Heterogeneous Nuclear Ribonucleoprotein H Is Required for Optimal U11 Small Nuclear Ribonucleoprotein Binding to a Retroviral RNA-processing Control Element

IMPLICATIONS FOR U12-DEPENDENT RNA SPlicing*

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An RNA-processing element from Rous sarcoma virus, the negative regulator of splicing (NRS), represses splicing to generate unspliced RNA that serves as mRNA and as genomic RNA for progeny virions and also promotes polyadenylation of the unspliced RNA. Integral to NRS function is the binding of U1 small nuclear ribonucleoprotein (snRNP), but its binding is controlled by U11 snRNP that binds to an overlapping site. U11 snRNP, the U1 counterpart for splicing of U12-dependent introns, binds the NRS remarkably well and requires G-rich elements just downstream of the consensus U11 binding site. We present evidence that heterogeneous nuclear ribonucleoprotein (hnRNP) H binds to the NRS G-rich elements and that hnRNP H is required for optimal U11 binding in vitro. It is further shown that hnRNP H (but not hnRNP F) can promote U11 binding and splicing from the NRS in vivo when tethered to the RNA as an MS2 fusion protein. Interestingly, 17% of the naturally occurring U12-dependent introns have at least two potential hnRNP H binding sites positioned similarly to the NRS. For two such introns from the SCN4A and P120 genes, we show that hnRNP H binds to each in a G-tract-dependent manner, that G-tract mutations strongly reduce splicing of minigene RNA, and that tethered hnRNP H restores splicing to mutant RNA. In support of this role, hnRNP H in both splicing pathways, hnRNP H antibodies co-precipitate U1 and U11 small nuclear ribonucleoproteins. These results indicate that hnRNP H is an auxiliary factor for U11 binding to the NRS and that, more generally, hnRNP H is a splicing factor for a subset of U12-dependent introns that harbor G-rich elements.

The genes of higher eukaryotes are normally interrupted by noncoding sequences (introns) that are transcribed into the primary transcript, generating a precursor-mRNA. These pre-RNAs are then matured through the process of RNA splicing whereby a large multicomponent machine termed the spliceosome recognizes and excises the introns (1). The vast majority of introns are recognized by a spliceosome that contains U1, U2, U4/U6, and U5 small nuclear ribonucleoproteins (snRNPs) and a large number of accessory factors (1). These are referred to as major class or U2-dependent introns. More recently, a lower abundance spliceosome was described that excises a rare class of introns (<1% of human introns) whose consensus sequences deviate from those of conventional introns (2–4). Interestingly, the minor class spliceosome (also called U12-dependent) contains functional analogs of four of the five snRNPs present in the major spliceosome (U11, U12, and U14atac/U6atac): U5 is utilized in both splicing pathways (5). Understanding the similarities and differences between the two splicing machines should lend insight into the mechanism of RNA splicing and evolutionary links between the two.

Despite the different snRNP compositions of the two spliceosomes, the assembly pathways and catalytic mechanisms are remarkably similar. Both pathways utilize a two-step mechanism in vitro that proceeds through a lariat intermediate, and each spliceosome assembles stepwise through the successive addition of snRNPs (1, 5, 6). U11 and U12 perform the early, analogous roles of U1 and U2 of the major pathway and interact by RNA base pairing with the 5′ss and branch point sequence, respectively, to form a prespliceosome (7–10). Subsequently, as in the major pathway, the U4atac/U6atac U5 tri-snRNP is integrated to form the mature spliceosome, at which time the extensive base pairing between U4atac and U6atac is disrupted and U6atac snRNA interacts with the 5′ss and with U12 snRNA to form the catalytic spliceosome (8, 11–14). A similar architecture between the active sites of the two spliceosomes was suggested by the functional replacement of a critical U6atac stem-loop with the analogous region from U6 (15). The model of stepwise assembly of spliceosomes is contrasted with the detection of a functional penta-snRNP in yeast extracts, which favors the action of a preassembled spliceosome (16, 17).

