U6atac complexes was evident (Fig. 5A, compare lanes 1 and 2).

The pathway for assembly of AT-AC spliceosomes (splicing complexes) is similar to that of the spliceosomes formed on the major class of GU-AG pre-mRNAs (Tarn & Steitz, 1996a). Figure 6 (lanes 1-4) shows the analysis by native gel electrophoresis of the splicing complexes formed at various time points during the splicing of P120 pre-mRNA. The splicing complexes with the lowest mobility, called the C complexes, have been shown to contain not only P120 pre-mRNA, but also splicing intermediates and spliced products, and hence have been considered to be the catalytically active form of splicing complexes, or spliceosomes (Tarn & Steitz, 1996a). In contrast, the B splicing complexes, which have greater gel mobility than C complexes, contain only unspliced P120 pre-mRNA (Tarn & Steitz, 1996a). We compared this spliceosome formation in the absence of the NS1 protein to that in the presence of $1.5 \mu\text{M}$ GST-NS1 protein, the same concentration used in the psoralen crosslinking experiment. During the first 60 min of the splicing reaction, the assembly of the B splicing complexes in the presence of the NS1 protein was slower than that in the absence of the NS1 protein (Fig. 6, compare lanes 5-8 to lanes 1-4). Nonetheless, at 60 min, a significant amount of the B complex was formed in the presence of the NS1 protein

