The role of overlapping U1 and U11 5' splice site sequences in a negative regulator of splicing

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ABSTRACT

Splicing of Rous sarcoma virus RNA is regulated in part by a *cis*-acting intronic RNA element called the negative regulator of splicing (NRS). An NRS mutant affecting nt 916–923 disrupts U11 snRNP binding and reduces NRS activity (Gontarek et al., 1993, *Genes & Dev* 7:1926–1936). However, we observed that a U1 5' splice site-like sequence, which overlapped the U11 site, was also disrupted by this mutation. To determine whether the U1 or the U11 site was essential for NRS activity, we analyzed twelve additional mutants involving nt 915–926. All mutations that disrupted the potential base pairing between U1 snRNA and the NRS reduced NRS activity, including single point mutations at nt 915, 916, and 919. The point mutation at nt 919 was partially suppressed by a compensatory base change mutation in U1 snRNA. In contrast, a mutation which strengthened the potential base pairing between the U1 site and the NRS increased NRS activity. Surprisingly, mutations that specifically targeted the U11 5' splice site consensus sequence increased the levels of unspliced RNA, suggesting U11 binding plays an antagonistic role to NRS activity. We propose that U1 snRNP binding to the NRS inhibits splicing and is regulated by U11 snRNP binding to the overlapping sequence. Competition between U1 and U11 snRNPs would result in the appropriate balance of spliced to unspliced RNAs for optimal viral replication. Further, a virus mutated in the U1/U11 region of the NRS was found to have delayed replication.

Keywords: Retrovirus, Rous Sarcoma Virus, snRNPs

INTRODUCTION

All retroviruses need to maintain a balance between levels of spliced and unspliced RNAs produced from a single primary transcript. The unspliced RNA is transported to the cytoplasm, where it serves as either mRNA for Gag and Gag-Pol proteins or as genomic RNA that is packaged into viral particles. Typically, over 50% of the primary transcripts are maintained as unspliced RNA, and the rest are spliced to generate subgenomic mRNAs including *env* (Coffin, 1996; reviewed by Banks et al., 1997; Rabson & Graves, 1997; Stoltzfus, 1988). Avian retroviral mutants with enhanced 3' splice sites, in which a majority of the total RNA transcripts are spliced, have diminished viral replication (Katz & Skalka, 1990; Zhang & Stoltzfus, 1995).

Avian retroviruses use several *cis*-acting elements to control their level of splicing. Like human immunodeficiency virus type 1 (HIV-1) (Purcell & Martin, 1993;

O'Reilly et al., 1995), Rous sarcoma virus (RSV) RNA has a strong 5' splice site and weak 3' splice sites (Katz et al., 1988; Stoltzfus, 1988; McNally & Beemon, 1992). In addition, avian viruses have a negative regulator of splicing (NRS) element in the intron, about 300 nt from the 5' splice site and 4,000 nt from the nearest 3' splice site (Arrigo & Beemon, 1988; Stoltzfus & Fogarty, 1989). When the NRS is deleted in viral constructs, increased splicing is observed (Arrigo & Beemon, 1988). The NRS is a bipartite element that is functional in vivo and in vitro when inserted into heterologous splicing constructs and is most active near the 5' splice site (Arrigo & Beemon, 1988; McNally et al., 1991; Gontarek et al., 1993).

Gontarek et al. (1993) demonstrated that U11 snRNP binds to the 3' end of the RSV NRS element in vitro. This region of the NRS contains the sequence GUAUC CUU, which is identical to the 5' splice site sequence of the majority of U12-dependent introns (Dietrich et al., 1997). Mutations in this region prevent U11 snRNP binding in vitro and inactivate the NRS in heterologous constructs tested in vivo (Gontarek et al., 1993). Thus, it was proposed that binding of U11 snRNP might be essential for NRS-mediated inhibition of splicing (Gontarek et al., 1993; McNally & McNally, 1998).

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U11 and U12 are minor snRNPs (reviewed in Baserga & Steitz, 1993), present in about 10,000 and 5,000 copies per cell, respectively, that are often complexed together in the nucleus (Wassarman & Steitz, 1992). U11 and U12 replace U1 and U2 snRNPs in the first step of spliceosome formation of certain rare introns, initially identified by their AT-AC intronic consensus sequences (Hall & Padgett, 1994, 1996; reviewed by Mount, 1996; Nilsen, 1996; Tarn & Steitz 1996a, 1996b, 1997; Wu et al., 1996; Sharp & Burge, 1997). Recently, it has been observed that the branchpoint sequence, which is complementary to U12 snRNA, is the most distinctive feature of these introns; thus, they are now called U12-dependent introns (Dietrich et al., 1997; Sharp & Burge, 1997). The 5' end of U11 snRNA binds the 5' splice site of U12-dependent introns at the highly conserved consensus sequence /RUAUCCUU (Dietrich et al., 1997; Yu & Steitz, 1997) much as U1 binds the 5' splice site of conventional (U2-dependent) introns at the less highly conserved consensus sequence AG/GURAGU (reviewed in Moore et al., 1993).