Further highlighting the similarity between the two spliceosomes, a surprisingly large number of proteins are shared between the two. For example, several subunits of SF3b, originally described as an essential U2-associated factor, are associated with the U11/U12 di-snRNP but not with the U11 mono-snRNP (18). Additionally, the protein composition of the U4/U6.U5 and U4atac/U6atac.U5 particles is very similar if not identical (19, 20). These observations support the notion that the splicing mechanism is conserved between the two systems, although splice site-bridging interactions are likely to be different (21). Despite the numerous similarities between the U2- and U12-dependent spliceosomes, there are significant differences. First, the 5′ss and branch point sequence consensus sequences are strictly adhered to in U12-dependent introns, and these introns lack the polypyrimidine tract that is an important feature of major class introns (3, 4). Furthermore, it has been shown that there are significant differences in the early events of splice site recognition. In the U2-dependent system, U1 snRNP can recognize 5′ splice sites as a mono-snRNP and initially interacts with SF1/mBBP and U2AF (probably bridged by SR proteins) at the far end of the intron. Subsequently, U2 is recruited, and a different set of interactions is...
formed between U1 and U2 (1). It was recently reported that hPrp5 is the bridging protein between U1 and U2 (22). This contrasts with the minor pathway in which U11 and U12 exist as a di-snRNP that binds to the 5'ss and branch point sequence simultaneously and cooperatively (23, 24). Presumably, protein components other than hPrp5 constitute the bridging factors, since hPrp5 was not found in the U11/U12 particle (21).

Although a large number of auxiliary cis sites and trans-acting factors are known to influence U1 binding to 5'ss splice sites and splice site choice (25), much less is known about factors that influence U11 binding to its respective 5'ss splice sites. Members of the SR protein family of splicing factors were shown to be required for basal and enhancer-stimulated minor-class splicing in vitro for two different substrates (26) and for enhancer effects on alternative splicing of model U12-dependent RNAs in vivo (27). An intronic purine-rich element was recently described in the Drosophila prospero "twintron" gene that influenced both the major and minor pathways; the trans-acting factor(s) that binds to and mediates the effect of the enhancer awaits identification (28).

An RNA-processing control element from Rous sarcoma virus (RSV), the negative regulator of splicing or NRS, harbors overlapping U1 and U11 binding sites and very efficiently binds U11 snRNP as well as U1 (29, 30). This element represses viral splicing and splicing of heterologous introns in vitro and in vivo (29, 31–33), and it is required for efficient viral RNA polyadenylation (34, 35). The NRS acts as a U1-type pseudo-5'ss that nonproductively interacts with and sequesters 3'ss splice sites from interacting with authentic 5'ss splice sites to accomplish splicing control (33, 36), and it is thought that the stalled splicesomelike complex that assembles on the NRS/3'ss promotes polyadenylation of unspliced RNA (34). U11 binding is thought to antagonize the activity of U1.