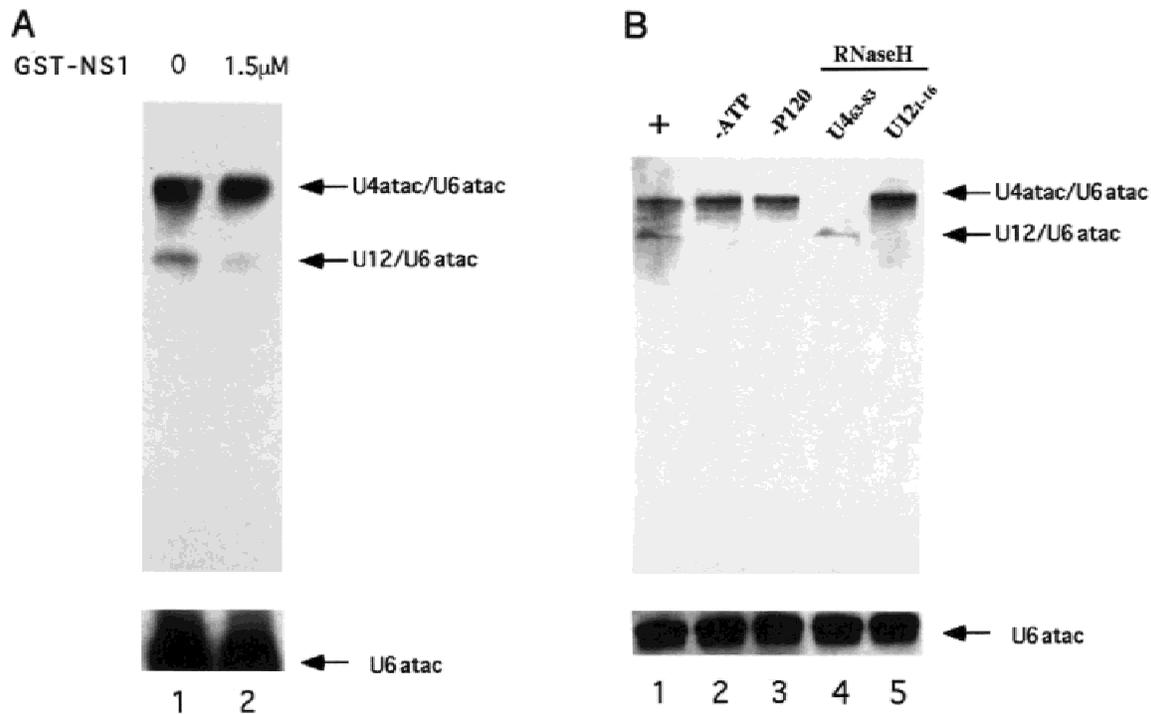


FIGURE 5. The NS1 protein inhibits the formation of U12/U6atac, but not U4atac/U6atac, complexes. **A:** Unlabeled P120 pre-mRNA (0.1 pmol) was incubated under standard splicing conditions (U2b oligoribonucleotide present) for 60 min in the absence (lane 1) or presence (lane 2) of the NS1 protein (1.5 μ M). After AMT psoralen treatment and UV irradiation, the RNA was subjected to denaturing gel electrophoresis, followed by northern blotting using a digoxigenin-labeled anti-U6atac RNA as probe. **B:** Determination of the identities of the crosslinked RNAs. Splicing reactions were performed as described above. Where indicated, ATP and the ATP-generating system (lane 2) or P120 substrate (lane 3) were omitted from the splicing reaction. After psoralen crosslinking, the RNAs of lanes 4 and 5 were incubated at 37 °C for 30 min with RNase H and deoxyoligonucleotides complementary to the U4atac₆₃₋₈₂ or U12₁₋₁₆ sequences, respectively. Northern blot analysis was performed as described above.

(Fig. 6, compare lanes 3 and 7). These B complexes most likely comprise the spliceosome precursors with which the NS1 protein is associated (Fig. 2). However, after 60 min, no detectable increase in the formation of B splicing complexes occurred in the presence of the NS1 protein, and the formation of C complexes was not detected (Fig. 6, lane 8). In contrast, in the absence of the NS1 protein, C complexes were formed (Fig. 6, lane 4). The lack of conversion of B splicing complexes to C splicing complexes in the presence of the NS1 protein is consistent with the block in the formation of U6atac/U12 snRNA complexes.

DISCUSSION

Of the snRNAs that function in the formation and activities of the major and minor mammalian spliceosomes, only two—U6 snRNA and U6atac snRNA—have been found to bind to the influenza virus NS1 protein. Thus, this protein specifically targets the two snRNAs that serve analogous functions in the two different spliceosomes. This specific targeting is remarkable in that these two snRNAs have highly divergent sequences (Tarn & Steitz, 1996b). In particular, the 3' halves of these two snRNAs, where the NS1 protein

binds, show very little sequence similarity. As shown in the present study, the NS1 protein binds to a stem-bulge near the 3' end of U6atac snRNA. The sequence of this stem-bulge differs significantly from the sequence of the stem-bulge to which it binds in U6 snRNA (Qiu et al., 1995; present study). The U6 snRNA bulge contains four nucleotides, all purines, whereas the U6atac snRNA bulge contains eight nucleotides, only half of which are purines. Nonetheless, it can be presumed that the U6 and U6atac stem-bulges share some common structural features that enable these two RNA sequences to bind to the NS1 protein. In contrast to U6atac snRNA, yeast U6 snRNA, which has more sequence homology with human U6 snRNA than does U6atac (Brow & Guthrie, 1988), fails to bind to the NS1 protein, indicating that yeast U6 snRNA lacks a stem-bulge with the common structural features presumed to be shared by U6atac and human U6 snRNAs.

Not only does the NS1 protein bind to specific stem-bulges in U6 and U6atac snRNAs (Qiu & Krug, 1994; Lu et al., 1995; present study), but it also binds to two other RNAs: poly(A) and double-stranded RNA (Qiu & Krug, 1994; Lu et al., 1995). Both mutational analysis of the protein and RNA competition experiments suggest that all four of these RNAs most likely share the