To assess the significance of the NRS for viral replication, we made a virus bearing a mutation in the U11 binding region of the NRS and found that viral replication was impaired. However, using compensatory U11 snRNA mutants, we were unable to prove that U11 snRNP binding was required for NRS activity. Because the U11 binding site partially overlaps a U2-dependent 5' splice site-like sequence, this mutant would be expected to block base pairing to the U1 site, also. To distinguish which interaction was functionally significant for NRS activity, NRS mutations specific for either the U1 or U11 site were generated in heterologous splicing constructs. Mutations that interfered with the predicted U1 base pairing abolished NRS activity. This activity could be partially rescued with a compensatory U1 snRNA mutant. Surprisingly, the mutations that prevented potential base pairing to U11 snRNA alone made the NRS stronger. We propose a model for RNA splicing regulation whereby U1 binding to the NRS blocks splicing at the viral 5' splice site, and U11 binding favors splicing. Competition between U1 and U11 snRNPs for this overlapping site would result in both spliced and unspliced retroviral RNAs, required for retroviral replication.

RESULTS

Mutations in the 3' end of the NRS destroy NRS activity

We observed previously that U11 snRNP binds to the NRS, and that mutant RG11 (which alters RSV nt 916–923) prevents this binding and inhibits NRS activity in vivo (Gontarek et al., 1993). Because this suggested that U11 snRNP binding may correlate with NRS activity, we wanted to extend this result by making addi-

tional mutations in this region. We also wanted to try to confirm the importance of U11 snRNP binding for NRS activity by rescuing these mutants with compensatory U11 snRNA mutants.

The NRS was originally proposed to bind loop 3 of U11 snRNA based on its sequence complementarity to this region of the NRS (nt 916–923; Gontarek et al., 1993); therefore, mutants NRS.1 through NRS.4 were designed to alter interactions between the NRS and U11 loop 3. More recently, the 5' end of U11 snRNA has been found to base pair with the 5' splice site consensus sequence RUAUCCUU in several U12-dependent introns in cellular genes (Dietrich et al., 1997). The 3' end of the NRS also contains the sequence GUAUCCUU (at nt 919–926; Fig. 1), and mutations RG11 and NRS.1 through NRS.4 also alter this U11 5' splice site consensus sequence. Like the previously characterized mutant RG11 (Gontarek et al., 1993), the mutants NRS.1, NRS.2, NRS.3, and NRS.4 (Fig. 2B) all prevented binding of U11 snRNP as demonstrated by an affinity selection assay (Gontarek, 1994).

To test the effect of these mutations on NRS activity, the mutant NRS elements were inserted into the intron (chicken *c-myc* intron 2) of a heterologous splicing construct pRSVNeo-int (Linial, 1987), as previously described (Arrigo & Beemon, 1988; McNally et al., 1991; Fig. 2A). After transient transfection into human embryonic kidney 293 cells, total RNA was harvested and splicing was analyzed by an RNase protection assay using a riboprobe spanning the 5' splice site. This allowed us to determine the level of splicing of these constructs in the presence of different NRS elements. The results are shown in Figure 2A. In this assay, the control construct lacking the NRS (termed *myc*) and the RG11 mutant resulted in $12 \pm 8\%$ and $12 \pm 5\%$ unspliced RNA, respectively (Fig. 2A, lanes 3, 5), as expected. The wild-type NRS partially inhibited splicing, so that $43 \pm 9\%$ of the RNA was unspliced (Fig. 2A, lane 4). The new mutants NRS.1, NRS.2, NRS.3, and

FIGURE 1. RSV NRS RNA is complementary to both U1 and U11 snRNAs. The 3' region of the NRS (nt 912–926) is depicted in the center and is flanked by U1 and U11 snRNAs. Note that the potential base pairing between the NRS and U1 or U11 snRNAs extends beyond the region of the respective 5' splice site consensus sequences. Residues in these U1 and U11 5' splice site consensus sequences that are present in the NRS are underlined or overlined, respectively.

FIGURE 2. Mutations in U1/U11 overlapping 5' splice site sequences. **A:** Wild-type (WT) or mutant NRS sequences were inserted into the intron of pRSVNeo-int, and the resulting constructs were transfected into human 293 cells. RNA was harvested and subjected to RNase-protection analysis using a probe designed to distinguish between spliced and unspliced RNAs. myc refers to the pRSVNeo-int plasmid without an NRS insert. RG11 was previously described by Gontarek et al. (1993). U: unspliced; S: spliced. **B:** NRS mutations are aligned under the wild-type sequence. Regions with potential for base pairing with U1 snRNA are underlined. The U1 snRNP 5' splice site consensus sequence is overlined.