We have used the NRS as a tool to study novel aspects of U11 binding. It was recently shown that a G-rich sequence downstream of the U11 consensus site is required for optimal U11 binding to the NRS in vitro and in vivo (30). Here, we show that the G-rich element specifically interacts with hnRNP H, a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family of RNA-binding proteins (37). Mutations that abolished hnRNP H binding reduced U11 binding to the NRS in vitro and in vivo as assessed by U12-dependent splicing from the NRS U11 site. A direct role for hnRNP H in U11 binding to and splicing from the NRS was demonstrated in vitro with tethering assays using an MS2-hnRNP H fusion protein. Interestingly, a number of authentic U12-dependent introns harbor potential hnRNP H binding sites, and we demonstrate that hnRNP H binds to intronic sites in the SCN4A and P120 minor class introns, that mutations in the sites decrease hnRNP H binding and splicing of minigenes, and that tethered hnRNP H stimulates SCN4A and P120 minigene splicing. Collectively, our data support a broader role of hnRNP H as an accessory factor for splicing of a subclass of U12-dependent introns that harbor hnRNP H binding sites.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—RSV sequences are from the PrC strain (38) with sequence coordinates as described by Schwart et al. (39). Plasmids described previously are p3ZMSxma (to generate full-length NRS RNA, nt 701–1011) and the G-tract mutations made in this background and described in the legend to Fig. 1A (30), and pP120, pNRS−P120, and p67−P120 (40). Plasmids containing just the NRS G-rich region (wild-type p3ZG1−4 and mutants p3ZmG1, etc.) were generated from the mutation series plasmids in the p3ZMSxma background by digestion with KpnI and XmaI to remove upstream NRS sequences 701–927, and the vectors were recircularized. p3ZMS/MS2 was made by replacing the Xmalt-Xbal fragment containing the NRS G-rich region with a PCR fragment harboring two MS2 binding sites. All primer sequences are available upon request. A pNRS−P120 chimera harboring the NRS mG1+2 mutation was made by overlap PCR between p3Z/SMG1+2 NRS sequences and the P120 region from pNRS−P120; the P120 intronic Bsu36I site was also converted to XhoI. Another chimera, pNRS−MS2, had the XmaI−Xhol NRS G-tract fragment replaced by a PCR product containing two MS2 binding sites. pP120 plasmids harboring mutations in the G-tracts (Fig. 9) were generated by overlap PCR. The MS2 binding sites were inserted into pP120mG1−3 by introducing an Xhol site just downstream of mG3 by overlap PCR, and blunt end insertion of a PCR product harboring two MS2 binding sites. The pSP64−SCN4A plasmid was a gift of Adrian Krainer. pCMV−SCN4A was made by moving a HindIII−BamHI fragment from pSP64−SCN4A into the same sites in p3×FLAG−CMV7.1 (Sigma). Mutations in or deletion of the G-tracts of the SCN4A intron (Fig. 6) were made by overlap PCR. These were cloned as HindIII−BamHI fragments to generate pCMV−SCN4AmG1−3, pCMV−SCN4AmG4−6, pCMV−SCN4AmG1−6, and pCMV−SCN4AAG. To replace the G-tracts in pCMV−SCN4A with the MS2 binding sites, overlap PCR was used to remove the G-tracts and introduce MroI and Sall sites. An Xmalt−HindIII MS2 fragment from p3ZSMS/MS2 was then cloned into the MroI−Sall intron sites. An hnRNP H expression vector (pcDNA−H) was made by PCR-amplifying the hnRNP H coding region with Xhol and BamHI ends from pET15b−H (gift of Douglas Black) and insertion into the same sites of pcDNA3.1 (+)−myc−HisA (Invitrogen). To make pcDNA−F, which expresses hnRNP F, the hnRNP F reading frame from pET15b−F (from D. Black) was amplified by PCR and cloned as an Xhol−BamHI fragment into pcDNA. pcDNA−MS2 was made by inserting a PCR product containing the MS2 coat protein reading frame into the Xbal/Xhol sites of pcDNA. pcDNA−MS2−H and pcDNA−MS2−F were made by inserting the MS2 coat protein reading frame into the Xhol/Xbal sites in pcDNA−H and −F. pcDNA−MS2−GFp was made by replacing the hnRNP H portion of pcDNA−MS2−H with a GFP PCR product.

HeLa Cell Nuclear Extracts and Recombinant Protein—HeLa S3 cells were either grown in spinner flasks in RPMI 1640 medium containing 10% bovine calf serum to no more than 5 × 10^5 cells/ml or obtained from the National Cell Culture Center (Minneapolis, MN). Nuclear extracts were made as described (41). For depletions, a 65-nt RNA containing five high affinity hnRNP H binding sites from the rat β-tropomyosin gene (or the antisense RNA, which was used as a negative control) (42) was attached to adipic acid dihydrazide beads as described below. Alternatively, the 106-nt NRS G-tract RNA was used. The beads were added to 50 µl of nuclear extract, and the mixture was incubated for 15 min, and then the beads were sedimented, and two additional incubations with fresh beads were performed. The amount of hnRNP H remaining in extracts was determined by Western blot on an aliquot of extract; bead-associated hnRNP H was determined by Western blot after elution from the beads with SDS sample buffer and SDS-PAGE. Recombinant hnRNP H was generated in Escherichia coli as described (43).