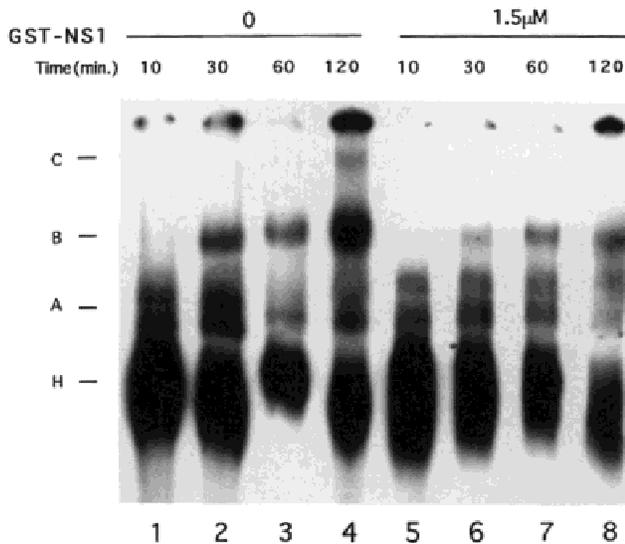


FIGURE 6. The NS1 protein blocks the formation of C spliceosome complexes. ^{32}P -labeled P120 pre-mRNA was incubated at 30 °C with HeLa cell nuclear extract under splicing conditions. At the indicated times, 5- μL aliquots of the reaction were treated with heparin, and then analyzed by electrophoresis on a 3.75% native polyacrylamide gel. The B and C spliceosome complexes are described in the text, and the A and H complexes were previously characterized by Tarn and Steitz (1996a).

same, or similar, binding sites on the protein (Qiu & Krug, 1994; Lu et al., 1995; Qiu et al., 1995; present study). The NS1 protein functions as a dimer when it binds to these RNAs, and the dimerization and RNA-binding domains of the protein appear to be coincident (Nemeroff et al., 1995). Clearly, structural studies of NS1-RNA complexes are needed to obtain a definitive picture of how the NS1 protein dimer binds to its several RNA targets. As the first step in such an analysis, both NMR and X-ray crystallography have shown that the dimeric NS1 RNA-binding domain in the absence of RNA adopts a novel six-helical chain fold that has the potential for several modes of interaction with RNA (Chien et al., 1997; Liu et al., 1997).

Although the U6atac and U6 snRNAs most likely bind to similar sites on the NS1 protein, our results indicate that U6atac snRNA binds with 5–10-fold lower affinity than human U6 snRNA. Consequently, the structures of the U6atac and human U6 stem-bulges probably differ. This difference may be reflected in the chemical modification/interference results obtained with the two RNAs. With U6 snRNA, strong interference was observed at essentially every nucleotide in the stem-bulge (Qiu et al., 1995), whereas, with U6atac snRNA, interference was observed at only a subset of the A and U nucleotides in the stem-bulge (present study). Perhaps this indicates that the NS1 protein is in close contact with all the nucleotides in the stem-bulge of human U6 snRNA, but with only a subset of the nucleotides in the stem-bulge of U6atac snRNA. Structural studies of the complexes between the NS1 protein and its RNA targets should resolve this issue.

As shown in the present study, the NS1 protein at concentrations of 0.5–1.5 μM completely inhibits AT-AC pre-mRNA splicing. At these concentrations, it is likely that all the U6 snRNA in the nuclear extract is bound to the NS1 protein (Qiu et al., 1995). Therefore, it is also likely that all the U6atac snRNA in the nuclear extract is bound to the NS1 protein: although the NS1 protein has a lower affinity for U6atac snRNA than for U6 snRNA (present study), U6atac snRNA is present in much lower amounts than U6 snRNA (Tarn & Steitz, 1996b). In fact, in nuclear splicing extracts, the binding of the NS1 protein to either U6 snRNA or U6atac snRNA is detected only when spliceosome formation occurs (Lu et al., 1994; present study), indicating that this snRNA binding site is accessible only when U6 or U6atac snRNA is undergoing its interactions with other snRNAs and the pre-mRNA substrate during the splicing reaction. In contrast, in nuclear extracts that had been preheated at 65 °C to inactivate splicing factors, U6 and U6atac snRNAs are rendered accessible to the NS1 protein in the absence of spliceosome formation (Lu et al., 1994; present study). In such preheated extracts, U6 and U6atac snRNAs are the only snRNAs that bind to the NS1 protein, demonstrating the specificity of the NS1 protein for these two snRNAs.

