Two Regions Promote U11 Small Nuclear Ribonucleoprotein Particle Binding to a Retroviral Splicing Inhibitor Element (Negative Regulator of Splicing)*

Lisa M. McNally, Lily Yee, and Mark T. McNally‡

From the Department of Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

The Rous sarcoma virus (RSV) negative regulator of splicing (NRS) is an RNA element that represses splicing and promotes polyadenylation of viral RNA. The NRS acts as a pseudo 5' splice site (ss), and serine-arginine (SR) proteins, U1snRNP, and U6 small nuclear ribonucleoproteins (snRNPs) are implicated in its function. The NRS also efficiently binds U11 snRNP of the U12-dependent splicing pathway, which is interesting, because U11 binds only poorly to authentic substrates that lack a U12-type 3' splice site. It is of considerable interest to understand how the low abundance U11 snRNP binds the NRS so well. Here we show that U11 can bind the NRS as a mono-snRNP \textit{in vitro} and that a G-rich element located downstream of the U11 site is required for efficient binding. Mutational analyses indicated that two of four G tracts in this region were important for efficient U11 binding and that the G-rich region did not function indirectly by promoting U1 snRNP binding to an overlapping site. Surprisingly, inactivation of U2 snRNP also decreased U11 binding to the NRS. The NRS harbors a branch point-like/pyrimidine tract sequence (BP/Py) just upstream of the U1/U11 site that is characteristic of 3' splice sites. Deletion of this region decreased U2 and U11 binding, and deletion of the G-rich region also reduced U2 binding. The G element, but not the BP/Py sequence, was also required for U11 binding to the NRS \textit{in vivo} as assessed by minor class splicing from the NRS to a minor class 3'ss from the \textit{P120} gene. These results indicate that efficient U11 binding to the isolated NRS involves at least two elements in addition to the U11 consensus sequence and may have implications for U11 binding to authentic splicing substrates.

An important step in the production of most mRNAs is the removal of intron sequences from pre-mRNA by the process of RNA splicing. Different combinations of exons can be combined via alternative splicing to generate numerous proteins from a single gene (1), and it is now appreciated that regulated splicing accounts for great protein diversity (1–3). RNA splicing is also used by many viruses to infect eukaryotic cells as a means to expand their coding capacity and to regulate gene expression (4). This is true for retroviruses, with HIV\textsuperscript{1} being an extreme example: it is estimated that more than 40 viral splice variants are produced in an HIV-infected cell (5–7). In addition to alternative splicing, retroviruses also exhibit incomplete splicing such that a large fraction of the primary transcripts remain unspliced and, in contrast to unspliced host cell mRNAs, are transported to the cytoplasm where they serve as mRNA and as genomes for progeny virions. These RNA processing events make retroviruses useful tools for studying the cellular RNA processing machinery, and determining how these events are controlled is required for understanding these important aspects of viral replication.

RNA splicing takes place in a large macromolecular complex termed the spliceosome in which five small nuclear ribonucleoprotein particles (snRNPs) and a large number of accessory proteins cooperate to identify splice sites and assemble the spliceosome (8, 9). A dynamic network of snRNA-snRNPs and snRNA-substrate interactions takes place to further identify the splice sites and to form the catalytic core of the spliceosome (10). Two splicing pathways have been identified (U2-dependent and U12-dependent), which excise introns with distinct cis splicing signals and differ in snRNP utilization (9, 11). Although U5 is utilized by both spliceosomes, the more abundant U2-dependent spliceosome contains U1, U2, U4, and U6 snRNPs, whereas the minor, U12-dependent pathway utilizes the low abundance U11, U12, U4atac, and U6atac snRNP counterparts. Non-snRNP splicing factors such as SR proteins are also shared between the two pathways (12, 13). The assembly pathways and catalytic properties of the two spliceosomes are quite similar despite the differences in splicing signals and spliceosome composition (11), yet important differences between the two pathways have been observed. Notably, U1 and U2 snRNPs are found as discrete particles in nuclear extracts and can independently bind to the 5'ss and branch point sequence (BPS), respectively, whereas U11 and U12 snRNPs can form a di-snRNP that cooperatively binds the 5'ss and BPS and only poorly associates with isolated splice sites (14–18).