UV Cross-linking/Immunoprecipitation—For UV cross-linking, plasmids harboring the wild-type and mutant NRS G-tracts (indicated in Fig. 3) were linearized with Xbal and transcribed in vitro with T7 RNA polymerase and [α-32P]UTP (44). The 106-nt-long RNAs contained 84 nt of RSV sequence (nt 928−1011) and 22 nt of vector sequence. SCN4A constructs were linearized with BamHI and transcribed into 374-nt RNAs with Sp6 RNA polymerase. pP120 constructs were linearized with EcoRI and transcribed into 518-nt RNAs with Sp6 RNA polymerase. Non-specific RNA was generated from Prull-digested pGEM-3Z
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DNA and Sp6 RNA polymerase. Approximately 200,000 cpm of RNA was incubated in HeLa nuclear extract for 20 min under splicing conditions (44), and the samples were irradiated with 260 nm light for 30 min at 4 °C, treated with 0.6 mg/ml RNase A, and subjected to SDS-PAGE and autoradiography. For the UV cross-link/immunoprecipitation, 10 µl of Protein A-Sepharose beads prebound with affinity-purified anti-hnRNP H antibodies or preimmune serum was added to standard cross-linking reactions and rotated for 1 h at 4 °C. After three washes in 0.5 ml of immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100), proteins were eluted in SDS sample buffer and subjected to SDS-PAGE and autoradiography. Affinity-purified, polyclonal rabbit anti-hnRNP H antibodies were generated by Bethyl Laboratories against a C-terminal peptide of hnRNP H (45).

For snRNP co-precipitation, Protein A-Sepharose beads were incubated with buffer, preimmune serum, or hnRNP H antibodies, and 10 µl of beads were added to 50 µl of nuclear extract under splicing conditions for 1 h. Samples were washed as above, treated with Proteinase K to release nucleic acids, and then phenol-extracted and subjected to Northern blot analysis using the polyclonal antibody to hnRNP H.

Western Blots—Proteins eluted from affinity selection beads in 1× SDS buffer or aliquots of nuclear extract mixed with 2× SDS buffer were boiled and subjected to electrophoresis in a 10% SDS-polyacrylamide gel followed by electrotransfer to nitrocellulose. The filters were blocked in 5% dry milk in TBST (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20), probed with anti-hnRNP H antibody, and, after several washes in TBST, incubated with a Protein A-horseradish peroxidase conjugate, and finally developed by chemiluminescence (Pierce Supersignal) by exposure to x-ray film or capture on a ChemiImager digital imager (Alpha Innotech).

Transfection and RNA Analysis—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 µg of DNA by the calcium phosphate method (Amersham Biosciences), and total RNA was isolated with Qiagen RNAeasy columns according to the manufacturer’s instructions. RT-PCR of P120 chimeras was performed as described (40), and product levels in the linear range were quantitated using an Alphalager (Alpha Innotech). For pCMV-SCN4A-based plasmids, reverse transcription was oligo(dT)-primed, and PCR was done as above with sense (CGGGTATTATAAAGATC) and antisense (GGCAACTTCCAGGGCCAGG) primers directed against transcribed vector sequences.

RESULTS

A ~55-kDa Protein Specifically Cross-links to the NRS G-tracts—U11 is a low abundance minor class splicing factor and binds to the RSV NRS element remarkably well (29, 30). It was recently shown that efficient U11 binding to the NRS in vitro and in vivo is dependent upon a G-rich region just downstream of the U11 consensus binding site (30). Mutations in G-tracts 1 and 2 substantially reduce U11 binding, whereas mutations mG3 and mG4 have little to no effect. The sequence of the G-rich region and mutations is shown in Fig. 1A. In an effort to identify trans-acting factors that might bind to the G-rich element and mediate its effect on U11 binding, we employed a UV cross-linking assay using HeLa nuclear extract and 32P-labeled G-tract RNA and utilized the G-tract mutants as specificity controls.
As shown in Fig. 1B, cross-linking with the wild-type 106-nt RNA representing only the G-tracts (84 nt of NRS plus vector sequences) produced an intense doublet or triplet of bands at \( \sim 55 \) kDa and a smaller band at \( \sim 36 \) kDa (lane 3); these bands were light or absent when a nonspecific RNA was used (lane 12) and were not seen in the absence of cross-linking or nuclear extract (lanes 1 and 2). The intensity of the upper 55-kDa band was reduced by mutations mG1 and mG2 (lanes 4 and 5), which also reduced U11 binding to the NRS (30), and the upper cross-link was also abrogated when mutations mG1 or mG2 were combined with mutations in G-tracts 3 and 4 (lanes 8–11). Cross-linking of the other \( \sim 55 \)-kDa bands was not consistently affected by the G-tract mutations. Thus, these experiments established a correlation between U11 binding and cross-linking of a \( \sim 55 \)-kDa protein to the G-tracts that are required for optimal U11 binding. Cross-linking of the \( \sim 36 \)-kDa band did not correlate with U11 binding (e.g. an efficient cross-link is observed with the mG1+2 mutant (lane 8)).