The NS1 protein does not affect the interactions of U6 and U6atac snRNAs with other spliceosomal snRNAs in exactly the same way. In the major spliceosome system, the NS1 protein inhibits the formation of both U4/U6 and U2/U6 complexes (Qiu et al., 1995). The formation of U4/U6 complexes is inhibited even though the NS1 protein binding site on U6 snRNA does not include the region of U6 snRNA that base pairs with U4 snRNA. However, this binding site does include the 3'-terminal region of U6 snRNA that has been shown to be important for the RNA transitions that are required for the formation of U4/U6 complexes (Wolff & Bindereif, 1993). In contrast, the NS1 protein has little or no effect on the formation of U4atac/U6atac complexes in the minor spliceosome system, as shown in the present study. The NS1 protein binding site on U6atac snRNA does not include the region of U6atac snRNA that base pairs with U4atac snRNA (Tarn & Steitz, 1996b), and our results indicate that this region of U6atac snRNA has no significant role during the process of formation of U4atac/U6atac complexes.

In the major spliceosome system, the NS1 protein inhibits the formation of U2/U6 complexes (Qiu et al., 1995), most likely because the NS1 protein binding site on U6 snRNA includes the U6 nucleotides that form one of the helices, helix II, with U2 snRNA (Nilsen, 1994; Sharp, 1994; Qiu et al., 1995). The NS1 protein also inhibits the formation of the comparable complexes in the minor AT-AC spliceosome system, i.e., U12/U6atac complexes. This inhibition most likely results in the observed lack of conversion of B splicing complexes to functional C spliceosomes. The block in

the formation of U12/U6atac complexes occurs even though the NS1 protein binding site on U6atac snRNA has not been implicated in any interaction with U12 snRNA (Tarn & Steitz, 1996b). Two interactions of U6atac snRNA with U12 snRNA that have been demonstrated or postulated involve the first 50 nt at the 5' end of U6atac. Our results suggest the possibility that a sequence in the 3' two-thirds of U6atac snRNA participates in an additional interaction with U12 snRNA.

The NS1 protein of influenza A virus is a unique posttranscriptional regulator that has several activities: it inhibits the nuclear export of poly(A)-containing spliced mRNAs (Alonso-Caplen et al., 1992; Fortes et al., 1994; Qian et al., 1994; Qiu & Krug, 1994); it binds to double-stranded RNA (dsRNA), thereby blocking the inhibition of translation caused by dsRNA activation of the dsRNA-activated protein kinase (Hatada & Fukuda, 1992; Lu et al., 1995); and it inhibits pre-mRNA splicing, both that catalyzed by the major (GU-AG introns) spliceosomes (Fortes et al., 1994; Lu et al., 1994; Qiu et al., 1995) and that catalyzed by the minor (AT-AC introns) spliceosomes (present study). The demonstration of a specific NS1 protein-U6 snRNA interaction in infected cells suggests that inhibition of pre-mRNA splicing by the NS1 protein does, in fact, occur in infected cells (Lu et al., 1994). However, in contrast to the other functions of the NS1 protein, the role that the NS1 protein-mediated inhibition of pre-mRNA splicing plays in infected cells has not yet been established. It is likely that all known pre-mRNA splicing systems would be inhibited in infected cells, but it is currently not clear how this would benefit virus gene expression, particularly in light of the fact that a fraction (about 10%) of two viral mRNAs, the NS1 and M1 mRNAs, also undergoes splicing (Krug et al., 1989; Shih et al., 1995). Consequently, some mechanism has to operate to allow the incomplete splicing of these two viral mRNAs to occur at the appropriate times of infection. One of the major challenges will be to elucidate how the inhibition of pre-mRNA splicing by the NS1 protein serves an important role in influenza virus gene expression.

MATERIALS AND METHODS

In vitro splicing

The pSP64 plasmid containing the P120 gene (provided by Joan Steitz) was linearized with *Hind* III and transcribed by T7 RNA polymerase in the presence of m7GpppG and [α -³²P] GTP. The conditions for the splicing reactions were essentially as described previously (Lu et al., 1994), except that the HeLa cell nuclear extract (10 μ L) was preincubated with 1.2 μ M U2b 2'-O-methyl oligoribonucleotide (complementary to nt 27-49 of U2 snRNA), plus, where indicated, another 2'-O-methyl oligoribonucleotide (1.2 μ M) under splicing conditions for 15 min at 30 °C. The labeled P120 pre-mRNA (25,000 cpm, approximately 10 fmol) was then added. Where

indicated, the GST-NS1 protein, GST-NS1RM protein, or the NS1(1-73) polypeptide was added. The mixtures, in a final volume of 25 μ L, were incubated for 2 h at 30 °C unless otherwise indicated. The reactions were stopped by the addition of pronase followed by phenol extraction and ethanol precipitation. The RNAs were analyzed by electrophoresis on 5% denaturing polyacrylamide gels.