For retroviruses, levels of unspliced RNA are controlled by the inefficient use of 3' splice sites and in avian retroviruses, through the action of a splicing inhibitor element within the \textit{gag} gene termed the negative regulator of splicing, or NRS, that primarily affects splicing to the src 3'ss (19–23). Interestingly, the NRS also is required for optimal polyadenylation of viral RNA, because deletions or mutations that disrupt NRS splicing inhibition also lead to read-through transcripts (19, 24, 25). For splicing inhibition, the NRS is thought to act as a

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† To whom correspondence should be addressed: Dept. of Microbiology and Molecular Genetics, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Tel.: 414-456-8749; Fax: 414-456-6535; E-mail: mtm@mcw.edu.

‡ The abbreviations used are: HIV, human immunodeficiency virus; snRNP, small nuclear ribonucleoprotein; snRNA, small nuclear RNA; 3'ss and 5'ss, 3' and 5' splice sites; BPS, branch point sequence; NRS, negative regulator of splicing; nt, nucleotide(s); RSV, Rous sarcoma virus; MS, NRS RNA at 701–1011; BP/Py, branch point-like/pyrimidine tract sequence.
pseudo 5′ss that non-productively associates with 3′ splice sites to prevent their interaction with the authentic 5′ss (26, 27). The NRS can also inhibit splicing of heterologous introns in vivo and in vitro (26). Important splicing regulators called SR proteins bind to high-affinity sites in the upstream portion of the ~230-nt NRS and serve to recruit U1 snRNP to a downstream binding site (28–30). U1 binding appears critical, because mutations that diminish U1 binding inactivate the NRS and abolish interactions with a 3′ splice site (27, 30, 31). Other mutations in the U1 binding site activate splicing from the NRS (30–32), suggesting that U1 binding is a required but not final step in the pathway leading to inhibition. These mutations and the potential for an extended base pairing interaction form the final step in the pathway leading to inhibition. These mutations were also generated in a background where the U1 site in the NRS was mutated (mU1 (30)), yielding the plasmid series pMSmU1mG1, etc. The 3′ deletion series pMSA4, pMSA5+4, and pMSA5G–4 were made by PCR and have the deletions indicated in Fig. 6. The 5′ deletion series, pMSA1, pMSA1+1, and pMSA1–3, were also made by PCR and are depicted in Fig. 7. The mG1, mG2, and mG1+2 mutations were incorporated into a variant of the chimeric expression plasmid pNRS-P120 (30) in which a unique XhoI site was introduced into the intron to facilitate cloning to generate pmG1-P120, pmG2-P120, and pmG1+2-P120.

In Vitro Transcription and Affinity Selection—pGEM-3Z plasmids containing NRS fragments were linearized with XbaI (MS version, 701–1011, 331 nt) except for pZBB, which was linearized with BamHI, to generate the 258-nt NRS RNA and with PvuII to generate BB-extended (497 nt) RNA and T7 RNA, polymerase was used. pZ2-P120 was linearized with BstXI to exclude the 5′ss half-substrate. For pSP64-SCNA4, XbaI was used for full-length RNA that included the U1 site, BstXI to exclude the U1 site, and BsaI for the 5′-half-substrate (sizes shown in Fig. 3). SP6 RNA polymerase was used for these plasmids. All plasmids were transcribed in vitro in the presence of biotin-11-UTP (20% of total UTP). RNAs were incubated under splicing conditions with ATP in HeLa nuclear extract for 20 min at 30 °C (36). Where indicated, 2′-O-methyl oligonucleotides to U1 (nt 1–14), U2 (nt 27–49), and U7 (nt 3–20) were added 15 min prior to addition of biotinylated substrate. For snRNA cleavage, extracts were pretreated for 15 min with 0.5 mg/ml RNase A and 0.5 mg/ml RNase T1 with or without glycerol (1 M). RNA was then electrophoretically transferred to a ZetaProbe membrane (Bio-Rad) and hybridized with a riboprobe to U11, U12, or U2 snRNAs and visualized and quantitated using a PhosphorImager.