NRS.4 impaired NRS activity, reducing the amount of unspliced RNA to $12 \pm 5\%$, $6 \pm 6\%$, $6 \pm 5\%$, and $7 \pm 6\%$, respectively (Fig. 2A, lanes 6–9). Thus, all of these two- and three-base-substitution mutations inactivated the NRS to within the same margin of error as the RG11 mutant.

In an attempt to confirm that U11 snRNP binding was required for NRS activity, we made U11 snRNA expression constructs with mutations compensatory to those in the NRS. These constructs contained flanking regulatory sequences from the U1 snRNA gene. Two series of U11 snRNA mutants compensatory to the mutations in NRS.1, NRS.3, and NRS.4 were constructed. The first series had mutations in loop 3 of U11 snRNA (Gontarek, 1994) and the second series had mutations in the region of U11 snRNA that interacts with the 5' splice site (C.S. Hibbert & K.L. Beemon, unpubl.). These mutant snRNA constructs were cotransfected with their respective compensatory mutant Neo-int-NRS constructs in both human 293 cells and in chicken embryo fibroblasts (CEFs). None of these compensatory mutants was able to restore the NRS activ-

ity, suggesting U11 binding may not be required for NRS activity.

U1 5' splice site-like sequence necessary for NRS activity

Gontarek et al. (1993) reported that U1 snRNP binds the NRS in vitro. This result was confirmed by psoralen crosslinking studies, but the U1 binding site(s) were not determined (Hou, 1998). We observed that the U1 5' splice site consensus sequence in the NRS at nt 919–926 overlaps a U1 5' splice site-like sequence at nt 914–920 (Fig. 1). The mutated nucleotides in RG11 and NRS.1 through NRS.4 are all in this overlapping region and would be predicted to alter interactions with both U1 and U11 snRNPs, making it impossible to determine which interaction is critical for NRS activity. Therefore, five new mutations were designed to test the possible base-pairing interactions of the NRS with U1 snRNA.

The three NRS point mutants, G915C, U916C, and G919A (Fig. 3B), were designed to destabilize the potential base pairing of U1 snRNA at positions +1, +2, and +5 of the U2-dependent 5' splice site consensus sequence. The G919A mutation may also stabilize binding of U11 snRNP, by converting a G-U to an A-U base pair at the +1 position of the consensus U12-dependent 5' splice site sequence (RUAUCCUU). These mutated NRS sequences were inserted into the intron of the pRSVNeo-int expression construct as described above. Splicing was analyzed by RNase protection analysis after transient transfection into CEFs, using a probe spanning the 5' splice site as shown in Figure 2A. A typical experiment is shown in Figure 3A, while the data from several experiments are averaged on the graph in Figure 3C. NRS mutants G915C, U916C, and G919A decreased levels of unspliced RNA from the wild-type level of $52 \pm 8\%$ to $20 \pm 5\%$, $24 \pm 5\%$, and $18 \pm 4\%$, respectively (Fig. 3A,C). These results are consistent with the proposal that interactions between the NRS and U1 snRNA are necessary for NRS activity.

The NRS has nonconsensus UU residues rather than the consensus RA at positions +3/+4 of the U2-dependent 5' splice site-like sequence (Fig. 1). Thus, an additional NRS mutant, UU917/918AA, was made to test the effect on NRS activity of increasing the potential base pairing between the NRS and U1 snRNA. A dramatic increase in unspliced RNA levels from the wild-type $52 \pm 8\%$ to $95 \pm 5\%$ was observed for this mutant in the Neo-int construct (Fig. 3A,C). Therefore, there appeared to be a positive correlation between the strength of U1 snRNA base pairing and NRS activity.

A fifth mutant, AU921/922UG, was designed to test the effect of altering the potential base pairing between U1 snRNA and the NRS at positions +7 and +8, extending beyond the 5' splice site consensus sequence. This mutant also changed positions +3 and +4 of the

FIGURE 3. Mutations specific for potential U1 or U11 binding sites. **A:** Wild-type or mutant NRS fragments were inserted into pRSVNeo-int, and the resulting constructs were transfected into CEFs and assayed by RNase protection as in Figure 2A. The mutants G915C, U916C, G919A, and AU921/922CA disrupt potential U1 base pairing while UU917/918AA strengthens U1 base pairing. The mutants C923A, CU924/925GA, and UU925/926AA disrupt potential U11 base pairing without affecting U1 base pairing. **B:** Sequence of NRS mutants aligned with U1 (underlined) and U11 (overlined) 5' splice site sequences. NRS mutants depicted with ++ activity are up-mutants. **C:** Summary of transfection data in chicken (CEF) and human (293) cells using these same constructs. Error bars represent the standard deviation from the average of three to five independent transfection experiments.

overlapping U12-dependent 5' splice site consensus sequence. The level of unspliced RNA for the mutant AU921/922UG was $22 \pm 7\%$ (Fig. 3A,C). This was similar to that of mutants G915C, U916C, and G919A. This suggested that base pairing between U1 snRNA and the two NRS residues downstream of the U1 5' splice site-like sequence was also important for NRS activity.