Immunoprecipitation of spliceosomes containing the NS1 protein

The volumes of the splicing reactions were increased to 35 μ L. Where indicated, ATP and the ATP-generating system (creatine phosphate and creatine phosphokinase) were omitted. After incubation at 30 °C for 2 h, the splicing reaction was diluted with 200 μ L Ipp₁₅₀ buffer (Hamm et al., 1987) containing 150 mM NaCl and 0.1% NP-40. This mixture was added to protein A-agarose beads, which had been equilibrated with Ipp₁₅₀ buffer and anti-NS1 antiserum, and incubated on a rotor for 90 min at 4 °C. The beads were then washed three times with Ipp₁₅₀ buffer and one time with Ipp₃₂₅ buffer containing 325 mM NaCl. RNA was extracted from the selected material and was analyzed by electrophoresis on denaturing polyacrylamide gels. The P120 pre-mRNAs were detected by autoradiography. For detection of snRNAs, the RNAs were transferred by electroblotting onto a nylon membrane for 16 h at a constant current of 150 mA at 4 °C. The membrane was then hybridized to digoxigenin-labeled RNA probes complementary to U11, U12, U4atac, U5, and U6atac snRNAs. The snRNAs were detected by chemiluminescence.

RNA-binding assays

The GST-NS1 fusion protein was purified, and the NS1 protein was cleaved from this fusion as described previously (Qiu & Krug, 1994). Where indicated, the NS1 protein with a mutation in its RNA-binding domain (RM, i.e., mutant 2 of Qian et al., 1994) was used. The purity of all proteins was established by gel electrophoresis followed by Coomassie blue staining. The N-terminal fragment of the NS1 protein [NS1(1-73)], which possesses the RNA-binding activity of the full-length protein, was purified as described previously (Qian et al., 1995). The pT3T7 U12, pGEM3Z-U4atac, and pGEM3Z-U6atac plasmids were kindly provided by Joan Steitz (Tarn & Steitz, 1996b). The psp64 U11 and the pEP6 yeast U6 plasmids were kindly provided by Mark McNally (Gontarek et al., 1993) and Christine Guthrie (Brow & Guthrie, 1988), respectively. All DNAs encoding U snRNA sequences were PCR-amplified using primers that resulted in a *Hind* III site at the 5' end and a *Bam*H I site at the 3' end. These sequences were then cloned into the pGEM1 vector, linearized by *Bam*H I, and transcribed by SP6 RNA polymerase in the presence of [α -³²P] UTP. The RNA-binding assays were performed essentially as described previously (Wang & Krug, 1996). The indicated concentration of cleaved NS1 protein was incubated with the labeled transcript (10,000 cpm, 1 pmol) on ice for 30 min in 20- μ L reactions containing 50 mM Tris-HCl, pH 8.8, 50 mM glycine, 8% glycerol, 2.5 mM dithiothreitol, 2.5 mg/mL *Escherichia coli* tRNA, and 2 units/ μ L RNasin. The protein-RNA complexes were resolved from free RNA on a 6% nondenaturing polyacrylamide gel as described previously (Wang & Krug, 1996).

Chemical modification/interference analysis

For A>>G modification, U6atac snRNA was 5' end-labeled with [γ - 32 P]ATP and treated with DEPC as described previously (Qiu et al., 1995). For U>C modification, U6atac snRNA was 3' end-labeled with [32 P]pCp and treated with 50% hydrazine/50% H₂O (Peattie, 1979). The modified U6atac (500,000 cpm, 10 pmol) was incubated with 10 μ M NS1 protein in a 50- μ L binding reaction. After nondenaturing gel electrophoresis, the free and bound U6atac snRNAs were recovered from the gel and subjected to aniline cleavage. Cleavage products were analyzed by denaturing gel electrophoresis (Wang & Krug, 1996).