**hnRNP H Binds the NRS G-tracts**—To identify the factor(s) responsible for the specific G-tract cross-link, we covalently attached to beads and incubated in nuclear extract, and bound proteins were eluted, subjected to SDS-PAGE, and visualized by Coomassie stain. M, markers; 3Z, nonspecific RNA. The arrowheads indicate the position of the \( \sim 50 \)-kDa band that was identified by mass spectrometry as hnRNP H and whose binding to the mutants is diminished. The asterisks denote two proteins that are specific to the G-tracts but do not respond to the mutations (identified by mass spectrometry as hnRNP A2/B1 and hnRNP A1). The sizes of the markers are shown at the left. B, anti-hnRNP H Western blot of bound proteins. An RNA affinity selection was performed as above, and eluted proteins were subjected to Western blot analysis with an anti-hnRNP H antibody. NE, an aliquot of nuclear extract; G1–4, mG1, mG1+3, and 3Z, samples eluted from RNA affinity beads.

50-kDa protein was selected less well with the mutants that showed diminished UV cross-linking and reduced U11 binding to the NRS. This protein was identified as hnRNP H.

The conclusion that hnRNP H binds the G-tract RNA and is sensitive to the mutations is supported by Western blots of affinity-selected proteins probed with an hnRNP H antibody, which showed a similar decrease in the amount of hnRNP H selected with the mutants (Fig. 2B). hnRNP H binding was also observed with the nonspecific RNA, but to a lesser degree than the wild-type or mutant G-tract RNA. The conclusion was further supported by experiments in which immunoprecipitation of UV cross-linking reactions with an anti-hnRNP H antibody precipitated the upper of the 55-kDa cross-links (Fig. 3, lane 2), confirming that hnRNP H binds the G-tract RNA and is responsible for the upper, specific cross-link. In further agreement with Fig. 2, immunoprecipitation of the cross-link was reduced to that observed with nonspecific RNA when NRS RNAs contained mutations in G-tract 1 or 2 but not mutants mG3 or mG4 (lanes 3–8). The precipitation was specific, since it was not observed in the absence of antibody or with preimmune serum (lanes 11 and 12), and no band was observed in the absence of extract or UV cross-linking (lanes 9 and 10).

**hnRNP H Promotes U11 Binding to the NRS in Vivo**—Having established a correlation between hnRNP H binding to the G-tract region and U11 binding, we performed depletion/add-back assays to demonstrate a requirement of hnRNP H for U11 binding to the NRS in vivo. Depletion was accomplished by passing nuclear extract three times over an RNA affinity column containing five iterations of the hnRNP H binding site from the rat \( \beta \)-tropomyosin gene (42, 48). Depletion was determined to be \( \sim 80\% \) as judged by Western blotting (Fig. 4A, lanes 2–7). A control RNA representing the antisense of the \( \beta \)-tropomyosin did not deplete (lanes 8–10). U11 binding to the full NRS was then assessed in control and depleted extracts with an affinity selection assay; biotinylated NRS RNA was incubated in extract to allow snRNP binding, RNAs selected on streptavidin beads were washed extensively, and snRNA was extracted and detected by Northern blot with a U11 probe. As shown in Fig. 4B, binding of U11 to NRS RNA was similar in nuclear extract and the control-depleted extract (lanes 2 and 3). In two independent hnRNP H depletions, U11 binding decreased \( \sim 40 \) or \( \sim 60\% \) (lanes 4 and 6), and
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FIGURE 4. hnRNP H promotes U11 snRNP binding to the NRS in vitro. A, depletion of nuclear extract of hnRNP H. Nuclear extract was treated three times in succession with beads that were covalently attached to RNAs containing either five high affinity binding sites for hnRNP H (H), the antisense of this sequence (AS), or the G1–G4 RNA. Equivalent aliquots of extract were reserved at each step and subjected to Western blot analysis with an hnRNP H antibody. Where indicated (+/−), 200 ng of recombinant hnRNP H was added to depleted extracts. snRNA associated with bound snRNPs was released by proteinase K digestion, phenol-extracted, and precipitated and then resolved on an 8M urea, 8%