To compare the effects of these mutations in mammalian cells with those in chicken cells, transient transfection experiments using these five mutants were repeated in human 293 cells. The wild-type NRS construct resulted in $46 \pm 4\%$ unspliced RNA in 293 cells, as compared with $52 \pm 8\%$ in CEFs. The mutants G915C, U916C, G919A, and AU921/922UG decreased unspliced RNA levels to $15 \pm 5\%$, $22 \pm 12\%$, $15 \pm 3\%$, and $22 \pm 5\%$, respectively (Fig. 3C). In contrast, mutant UU917/918AA resulted in $82 \pm 12\%$ unspliced RNA (Fig. 3C). As in CEFs, mutants that interfered with potential base pairing between the NRS and U1 snRNA at the 5' splice site consensus positions +1, +2, +5, or +7/+8 (downstream of the consensus sequence) all decreased NRS activity to levels near background (no NRS). In contrast, mutant UU917/918AA, which strengthened the potential U1/NRS interaction at positions +3/+4, was an up-mutant in both CEFs and 293 cells. In conclusion, these five mutants, as well as mutants RG11 and NRS.1 through NRS.4 (Fig. 2), all give a positive correlation between NRS activity and the potential for base pairing of U1 snRNA to this U1 splice site-like sequence. These results provide evidence that binding of U1 snRNP may be required for NRS activity.

Mutations in the U11 5' splice site consensus sequence increase NRS activity

To test whether the U11 binding site in the NRS was also critical for NRS activity, as had been proposed previously (Gontarek et al., 1993; McNally & McNally, 1998), three additional NRS mutations were made that would disrupt potential base pairing to the U11 site but not to the U1 site (Fig. 3B). These NRS mutants were inserted into the pRSVNeo-int construct, and splicing of Neo-int pre-mRNA was analyzed as described above.

Three different U11-specific NRS mutants were tested, altering the NRS at nt 923, 924/925, and 925/926, corresponding to positions +5, +6/+7, and +7/+8, respectively, of the U12-dependent 5' splice site consensus sequence (Fig. 3B). Mutants CU924/925GA and UU925/926AA were modeled after the U11 splice site mutants of Kolosova and Padgett (1997). Mutant C923A changed the C at +5 to an A, altering the U11 binding site at this position. Surprisingly, none of these mutants, which disrupted the potential base pairing to U11 but not to U1 snRNA, reduced NRS activity (Fig. 3). In fact, all three mutants increased NRS activity above wild-type levels, with C923A and CU924/925GA showing the most dramatic increases. In CEFs,

the wild-type NRS construct resulted in $52 \pm 8\%$ unspliced RNA, while the C923A and CU924/925GA mutants yielded $82 \pm 11\%$ and $79 \pm 7\%$ unspliced RNA, respectively (Fig. 3). Mutant UU925/926AA increased activity only slightly above wild-type NRS levels to $56 \pm 7\%$ unspliced RNA (Fig. 3A,C).

These U11-specific constructs were also tested by transient transfection into human 293 cells. In 293 cells, the wild-type NRS construct resulted in $46 \pm 4\%$ unspliced RNA, while the mutants C923A, CU924/925GA, and UU925/926AA yielded $81 \pm 12\%$, $69 \pm 21\%$, and $60 \pm 8\%$ unspliced RNA, respectively (Fig. 3C). Thus, similar results were obtained in human and chicken cells, showing that all of the U11-specific NRS mutants were up-mutants.

The surprising result that the mutations which disrupt the potential base pairing between U11 snRNA and the NRS all increase NRS activity suggests three points. First, U11 snRNP binding is not required for NRS activity. Second, the interaction with the NRS involves the 5' end of U11 snRNA rather than U11 loop 3, as these three mutations are all downstream of the NRS region complementary to loop 3 (nt 916–923). Third, the binding of U11 snRNP appears to play an antagonistic role to the inhibitory effect of the NRS.