Psoralen crosslinking and spliceosome assays

Unlabeled P120 pre-mRNA (approximately 0.1 pmol) was incubated with the HeLa cell nuclear extract in a splicing reaction as described above. After a 60-min incubation at 30°C, 20 μ g/mL AMT (4'-aminomethyl-4, 5', 8-trimethyl) psoralen was added, followed by UV irradiation at 365 nm for 15 min on ice (Qiu et al., 1995). The mixtures were treated with pronase at 37°C for 30 min. The RNAs were then extracted and subjected to electrophoresis on a 5% denaturing polyacrylamide gel. The separated RNAs were transferred by electroblotting onto a nylon membrane for 16 h at a constant current of 150 mA at 4°C. The membrane was then hybridized to a digoxigenin-labeled RNA probe complementary to U6atac snRNA, and the labeled RNA bands were detected by chemiluminescence. Spliceosome assays were performed essentially as described previously (Konarska, 1989; Qiu et al., 1995). The splicing reaction was terminated by the addition of 0.25 mg/mL heparin to 5 μ L of the splicing reaction at the indicated time points. The mixtures were kept on ice for 30 min and were then subjected to electrophoresis on a 3.75% nondenaturing polyacrylamide gel for 4 h at 250 V at room temperature.

ACKNOWLEDGMENTS

This investigation was supported by NIH grant AI11772 (Merit) to R.M.K.

Received August 4, 1997; returned for revision August 27, 1997; revised manuscript received October 24, 1997