FIGURE 5. hnRNP H is required for optimal U11 splicing from the NRS in vivo. A, schematic of the NRS/P120 minigenes used in transfections (40). Open boxes, P120 exons; thin line, P120 intron (not to scale). Shaded boxes, NRS sequences upstream of the U11 site; thin shaded boxes are NRS sequences downstream of the U11 site. The single X represents a mutation in the NRS U11 site, and the double X indicates the mG1 + 2 mutation. The stem-loop structures represent the two binding sites for the MS2 coat protein. B, RT-PCR analysis for splicing of minigenes. 293 cells were transfected with the indicated minigenes or cotransfected with the NRS-MS2 construct and expression vectors for free hnRNP H or for fusion proteins of the MS2 protein to GFP, hnRNP H, or hnRNP F. RNA was isolated and subjected to RT-PCR. The products were resolved on an agarose gel, and a representative experiment is shown. C, quantitation of the data from three independent experiments.

upon the addition of 200 ng of recombinant hnRNP H, U11 binding was restored to 75 and 60%, respectively (lanes 5 and 7).

Nuclear extract was also depleted with the NRS G-tract region, since hnRNP H appears to bind this RNA well. Western blots showed that depletion was as good as if not better than depletion with the β-tropomyosin RNA (Fig. 4A, bottom). U11 binding was decreased ~60% in extract depleted with the G-rich element, and the addition of recombinant hnRNP H restored binding to 70% (Fig. 4B, lanes 8 and 9). Thus, depletion with two different RNAs containing binding sites for hnRNP H decreased U11 binding to the NRS, and recombinant protein partially reversed this effect. Collectively, these results indicate that hnRNP H contributes to U11 binding to the NRS in vitro.

hnRNP H Promotes Splicing from the NRS in Vivo—The data above indicated that hnRNP H binding to the NRS G-rich region is required for optimal U11 binding in vitro. We previously showed that the G-tracks also contribute to U11 binding and function in vivo by taking advantage of a previous finding that splicing occurs from the NRS U11 site when the P120 U12-dependent 3′ss is provided (Fig. 5A) (40); G-track mutations that diminished hnRNP H and U11 binding in vitro likewise decreased splicing from the NRS in vivo (30). This system serves as a reporter for U11 binding to the NRS. As observed previously, splicing of the NRS-P120 chimeric RNA was as efficient as the authentic P120 minigene RNA (Fig. 5B, lanes 2 and 3; quantification in Fig. 5C), and splicing was by the U12-dependent pathway, since a mutation in the U11 site virtually abolished splicing (Fig. 5C) (40). Also as reported, the mG1 + 2 mutation substantially reduced NRS splicing (lane 4) (30).

To determine whether the effect of the mutations on U11 binding and splicing from the NRS was mediated by hnRNP H, we substituted the NRS G-tracks in the P120 chimera with two binding sites for the MS2 coat pro-
tein (Fig. 5A) and tethered hnRNPH to this RNA in vivo as an MS2-hnRNPH fusion protein. It was expected that splicing of the NRS-MS2 RNA would be poor but that recruitment of hnRNPH to the substrate RNA via MS2 would restore splicing. RNA in which the NRS-G-tracts were substituted with the MS2 binding sites showed very little splicing, and expression of nonfused hnRNPH or an MS2-GFP fusion protein had little effect (lanes 5–7). However, splicing was restored to ~30% when the MS2-hnRNPH fusion protein was expressed, and this effect was not observed when a closely related protein, hnRNPF, was tethered to the RNA (lanes 8 and 9). This shows that the restoration of splicing is specific to hnRNPH tethering and provides strong evidence that hnRNPH can promote U11 binding to the ensuing splicing event.
G-rich Elements in the SCN4A U12-dependent Intron Are Required for Efficient Minigene Splicing—Having shown that hnRNP H promotes U11 binding to the U11-type 5’ss in the NRS, we considered whether hnRNP H might have a more general role in U11 binding to and splicing of authentic U12-dependent introns. Interestingly, inspection of intron sequences near and downstream of U11 sites in a data base of human U12-dependent introns developed by Levine and Durbin (2) revealed a significant number of introns with at least two potential hnRNP H binding sites (GGGN) and several with four or more (Table 1). In most instances, the homologous introns in mice also have GGGN elements clustered near the 5’ss, although the intron sequence and G element number and position vary (data not shown).