A U1 snRNA compensatory mutant partially rescues mutant NRS activity

To further test whether U1 snRNP binding is critical for NRS activity, we coexpressed an NRS mutant with a U1 snRNA compensatory mutant. The U1-4u mutant snRNA (Zhuang & Weiner, 1986) was cotransfected with the NRS.5 (G919A) mutant in the pRSVNeo-int construct in an attempt to rescue NRS activity. The U1-4u mutant has a substitution of U for C in the U1 snRNA (Zhuang & Weiner, 1986), rendering it compensatory to the mutated U1 binding site in NRS.5, which has a G to A substitution at position +5 (Fig. 4C). The results of cotransfection experiments in HeLa cells are shown in Figure 4.

Both the wild-type NRS and the NRS.5 mutant pRSVNeo-int constructs were cotransfected with either a control wild-type U1 snRNA expression construct or with the U1-4u compensatory mutant. Although the percentage of unspliced RNA for both the wild-type NRS (95%) and for the NRS.5 mutant ($46 \pm 3\%$) were significantly higher in the transfected HeLa cells than in CEFs or 293 cells, there was still a substantial difference between the two constructs (Fig. 4A,B, lanes 1 and 3). In the presence of the compensatory U1 mutant, the NRS.5 mutation was partially suppressed relative to the control cotransfection with wild-type U1, partially rescuing NRS activity (from $46 \pm 3\%$ to $59 \pm 3\%$ unspliced RNA; Fig. 4A,B, lanes 3 and 4). As expected, no difference in NRS activity was observed when the wild-type NRS construct was cotransfected

FIGURE 4. U1 compensatory mutant partially restores NRS activity. **A:** Both wild-type NRS and NRS.5 (G919A) in pRSVNeo-int were cotransfected with either a wild-type U1 expression construct or with the compensatory U1 snRNA mutant, U1-4u (Zhuang & Weiner, 1986) in HeLa cells. **B:** Bar graph showing results of cotransfection experiments. The lane numbers 1–4 in **A** correspond to the column numbers 1–4 in **B**. Error bars represent the standard deviation from the average of three independent cotransfection experiments. **C:** Sequence of U1-4u snRNA and the NRS.5 (G919A) mutant.

with either the wild-type U1 snRNA construct or with the mutant U1-4u (Fig. 4A, lanes 1 and 2). These results support the proposal that binding of the U1 snRNP is necessary for NRS activity.

Mutations in the overlapping U1/U11 5' splice site sequences delay viral replication

To determine whether mutations in the U1/U11 overlapping sites affect viral replication, we cloned mutant NRS.1 (Fig. 2B) into a nonpermuted, full-length RSV

viral construct, pAPrCΔpBR (Barker & Beemon, 1991) to generate pRSV.NRS.1. This three-base-substitution mutation does not alter the amino acid sequence of the viral Gag polypeptide; however, it does mutate the potential binding sites for both U11 and U1 snRNPs in the NRS. This mutation was shown to block binding of U11 snRNP to the NRS in an *in vitro* binding assay (Gontarek, 1994) and to abolish NRS activity in a heterologous construct assayed *in vivo* (Fig. 2).

The RSV wild-type and mutant constructs were transfected into CEFs, and virus was recovered from both culture supernatants 48 h later. The viral titer was determined by a reverse transcriptase (RT) assay. Equivalent RT units of wild-type and mutant virus were then used to infect fresh CEFs. Viral replication in these infected cells was determined by an RT assay using culture supernatants, and the results are shown in Figure 5. We observed a two-day delay in viral replication for the NRS.1 mutant virus compared to the wild-type virus (Fig. 5). The wild-type virus showed significant RT activity two days after infection; however, the RSV.NRS.1 mutant virus did not show RT activity until four days after infection. At five days after infection there was about five times more RT activity in the supernatants from the wild-type infected cells than in those infected with the RSV.NRS.1 mutant virus (Fig. 5). Similarly, RT activity six days after transfection was about four times higher for the wild-type RSV plasmid compared to RSV.NRS.1 (data not shown). Thus, this mutation that would affect binding of both U1 and U11 snRNPs delays viral replication in infected cells.

FIGURE 5. A mutation in the U1/U11 snRNP overlapping binding sites delays viral replication. Viral levels were measured by reverse transcriptase assays, using virus particles isolated from the culture supernatants of CEFs infected with each virus. Mutant virus RSV.NRS.1 contains the three-base substitution of the NRS.1 construct, which alters the nucleotide sequence within this region without changing the *gag* gene amino acid sequence. Error bars represent the standard deviation of the average of four independent infection experiments.