REFERENCES

- Alonso-Caplen FV, Nemeroff ME, Qiu Y, Krug RM. 1992. Nucleocytoplasmic transport: The influenza virus NS1 protein regulates the transport of spliced NS2 mRNA and its precursor NS1 mRNA. *Genes & Dev* 6:255-267.
- Brow DA, Guthrie C. 1988. Spliceosomal RNA U6 is remarkably conserved from yeast to mammals. *Nature* 334:213-218.
- Chien C, Tejero R, Huang Y, Zimmerman DE, Rios CB, Krug RM, Montelione GT. 1997. A novel RNA-binding motif in influenza A virus non-structural protein 1. *Nature Struct Biol*. Forthcoming.
- Fortes P, Beloso A, Ortin J. 1994. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. *EMBO J* 13:704-712.
- Gontarek RR, McNally MT, Beemon K. 1993. Mutation of an RSV intronic element abolishes both U11/U12 snRNP binding and negative regulation of splicing. *Genes & Dev* 7:1926-1936.
- Hall SL, Padgett RA. 1994. Conserved sequences in a class of rare eukaryotic nuclear introns with non-consensus splice sites. *J Mol Biol* 239:357-365.
- Hall SL, Padgett RA. 1996. Requirement of U12 snRNA for in vivo splicing of a minor class of eukaryotic nuclear pre-mRNA introns. *Science* 271:1716-1718.
- Hamm J, Kamaier M, Mattaj JW. 1987. In vitro assembly of U1 snRNPs. *EMBO J* 6:3479-3485.
- Hatada E, Futada R. 1992. Binding of influenza A virus NS1 protein to dsRNA in vitro. *J Gen Virol* 73:3325-3329.
- Jackson JJ. 1991. A reappraisal of non-consensus mRNA splice sites. *Nucleic Acids Res* 19:3795-3798.
- Konarska MM. 1989. Analysis of splicing complexes and small nuclear ribonucleoprotein particles by native gel electrophoresis. *Methods Enzymol* 180:442-453.
- Krug RM, Alonso-Caplen FV, Julkunen I, Katze MG. 1989. Expression and replication of the influenza virus genome. In: Krug RM, ed. *The influenza viruses*. New York: Plenum Press. pp 89-152.
- Liu J, Lynch PA, Chien CY, Montelione GT, Krug RM, Berman HM. 1997. Crystal structure of the unique multifunctional RNA-binding domain of the influenza virus NS1 protein. *Nature Struct Biol*. Forthcoming.
- Lu Y, Qian X, Krug RM. 1994. The influenza virus NS1 protein: A novel inhibitor of pre-mRNA splicing. *Genes & Dev* 8:1817-1828.
- Lu Y, Wambach M, Katze MG, Krug RM. 1995. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the eIF-2 translation initiation factor. *Virology* 214:222-228.
- Madhani HD, Guthrie C. 1992. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell* 71:803-817.
- Nemeroff ME, Qian X, Krug RM. 1995. The influenza virus NS1 protein forms multimers in vitro and in vivo. *Virology* 212:422-428.
- Nilsen TW. 1994. RNA-RNA interactions in the spliceosome: Unraveling the ties that bind. *Cell* 78:1-4.
- Nilsen TW. 1996. A parallel spliceosome. *Science* 273:1813-1813.
- Peattie D. 1979. Direct chemical method for sequencing RNA. *Proc Natl Acad Sci USA* 76:1760-1764.
- Qian X, Alonso-Caplen F, Krug RM. 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J Virol* 68:2433-2441.
- Qian X, Chien C, Lu Y, Montelione GT, Krug RM. 1995. An amino-terminal polypeptide fragment of the influenza virus NS1 protein possesses specific RNA binding activity and largely helical backbone structure. *RNA* 1:948-956.
- Qiu Y, Krug RM. 1994. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J Virol* 68:2425-2432.
- Qiu Y, Nemeroff ME, Krug RM. 1995. 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. *RNA* 1:304-316.
- Sharp PA. 1994. Split genes and RNA splicing. *Cell* 77:805-815.
- Shih S, Nemeroff ME, Krug RM. 1995. The choice of alternative 5' splice sites in influenza virus M1 mRNA is regulated by the viral polymerase complex. *Proc Natl Acad Sci USA* 92:6324-6328.
- Tarn W, Steitz JA. 1996a. A novel spliceosome containing U11, U12, and U5 snRNPs excises a minor class (AT-AC) intron in vitro. *Cell* 84:801-811.
- Tarn WY, Steitz JA. 1996b. Highly diverged U4 and U6 small nuclear RNAs required for splicing rare AT-AC introns. *Science* 273:1824-1832.
- Wang W, Krug RM. 1996. The RNA-binding and effector domains are conserved to different extents among influenza A and B viruses. *Virology* 223:41-50.
- Wolff T, Bindereif A. 1993. Conformational changes of U6 during the spliceosome cycle: An intramolecular helix is essential both for initiating the U4-U6 interaction and for the first step in splicing. *Genes & Dev* 7:1377-1389.

ERRATUM

In an article by Weirong Wang and Robert Krug, "U6atac snRNA, the highly divergent counterpart of U6 snRNA, is the specific target that mediates inhibition of AT-AC splicing by the influenza virus NS1 protein," that appeared in the January issue of *RNA* (Vol. 4, No. 1) on pages 55–64, an incorrect Figure 2B was printed by mistake. The correct figure in its entirety appears below. We regret the error.