A particularly attractive candidate for involvement of hnRNP H is the U12-dependent intron present in the SCN4A gene. Similar to the NRS, this intron has six potential hnRNP H binding sites within 52 nt of the U11 consensus site (Fig. 6A, sites G1–G6). To determine if these sites were required for optimal splicing of this intron, we constructed wild-type and mutant SCN4A minigenes that contained mutations in putative hnRNP H binding sites 1–3, 4–6, and 1–6 or lacked this region (Fig. 6). These constructs were expressed in 293 cells, and splicing was assayed by RT-PCR. Over 85% of the wild-type RNA was spliced correctly (Fig. 6C, lane 2, and Fig. 6D), but mutations in the first three sites (G1–G3) reduced splicing by ∼35% (lane 3). The G4–G6 mutation had a less pronounced but significant effect, and mutation of all six sites was roughly additive; deletion of the entire G-rich region had the most severe effect (lanes 4–6). Thus, as with the NRS, mutation of putative hnRNP H binding sites had a detrimental effect on SCN4A splicing.

hnRNP H Binds SCN4A and P120 RNAs in Vivo and Promotes Minigene Splicing in Vivo—We next used the UV cross-linking/immunoprecipitation assay to determine whether hnRNP H binds SCN4A RNA in a G element-dependent manner in vitro. Again, an intense cross-link at ∼55 kDa was observed with the SCN4A substrate (Fig. 7, lane 1) that could be specifically and efficiently immunoprecipitated with anti-hnRNP H antibodies (lane 2). The immunoprecipitated cross-link was diminished by mutants mG1–3 and mG4–6 (lanes 3 and 4; ∼70 and 55% of wild type, respectively), and the effect of the combined mutations was additive (mG1–6, lane 5; ∼25% of wild type). Significantly, the diminution of the cross-link roughly correlated with the severity of the splicing defect in vivo.

To more directly implicate hnRNP H in SCN4A splicing, we replaced the hnRNP H binding sites in the SCN4A intron with MS2 binding sites (Fig. 8A) and expressed this RNA in 293 cells or coexpressed it with either unfused hnRNP H or the MS2-hnRNP H fusion protein and determined splicing efficiency by RT-PCR. As shown in Fig. 8B and quantitated in Fig. 8C, splicing of the SCN4A-MS2 RNA was virtually eliminated when the G elements were replaced by MS2 binding sites (Fig. 8B, lanes 2 and 3), and expression of MS2, MS2-GFP, or free hnRNP H had little effect (Fig. 8, B (lanes 4 and 5) and C). Significantly, expression of the MS2-hnRNP H fusion, but not MS2-hnRNP F,
FIGURE 8. hnRNP H promotes splicing of the SCN4A U12-dependent intron when tethered to RNA. A, schematic of the SCN4A minigene used to tether hnRNP H. Open boxes, SCN4A exons; thin line, the SCN4A intron (not to scale). The stem-loop structures represent the high affinity binding sites for the MS2 coat protein. B, RT-PCR analysis of minigene splicing. Wild-type and SCN4A-MS2 minigenes were transfected into 293 cells or cotransfected with the indicated expression vectors, and RNA was subjected to RT-PCR. M, size markers. Products were resolved on an agarose gel, and representative results are shown. Bands corresponding to unspliced (Un) and spliced (Sp) RNA are indicated to the right. C, quantitation of the data from three independent experiments.

resulted in a 4-fold increase in SCN4A splicing over control (lanes 6 and 7). These data indicate that hnRNP H is an auxiliary factor for splicing of the SCN4A U12-dependent intron.