DISCUSSION

U1 binding to the NRS inhibits splicing and is regulated by U11 binding

We have identified a U1 5' splice site-like sequence in a critical region of the RSV NRS splicing inhibitory element. All mutations that disrupted U1 base pairing to this sequence reduced NRS activity, whereas a mutation that improved U1 base pairing increased NRS activity. In addition, a U1 snRNA mutant partially suppressed a compensatory mutation in this U1 splice site-like sequence. These results suggest U1 snRNP binding to the NRS is required for maintenance of unspliced RNA. This U1 binding site overlaps a U11 binding site in the NRS. Surprisingly, mutants that disrupted the U11 site increased rather than decreased NRS activity, as was previously predicted. Therefore, we propose that U11 snRNP binding promotes splicing by blocking or displacing U1 snRNP binding at the overlapping site.

A splicing regulation model is proposed in Figure 6 to explain our data. We propose that competition between U1 and U11 snRNPs for binding to this overlapping region is important in determining the balance between spliced and unspliced viral RNAs. When U1 snRNP binds the NRS, splicing is inhibited, resulting in production of full-length genomic RNA and mRNA for Gag-Pol polyproteins (Fig. 6B). When U11 binds the NRS, U1 binding to the NRS is prevented, resulting in splicing of the *env* and *src* mRNAs (Fig. 6C). According to this model, it would be expected that mutations in the U11 site would have no effect on NRS activity if the binding of U1 had already been abrogated by a mutation in the U1 binding site. In fact, all the mutations directed at both U1 and U11 snRNPs had an NRS phenotype similar to that of mutations specific for the U1 binding site.

The importance of snRNP binding to the NRS was demonstrated by our finding that a virus bearing three base changes in the U1/U11 overlapping binding sites showed delayed replication. In the future, it will be in-

FIGURE 6. Model for regulation of RSV splicing by competition between U1 and U11 snRNPs for binding to the RSV NRS sequence. **A:** RSV has three mRNAs: the full-length transcript is *gag/pol* mRNA, and two alternatively spliced mRNAs encode *env* and *src* products. The NRS is within the *gag* gene, which is part of the intron for the spliced mRNAs. Binding sites of the SR protein ASF/SF2 and U1/U11 on the NRS are shown. **B:** The active form of the NRS binds U1 snRNP and ASF and inhibits splicing of the primary transcript. It is not known whether U1 snRNP binds the authentic 5' splice site in this case. **C:** The inactive form of the NRS binds U11 snRNP, preventing U1 snRNP from binding to the NRS, and allowing splicing to occur. It is not known whether ASF binding is occurring in this case.

teresting to see what effect mutations specific for U1 or U11 binding have on viral replication and splicing. This is the first report of the effect of an NRS mutation on viral growth rate. However, altered pathogenesis of a related avian leukosis virus with a deletion in the exonic splicing enhancer (ESE) region of the NRS (nt 735–776) has been observed (Jiang et al., 1997; Smith et al., 1997). It is not known whether these consensus (U11) and near-consensus (U1) sequences in the NRS are ever used as real splice sites. Although splicing to this region of the NRS has not been seen when the NRS is in the pRSVNeo-int construct (M.T. McNally & K.L. Beemon, unpubl.), Weldon and Wills (1993) observe some alternative splicing from this region in a different heterologous construct. An important question is whether these potential splice sites are ever used in the virus.

An ESE in the NRS, which binds ASF/SF2 and is necessary for maximal NRS activity (McNally & McNally, 1996, 1998; Smith et al., 1997), may recruit U1 snRNP to the NRS as shown in the model in Figure 6B. Although there are more than 140 nt between the ESE and the snRNP binding sites (Fig. 6A), the NRS has been proposed to form a hairpin loop bringing the ESE and the snRNP binding sites together near the bottom of the loop (McNally et al., 1991). The recruitment of U1 snRNP to 5' splice sites by ASF has been previously demonstrated (reviewed by Manley & Tacke, 1996). In addition, ASF/SF2 has recently been implicated in the negative control of retroviral splicing. The depletion of ASF/SF2 in vivo results in the increase of splicing of an HIV-1 *tat* pre-mRNA (Wang et al., 1998). A possible role for ASF recruitment of U11 to the NRS has not yet been established and is shown by the question mark in Figure 6C.

Once recruited to the NRS, the U1 snRNA could base pair at positions –1, +1, +2, +5, and +6 of the 5' splice site-like sequence. Point mutations at +1, +2, and +5 all inactivated NRS activity. There is also potential base pairing between the NRS and U1 snRNA at positions +7 and +8; mutation of both of these nucleotides also inactivated the NRS, suggesting this extension to the consensus sequence is important. At positions +3 and +4, the NRS has UU residues instead of the consensus RA. When the UU sequence was converted to the consensus AA in the NRS, almost complete inhibition of splicing was observed. These UU residues may form non-Watson–Crick base pairs with two pseudouridines in the U1 snRNA, as has been observed for UU duplexes (SantaLucia et al., 1991). It is interesting that a single U–U interaction is present and selected for in the yeast U1 5' splice site consensus sequence (D. Libri, pers. comm.).