Transfection of 293 Cells and Analysis of RNA—293 cells were grown in minimal essential medium supplemented with 10% fetal calf serum. Cells grown to about 40–60% confluence in 6-cm dishes were transfected with 2–3 mg of DNA by the calcium-phosphate method (Amersham Biosciences), and total RNA harvested 40 h later was isolated with Qiagen RNeasy columns according to the manufacturer’s instructions. For reverse transcription–PCR, 1–2 μg of total RNA was reverse-transcribed with an antisense primer directed to the 5′-end of the transcription unit (GCAGACACTCTATGCCTGTGG) and common to all RNAs in 20 μl using 200 units of reverse transcriptase (Invitrogen) and the manufacturers recommended reaction conditions. For PCR, 2 μl of the reverse transcription reaction was subjected to 18–25 cycles of PCR as described (30), and product levels in the linear range were quantitated using an AlphaImager. Alternatively, PCR was performed with a radiolabeled 5′ primer, and products were quantitated on acrylamide gels with a PhosphorImager (with similar results).

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Rsv sequences are from the Prc strain (34) with sequence coordinates as described by Schwartz et al. (35). Plasmids described previously are pZBB (to generate the short NRS RNA, nt 701–930) (28), pZ2xKMS (to generate the long NRS RNA, nt 701–1011) (30), and pP120 and pNRS-P120 for expression of the P120 splicing cassette (30). The pSP64-SCNA4 plasmid was a gift of Adrian Krainer. pZ2-P120 was made by inserting a HindIII-BglIII PCR fragment containing the first 5′ end of exon 1 through nt 180 of exon 7 into the HindIII-BamHI sites of pGEM-3Z (Promega). Point mutations in the G

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2 Y. Wang and M. T. McNally, unpublished data.

**RESULTS**

Fig. 1 depicts the major features of the NRS, including the upstream SR protein binding sites and important sequences for snRNP binding. The consensus U1 and U11 5′ss sequences are shown over the experimentally determined snRNP binding sites within the NRS. U1 binding to its suboptimal site can be substantially improved by mutating the U11 site, whereas only a modest improvement in U11 binding was observed upon mutation of the U1 site (30). Thus, U11 binds the NRS very efficiently and out-competes U1. The efficient U11 binding is surprising, because the NRS does not harbor the highly conserved U12-type BP consensus signal, and it is known that efficient U11 binding to bona fide 5′ splice sites requires a corresponding U12-type 3′ss (17).

U11 Binding to the NRS Does Not Require U12—Although U11 and U12 snRNPs are thought to exist primarily as a di-snRNP in cells, between 60 and 80% of U11 snRNP exists as
a mono-snRNP in extracts while the rest is complexed with U12 (16). Thus, it is possible that U11 snRNP binds the NRS as a free snRNP or as a di-snRNP in vitro. To determine if U12 is required for U11 binding to the NRS, U12 snRNA in nuclear extracts was cleaved with oligonucleotides directed toward nt 2–23 (the di-snRNP remains intact) or nt 53–71 (which destroys U12 snRNA and thus, the di-snRNP). U11 binding to the NRS (nt 701–1011) in these extracts was then assessed by affinity selection on biotinylated NRS RNA and Northern blotting for U11 snRNA. As shown in Fig. 2A and consistent with a previous study (16), treatment of extract with the U12-23 oligonucleotide cleaved off the 5' end of U12 snRNA, whereas U12-53–71 caused complete degradation (lanes 5 and 6). A control oligonucleotide to U6 snRNA had no effect on U12 snRNA (lane 7). None of the oligonucleotides had an effect on U11 snRNA (data not shown). As shown in Fig. 2B, in untreated extracts NRS RNA selected U11 snRNP well (lane 3), whereas binding to a substrate with mutations at the +6 and +7 positions in the U11 site was very low (lane 2). U11 binding to the NRS was not affected by treatment with the U6 control oligonucleotide (lane 7), nor was there a significant effect upon treatment with either of the U12 oligonucleotides (lanes 5 and 6). These results indicate that, in contrast to authentic splicing substrates, U11 snRNP need not be complexed with U12 to bind the NRS.