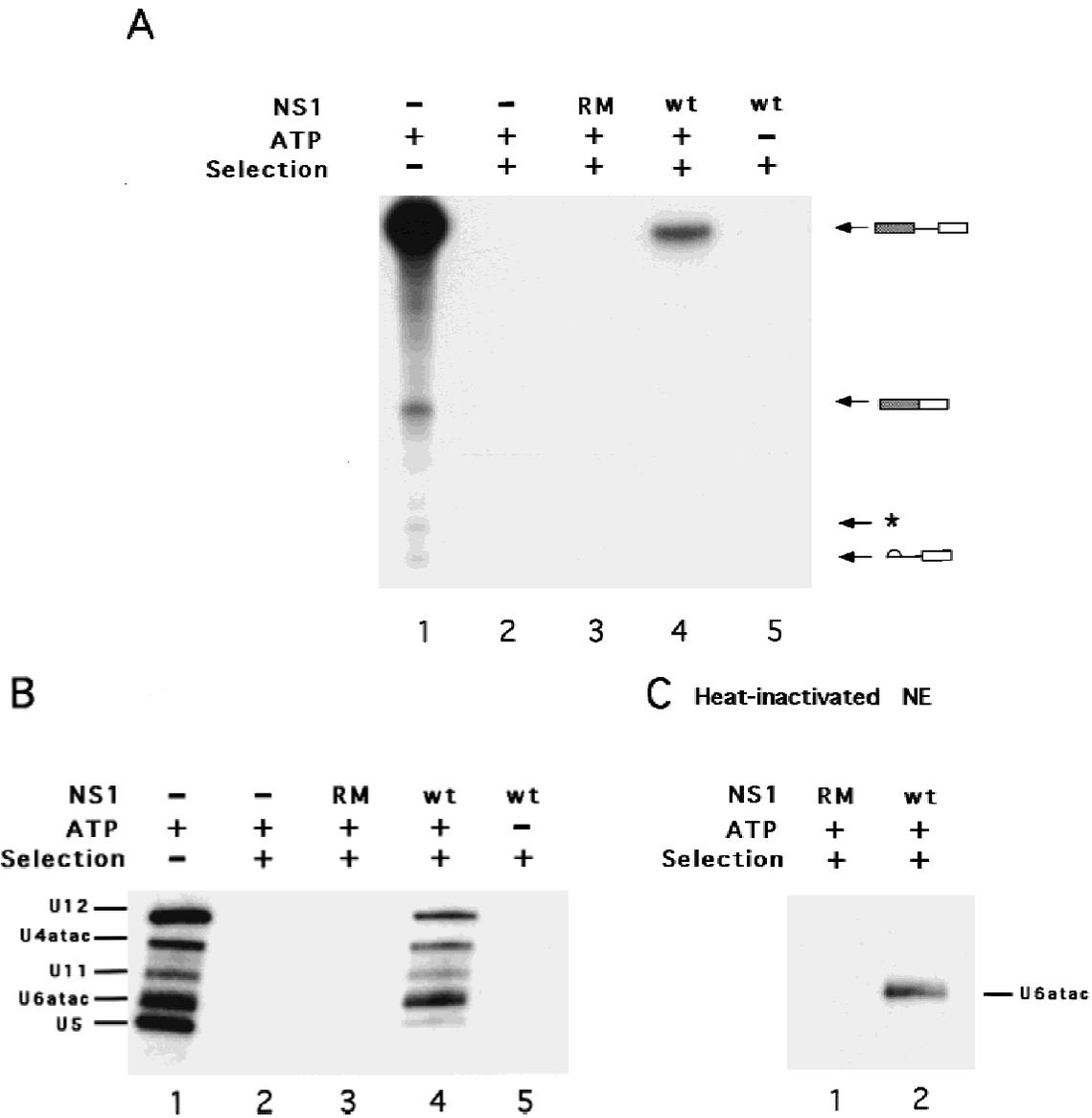


FIGURE 2. Binding of the NS1 protein to RNA(s) in the minor class spliceosomes. **A,B:** ³²P-labeled P120 pre-mRNA was incubated for 2 h at 30 °C with the HeLa cell nuclear extract under splicing conditions (U2b oligoribonucleotide present) in the absence of the NS1 protein (lane 2), in the presence of 1.5 μM GST-NS1RM protein (lane 3), or in the presence of 1.5 μM GST-NS1 protein (lanes 4 and 5). ATP and the ATP-generating system were omitted from the splicing reaction of lane 5. After incubation, the mixtures were subject to antibody selection. Lane 1, same splicing reaction as lane 2 (no NS1 protein) prior to antibody selection. In A, the RNA was extracted from the selected material and electrophoresed on a 5% denaturing gel. In B, the RNAs were separated on a 7% denaturing gel and electrotransferred onto a nylon membrane. The membrane was probed with digoxigenin-labeled probes specific to U11, U12, U4atac, U5, and U6atac snRNAs. The identities of the individual snRNA bands were verified by using individual riboprobe in separate assays. **C:** The HeLa cell nuclear extract was inactivated by preheating at 65 °C for 5 min before its addition to the splicing reactions containing either GST-NS1RM or GST-NS1 protein. Antibody selection and Northern blot assays were performed as described in B.