If U11 snRNP binding to the NRS affects U1 snRNP binding, the competition could be regulated by the amount of available snRNPs and other protein factors and by the affinities of the two snRNPs for their respec-

tive binding sites. The 100-fold excess of U1 to U11 snRNP found in the HeLa nucleus (Baserga & Steitz, 1993), might be expected to favor U1 binding over U11 binding to the NRS. In vivo the ratio of available U1 to U11 could be far less than a 100-fold excess since the majority of nuclear splicing involves U1 rather than U11 snRNPs. Nevertheless, after incubation of an RNA transcript containing the NRS in the adenovirus major late intron in HeLa nuclear extract, affinity selection data suggest that at least as much U11 as U1 bound to this RNA (Gontarek et al., 1993). However, significantly less U11 binds to the free NRS RNA (Gontarek et al., 1993; Cook & McNally, 1998). Since U11 binding to a 5' splice site can be stabilized by U1 bound elsewhere to the same pre-mRNA (Wu & Krainer, 1996), U1 bound to the RSV 5' splice site (at nt 397) might stabilize U11 binding to the NRS.

How does U1 snRNP binding to the NRS inhibit splicing?

There is increasing evidence for the role of U1 5' splice site-like sequences in the regulation of RNA metabolism. For example, an intron enhancer which binds U1 snRNP promotes polyadenylation at an upstream site in the calcitonin gene (Lou et al., 1996). In addition, the 3' untranslated region of papillomavirus late mRNA contains a 5' splice site-like sequence that binds U1 snRNP and appears to regulate polyadenylation efficiency (Furth et al., 1994). 5' splice site sequences have also been implicated as crucial components of splicing regulatory elements which interact with U1 snRNA but are not used as splice sites. For example, the U1 snRNA plays an essential role in the formation of an unspliced HIV-1 *env* mRNA that is subject to Rev regulation (Lu et al., 1990). Moloney and Friend murine leukemia viruses also have an alternative 5' splice site consensus sequence within the *gag* gene, which may play a role in regulation of splicing (Oshima et al., 1996; M. Mougél & M. Sitbon, pers. comm.). In addition, Siebel et al. (1992) observed that the binding of U1 snRNP and protein complexes to two exonic pseudo-5' splice sites prevents U1 snRNP binding to the accurate 5' splice site and thus prevents splicing. It is possible that the NRS may be involved in polyadenylation regulation or in interfering with U1 binding to the authentic 5' splice site. Since the NRS can form a complex with a downstream 3' splice site in vitro (Gontarek, 1994), we also think that it may compete with the bona fide 5' splice site 300 nt upstream of the NRS for interaction with this same 3' splice site.

In conclusion, we propose that negative regulation of splicing in RSV is due to an interaction between U1 snRNA and a 5' splice site-like consensus sequence. U11 snRNPs regulate splicing inhibition by blocking or displacing U1 snRNP. Competition between U1 and U11 snRNPs for 5' splice sites may be part of a more

general splicing regulation mechanism. It is possible that overlapping U1 and U11 5' splice sites are a common evolutionary feature, allowing for more flexibility of exon shuffling involving different reading frames. It is possible, also, that U11 snRNP binding to 5' splice sites in cellular pre-mRNAs may be regulated by competition for binding to U1 snRNP, as we have seen in RSV RNA.

MATERIALS AND METHODS

DNA constructs

Viral DNA restriction fragments containing the NRS from the Prague-C strain of RSV were obtained by PCR amplification of plasmid pAPrCΔpBR (Barker & Beemon, 1991), using the sequence coordinates determined by Schwartz et al. (1983). The RSV fragments were cloned into the chicken *c-myc* intron of pRSVNeo-int (Linial, 1987) at the *SacII* site, approximately 300 nt from the 5' splice site.

NRS mutants NRS.1–NRS.4 were generated by PCR amplification of a *Bst*NI fragment from RSV with a 5' primer corresponding to nt 703–726 (primer 703) and a 3' primer spanning nt 907–930. Mutants were amplified using primer 703 in conjunction with either of the following primers:

(NRS.1) (5'-AGGGAAGCCTGAGTTCCACTCCCC-3'),
(NRS.2) (5'-AGGGAAGGATAGTACCACTCCCC-3'),
(NRS.3) (5'-AGGGAAGGCTGCAAACCACTCCCC-3'), and
(NRS.4) (5'-AGGGAAGGATGAGAACCACTCCCC-3').