A G-rich Downstream Element Promotes U11 Binding to the NRS—To determine the efficiency of U11 snRNP binding to the NRS versus authentic minor class 5' splice sites, biotinylated NRS or P120 5'ss and SCN4A RNAs were subjected to affinity selection. P120 and SCN4A represent two U12-dependent substrates that have been well characterized (12, 37, 38). The NRS RNA contained RSV nt 701–1011, the P120 5'ss substrate contained the entire exon 6 through position 66 of the 99 nt intron, and the SCN4A 5'ss RNA included 119 nt of exon (plus 12 nt from the vector) through nt 66 of the intron. As expected, binding of U11 was well above background for the NRS (Fig. 3, compare lanes 2 and 3) but was low to undetectable with isolated 5' splice sites from the two U12-dependent genes (lanes 4 and 5). The difference in U11 binding could not be attributed to the presence of an adenosine rather than a guanosine at position +1 of the 5'ss of the P120 transcript, because conversion to guanosine did not improve U11 binding (data not shown). Furthermore, U11 binding was also low for a complete SCN4A substrate and one that contains a downstream U1-type 5'ss, which has been reported to enhance splicing of this substrate (lanes 6 and 7) (12, 37, 38). The low level of U11 binding likely reflects the short time of incubation (20 min). These results indicated that one or more features of the NRS distinguish it from normal U11-type 5' splice sites concerning the efficiency of U11 binding.

Examination of the NRS revealed a G-rich region just downstream of the U11 binding site. The prevalence of G residues in this region is significantly higher than in surrounding regions, and the density of G triplets per kilobase is also non-random and substantially higher (Table I). This suggested a possible role for the G tracts and associated factors in mediating efficient binding of U11 to the NRS. To test this hypothesis, U11 binding to two NRS substrates differing in the presence (MS) or
The data were generated with DNASTar software.

| Sequence boundary | Length | Number of residues (% of total) | GGG number | G triplets per kb |
|------------------|-------|--------------------------------|------------|------------------|
|                  |       | G (%) | A (%) | U (%) | C (%) |                  |
| 1–700            | 700   | 222 (32)| 160 (23) | 152 (22) | 166 (23) | 32 (4.6) | 46 |
| 701–930          | 230   | 69 (30) | 57 (25) | 43 (19) | 61 (26) | 5 (2.2) | 22 |
| 931–1011         | 81    | 41 (51) | 16 (20) | 5 (6) | 19 (23) | 14 (18) | 173 |
| 1012–1111        | 100   | 40 (40) | 15 (15) | 20 (20) | 25 (25) | 5 (5.1) | 50 |
| 1112–2111        | 1000  | 300 (30) | 234 (23) | 201 (20) | 265 (27) | 33 (3.3) | 33 |
| 2112–4111        | 2000  | 568 (28) | 451 (23) | 485 (24) | 497 (25) | 61 (3.1) | 31 |
| 1–9312           | 9312  | 2681 (29) | 2212 (24) | 2060 (22) | 2359 (25) | 284 (3.1) | 30 |

* Number of GGG triplets within a region as the percentage of all trinucleotides.

**TABLE I**

Nucleotide distribution in RSV genomic RNA

absence (BB) of the G tracts was assessed. Both of these constructs are capable of splicing inhibition (39) and bind U1 predominantly involved in optimal U11 binding, that neither G3 and G4 do not play a prominent role in efficient U11 binding to the NRS.

G Tracts Are Not Interchangeable—The observations that mutations in G tracts 3 and 4 had little effect (Fig. 5B) on U11 binding suggested that close positioning to the U11 site might be required for function such that G3 and G4 are suboptimally effective due to distance, and thus their mutation shows little effect. Alternatively, the sequence of G3 and G4 may not support efficient U11 recruitment. To determine if G3 and G4 might function when repositioned closer to the U11 site, the G tracts were sequentially deleted from the 5’ end (Fig. 7). Like the mG1 point mutation, deleting G1 reduced U11 binding to the level of the NRS that lacks the G region (compare lanes 4 and 7), again indicating a requirement for G1 in optimal U11 binding. U11 binding remained inefficient upon deletion of G1 plus G2 and G1 minus G3 (lanes 5 and 6). These results show that placing G3 and G4 at the position of G1 and G2 is not sufficient for efficient U11 binding. We conclude that G3 and G4 do not play a prominent role in efficient U11 binding to the NRS.

U2 snRNP Inactivation Decreases U11 Binding to the NRS in Vitro—As a complement to the data in Fig. 5B that indicated a direct role for the G tracts in U11 binding rather than an indirect effect of competition with U1 binding, we inactivated U1 snRNP in nuclear extract with a 2’-O-methyl oligonucleotide to the 5’ end of U1 snRNA. Consistent with the data in Fig. 5, U11 binding was variably but mildly affected upon inactivating U1 snRNP or by a control oligonucleotide to the non-spliceosomal U7 snRNP (Fig. 8, lanes 4–7 and data not shown).
U1 snRNP Binding to the NRS

Fig. 5. Mutation of G tracts 1 and 2 diminishes U11 binding to the NRS. A, schematic representation of the NRS (not to scale), showing the U11 binding site and the position of the downstream G tracks 1–4. Expanded below is the sequence of the U11 binding site (shaded) and the G-rich region, with the G tracts underlined. Above the sequence are the mutations introduced into each G tract. B, representative affinity selection experiment using substrates with the U1 site mutated (mU1). To the left is a schematic of RNAs used (nt 701–1011) with a small “x” indicating the U1 mutation, and the heavy “X” depicts the position of G tract mutations; mG1 through mG4 are the mutations shown in A, and mG1+2 and mG1+3 have the indicated mutations combined. The marker is a sample of nuclear extract RNA for the positions of U11 and U1 snRNA. The percentage of NRSu1 signal for U11 and U1 is shown beside each lane. U1 binding to mU1 was 40% of the wild-type NRS RNA. The data are representative of at least three independent experiments.

Fig. 6. Analysis of U11 binding to G tract 3′ deletion series. To the left are schematic representations of NRS G tract deletions (not to scale) with the 3′ end nucleotide noted, and a representative affinity selection experiment is on the right. The marker is a sample of nuclear extract RNA for the position of U11 snRNA. The percentage of NRS signal is shown beside each lane. The data are representative of three independent experiments.

Unexpectedly, a control oligonucleotide to U2 snRNP reduced U11 binding in a dose-dependent manner (lanes 8–11). This oligonucleotide is directed to nt 27–49, which are involved in binding to the branch point of splicing substrates. These data suggested an unexpected role for U2 snRNP in U11 binding to the NRS in vitro.

Deletion of the NRS Branchpoint-like/Polyprymidine Tract Sequence Decreases U2 and U11 Binding to the NRS in Vitro—U2 snRNP was shown by immunoprecipitation to interact with the NRS (26), presumably to a branchpoint-like/pyrimidine tract (BP/Py) region just upstream of the U1/U11 sites. This region resembles splicing signals associated with 3′ splice sites that are required for U2 binding, but the precise U2 binding site within the NRS has not been determined. To ask if these cis signals might mediate the U2 effect and if they are required for optimal U11 binding in vitro, the BP/Py tract region was deleted and U2 and U11 binding to the NRS was assayed. As shown in Fig. 9, U2 binding as assessed by affinity selection was weak but detectable on the wild-type NRS (lane 3), and the BP/Py deletion reduced U2 binding by ~60% (lane 4). The BP/Py deletion also reduced U11 binding to the level observed for the NRS lacking the G tracts (compare lanes 4 and 5), indicating that this region is also required for optimal U11 binding. Interestingly, U2 binding was also lower on the substrate lacking the G tracts (lane 5). These data suggest that the binding of U2 and U11 are mutually beneficial. In contrast to these results, however, the BP/Py deletion did not appear to affect U11-type splicing (and therefore, binding) from the NRS in vivo (data not shown, see below and “Discussion”).

G Tracts 1 and 2 Are Required for U12-dependent Splicing from the NRS in Vivo—To address whether the G element was required for U11 binding to the NRS in vivo, we took advantage of an earlier observation that splicing occurs efficiently and accurately from the NRS U11 site when an authentic U12-dependent 3′s is provided (30), and thus, NRS minor class splicing...
FIG. 9. Analysis of U2 and U11 snRNP binding to ΔBP/Py and NRS RNA lacking the G-rich region. At the top is the sequence of the branch point-like/pyrimidine tract region just upstream of the U1 (overlined) and U11 (underlined) binding sites. The region from nt 875 to 901 (between the arrows) was deleted in ΔBP/Py. A schematic (not to scale) of the RNAs is at the left, and a representative affinity selection experiment is shown on the right. M, nuclear extract RNA as a marker for U2 and U11 snRNA (positions of U2 and U11 are indicated on the right); non, non-biotinylated NRS RNA. The percentage of NRS signal for U2 and U11 is shown below each lane.