The eight NRS mutants shown in Figure 3B were generated by PCR amplification of a *Bst*NI fragment from RSV with engineered *SacII* sites on both ends. In all cases, the 5' primer corresponded to RSV nt 703–726 (primer 5'BB) and had the sequence (5'-ATCTTCCGCGGAGGTCCGGAGTG CATCGAG-3'). Each mutant was generated with a unique 3' primer spanning nt 907–930 that changed nt 915, 916, 917/918, 919, 921/922, 923, 924/925, and 925/926 respectively. Mutants were amplified using primer 5'BB in conjunction with either of the following 3' primers: (G915C: 5'-TAGTACCGCGGAGGGAAGGATACAAAGCAC-3'), (U916C: 5'-TAGTACCGCGGAGGGAAGGATACAAAGCCAC-3'), (UU917/918AA: 5'-TAGTACCGCGGAGGGAAGGATACCTTACCAC-3'), (G919A: 5'-TAGTACCGCGGAGGGAAGGATATAAAC CAC-3'), (AU921/922UG: 5'-TAGTACCGCGGAGGGAAG GCAACAAACCAC-3'), (C923A: 5'-TAGTACCGCGGAGGG AAGTATACAAACCAC-3'), (CU924/925GA: 5'-TAGTACCG CGGAGGGATCGATACAAACCAC-3'), and (UU925/926AA: 5'-TAGTACCGCGGAGGGTTGGATACAAACCAC-3'). The unique *SacII* site in the intron of pRSVNeo-int was blunt-end repaired to accept the blunt-end repaired fragments for NRS.1–4. For the other eight mutants the pRSVNeo-int vector and PCR product inserts were digested with *SacII* and ligated. Clones were verified by sequencing by the dsCycle Sequencing Kit method (Life Technologies).

Cell culture and DNA transfection

Primary CEFs were prepared according to Rein & Rubin (1968) and maintained in Medium 199 containing 2% tryptose phosphate broth, 1% calf serum, and 1% heat-treated

chick serum (Life Technologies). CEFs on 6-cm plates were transfected with 2–5 μ g plasmid DNA in serum-free Medium 199 containing 200 μ g/mL DEAE-dextran. After 5 h, cells were subjected to a 2- to 3-min shock with 10% dimethyl sulfoxide (Arrigo et al., 1987).

Human 293 cells were maintained in Minimal Essential Medium (Life Technologies) supplemented with 10% fetal calf serum. HeLa cells were grown in Dulbecco's Modified Eagle Medium (Life Technologies) plus 10% fetal calf serum. Cells on 6-cm plates were transfected with a total of 3–5 μ g plasmid DNA by the calcium phosphate (Profectin kit, Promega Biotec) or lipofectamine (Life Technologies, Inc.) method. The procedures of the manufacturers were used, except that no glycerol shock was used for the calcium phosphate procedure. Independent transfection experiments in CEFs and mammalian cells were performed a total of 5 to 10 times for each mutant.

When cotransfections were performed for the genetic suppression analysis, HeLa cells in 6-cm culture plates at 60% confluency were transfected with 5 μ g of either the wild-type or mutant NRS construct and 5 μ g of either wild-type U1 snRNA control plasmid or mutant U1-4u snRNA test plasmid. Results are the average of three independent transfection experiments.

RNase protection

RNA isolation, riboprobes, and RNase-protection analyses were as previously described (McNally et al., 1991). Briefly, total RNA was extracted 48 h posttransfection using RNAzol B (Tel-Test), treated with 40 U of RNase-free DNase (Boehringer Mannheim), phenol extracted and hybridized at 55°C for 16 h. Quantitation of radiolabeled RNA in polyacrylamide gels was determined by analysis on an InstantImager (Packard).

Viral infectivity assay

The 3-nt substitution mutation NRS.1 (Fig. 2B), which does not alter the *gag* coding sequence, was introduced into a full-length nonpermuted RSV genomic construct pAPrCΔpBR (Barker & Beemon, 1991) by a two-step cloning procedure. First, the mutation was introduced into an NRS subclone (*MroI*–*SphI*, nt 706–1006) by recombinatorial PCR. This subclone was cloned into the *SacII* *gag* gene subclone (containing RSV nt 543–1806), which was introduced into pAPrCΔpBR to generate pRSV.NRS.1. CEFs on 50% confluent 10-cm culture dishes were transfected with 8 μ g of DNA of the RSV genomic plasmids pAPrCΔpBR (RSV.wt) or pRSV.NRS.1. After 48 h, viral titer in the supernatant was determined by RT assays as previously described (Ogert et al., 1996). To assay viral infectivity, equal virus titers (6,000 cpm by RT assay) of the 48 h supernatant from the transfected CEFs were directly used to infect CEFs which were about 15% confluent on 10-cm culture dishes. RT assays of the infected cells were determined every 24 h following the initial assay at 48 h after infection. Results are the average of four independent infection experiments.

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