FIG. 10. G tract mutations diminish U12-dependent splicing from the NRS in vivo. A, schematic diagram of the RNAs expressed in cells. Open boxes represent P120 exons, and the black line depicts the P120 intron. Large hatched boxes represent NRS sequence upstream of the U11 site, and the smaller hatched box indicates sequences spliced out (intron). The coordinates of the NRS used in the chimera are shown. The large "X" depicts the U11-site mutation, and small "x" indicates mutations in the G tracts. B, analysis of minor class splicing from the NRS. The constructs in A were expressed in 293 cells, and RNA was analyzed by reverse transcription-PCR. PCR samples were obtained in the linear range of the reaction. mock is a PCR reaction from untransfected cells. The positions of products from unspliced (Un) and spliced (Sp) RNA are shown at the right. C, quantitation of the data in B.

DISCUSSION

The RSV NRS element is distinct from the viral splice sites yet functions to suppress splicing and promote polyadenylation. Considerable evidence suggests that binding of U1 snRNP to the NRS commits RNAs to the inhibition pathway whereby a non-functional spliceosome complex assembles on the NRS and an authentic 3’ss, which sequesters the 3’ss from the normal interaction with the authentic 5’ss (27). It is likely that inhibition takes place at steps subsequent to U1 binding and may involve U6 snRNP.5 This stalled splicing complex is also proposed to promote 3’ end processing at the distant RSV polyadenylation site (24). Although U11 snRNP appears to play no direct role in splicing inhibition (30, 31), its efficient binding to the NRS may regulate the degree to which the NRS functions and provides an interesting model to investigate novel cis and trans factors that contribute to U11 snRNP binding to 5’splice sites. In this report we have identified two regions that are required for optimal binding of U11 to the NRS in vitro: an upstream 3’ss-like sequence and a downstream G-rich element. The G-rich element also promotes U11 binding in vivo as assessed by U12-dependent splicing from the NRS.

The major and minor splicing pathways are remarkably similar, but there are important differences between the two (11). First, the 5’ss and BPS of the U12-dependent introns are highly conserved, which contrasts with U2-dependent introns whose 5’ss and BPS consensus sequences are not strictly adhered to. The minor class introns also lack a polypyrimidine tract between the BPS and 3’ss, which is a hallmark of major class introns (40). Additionally, the minor class snRNPs are of quite low abundance and, unlike U1 and U2 snRNPs of the major pathway, U11 and U12 exist as a di-snRNP and act

<sup>5</sup> M. T. McNally, unpublished data.
cooperatively in early steps of 5’ss and BPS recognition (16–18). Thus, U11/U12 binds poorly to isolated splice sites. It is therefore novel that the NRS binds U11 so well in the apparent absence of a standard U12-type BPS (UUCUUAAC). Consistent with previous reports (17), little U11 associated with authentic U11-type 5’ss substrates in an affinity selection assay where efficient binding is observed with the NRS. Although di-snRNP does interact with the NRS as exemplified by co-selection of 12 snRNPs (26) (data not shown), surprisingly, U11 binding to the NRS was not affected when U12 snRNA was destroyed, indicating that U11 can bind the NRS efficiently as a mono-snRNP. One possibility is that the high affinity SR protein binding sites in the 5’ half of the NRS and associated SR proteins account for the differences in U11 binding to the NRS and authentic substrates. It has been shown that SR proteins are required for in vitro splicing of minor class introns (12). However, the activity of a distinct cis element was indicated, because efficient U11 binding is not dependent upon the upstream SR protein binding sites (30).³

Our results show that a G-rich region just downstream of the U11 site contributes to efficient U11 binding. The 80-nt region exhibits a non-random, increased frequency of G residues and G triplets that roughly segregate into four blocks and whose deletion results in substantially decreased U11 binding. Curiously, the four G blocks are not functionally redundant. The first two G tracts are required for efficient U11 binding, but neither is sufficient, and blocks 3 and 4 cannot substitute even when moved to the position of blocks 1 and 2. Furthermore, the action of G tracts 1 and 2 does not appear to be additive, because mutation or deletion of either one reduces U11 binding to levels near that observed when the region is completely removed. It may be that both G1 and G2 must be occupied by a trans-acting factor, or alternatively, they may comprise a single element. It is not obvious what distinguishes between active and inactive G tracts. Additional mutagenesis studies will be required to understand the subtle differences that differentiate active G1 and G2 from inactive G3 and G4.

The decrease in U11 binding upon mutational inactivation of the G region does not appear to be an indirect effect of increasing the binding of U1 snRNP to the NRS, which competes with U11 for binding to an overlapping site. A more direct role of the G tracts in U11 binding is supported by the finding that U11 binding to the G-tract mutants was not restored by a mutation in the U1 binding site or by 2’-O-methyl oligonucleotide inactivation of U1 snRNP. Surprisingly, a control oligonucleotide to U2 snRNP decreased U11 binding in a dose-dependent manner. This is an interesting observation, because it was shown previously by immunoprecipitation that U2 interacts with the NRS (26), and upstream of the U1/U11 site is a sequence that resembles the rat calctinin-specific BPS followed by a pyrimidine tract that is a strong candidate for the U2 binding region (39, 41). Weak binding of U2 to the NRS was eliminated when the BP/Py tract was deleted. This suggests that U2 can associate with this region but additional experiments are required to confirm this possibility. The BP/Py tract also appears important for U11 binding, because its deletion reduced U11 binding to levels seen with substrates lacking the downstream G-rich region. These observations establish a correlation between the BP/Py sequence and U2 and U11 binding. One possibility is that pyrimidine tract-binding splicing factors bind the NRS BP/Py tract and assist downstream binding of U11 in a manner similar to exon definition interactions described for U2AF and U1 snRNP (42, 43). However, the observation that the U11 interaction was decreased by the U2 snRNA inactivating 2’-O-methyl oligonucleotide suggests a more direct role for U2 in U11 binding. U2 and U11 binding appears to be mutually beneficial, because the substrate lacking the G region and that binds U11 poorly also showed reduced U2 binding. It is possible that U2 and U11 interact through a bridging protein such as hPrp5, as has been proposed for cross-intron and/or exon interactions between U2 and U1 snRNPs (44). Alternatively, the G tracts might also influence U2 binding directly in this context. The influence of the G tract on U11 is also not restricted to in vitro binding. The U11 site in the NRS is normally not used, but splicing is activated when the minor class 3’ss from the P120 gene is provided (30). The NRS fragment used in the P120 chimera lacks G tracts 3 and 4, which is consistent with the in vitro observation that these motifs are not required for efficient U11 binding. Importantly, the same mutations that diminished U11 binding in vitro caused a marked decrease in minor class splicing from the NRS in vitro. A decrease was not observed when the upstream SR protein binding sites³ or the BP/Py-like region was removed (data not shown), indicating a direct effect of the G tracts on U11 binding. It is possible that the BP/Py region is required for optimal U11 binding to the isolated NRS but is dispensable in the presence of a minor class 3’ss.

A large number of cis elements have been identified that influence splicing efficiency in the major splicing system but that are distinct from the splice sites (e.g. intronic and exonic splicing enhancers and silencers) (9). In contrast, aside from the 5’ss and BPS, descriptions of regulatory cis elements have been few for U12-dependent introns, enhancers being the exception (13). Thus, identification of novel elements that promote U11 binding to the NRS may have implications for splicing of authentic minor class introns. Interestingly, like the NRS, intron sequences just downstream of the SCN4A U11 5’ss are rich in G residues and G triplets, although this is not a general feature of all U12-dependent introns (45). This raises the possibility that aspects of efficient U11 binding to the viral NRS element will be applicable to cellular U12-dependent genes and further our understanding of minor class splicing. Identification of factors that associate with the G-rich element to influence U11 binding is an important future goal.

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