To ensure that the effects observed with SCN4A were not unique to this transcript, similar analyses were performed with the P120 U12-dependent intron, which harbors three G elements predicted to bind hnRNP H (Fig. 9A). hnRNP H binding to the full transcript (diagrammed in Fig. 9B) was detected in vitro by UV cross-linking/immunoprecipitation (Fig. 9C, lanes 1 and 8), and mutation of all three or G-tracts 2 and 3 decreased the hnRNP H cross-link; no effect was observed when the proximal G triplet was mutated (lanes 2–4 and 9–11). The cross-linking data also correlated with in vivo splicing efficiency of the various minigenes (Fig. 9, D and F). Mutation of the first G triplet, which at +12 relative to the splice junction is positioned closer to the splice junction than those for the NRS or SCN4A (+16 and +17), caused a mild but reproducible increase in splicing (lane 2 versus lane 3), whereas mutants mG2–3 or mG1–3 both showed considerably less splicing (lanes 4 and 5). Thus, G1 may be inappropriately close to the 5’ss and repress splicing, whereas G2 and/or G3 are required for optimal splicing.

To implicate hnRNP H directly in P120 splicing, the MS2 binding sites were incorporated just downstream of the third G triplet in the mG1–3 minigene, and various proteins were coexpressed with the RNA. As shown in Fig. 9, E and F, the MS2-containing substrate was very poorly spliced, and expression of hnRNPH and F or MS2-hnRNPF and MS2-GFP had little or no effect. However, expression of the MS2-hnRNPH fusion restored splicing to wild-type levels or better. Collectively, these data show that hnRNP H is also a splicing factor for the P120 U12-dependent intron. The presence of at least two potential hnRNPH binding sites close to the 5’ss in numerous other U12-dependent introns (Table 1) suggests the possibility that hnRNP H plays a more general role in splicing these introns.

U11 and U1 snRNPs Co-precipitate with hnRNP H—Having shown that hnRNP H is required for optimal splicing of the NRS, SCN4A, and P120 minigenes and promotes binding of U11 snRNP to the NRS, we considered the possibility that hnRNP H might do so through an interaction with U11 snRNP. This was addressed in a co-immunoprecipitation assay whereby antibodies to hnRNP H were added to nuclear extracts and RNA was extracted from co-precipitating material and subjected to Northern blotting with a U11 probe. As shown in Fig. 10, U11 was specifically precipitated (lane 3), although a background was consistently observed with preimmune serum but not beads alone (lanes 4 and 2, respectively). Because it was reported previously that hnRNP H is required for a U1 snRNP association with an HIV splicing enhancer (49) and, more recently, that hnRNP H is required for splicing of some U2-dependent introns (50, 51), we also probed the blot for U1 snRNA to determine whether U1 is also associated with hnRNP H. Again, this was the case. The difference in levels of coprecipitating U11 and U1 snRNPs probably reflects their relative abundance in nuclear extracts; U11 is ~100 times less abundant than U1 (52). Thus, hnRNP H associates, directly or indirectly, with both U1 and U11 snRNPs in vitro, which is compatible with a role in U11 binding to the NRS and U12-dependent splicing, and in U1 recruitment to the HIV splicing enhancer and U2-dependent splicing.

DISCUSSION

The NRS is thought to block splicing and promote polyadenylation in RSV by acting as a pseudo-5’ss that interacts with and sequesters the 3’ss splice sites in an abortive splicesome, and it is this complex that promotes efficient polyadenylation of the unspliced viral RNA (34). Binding of U1 snRNP to the NRS is required to send transcripts into the inhibition pathway, but this event can be antagonized by U11 snRNP, which binds remarkably well to an overlapping site (30, 40, 53). U1 binding to its suboptimal site is promoted by a strong upstream enhancer that binds members of the SR protein family of splicing factors, but this region is not required for U11 binding and cannot explain efficient U11 binding (40, 44, 47, 54). U11 would appear to be at a considerable competitive disadvantage, since it is ~100-fold less abundant than U1 (52). Thus, it might be expected that auxiliary elements exist to augment U11 binding. Two such elements in RSV were recently described: an upstream branch point/pyrimidine tract-like sequence and a G-rich tract downstream (30). We present evidence here that hnRNP H binds the G-tracts near the NRS and promotes U11 binding and splicing and extend this observation to two authentic U12-dependent introns from the SCN4A and P120 genes.

G-rich sequences like those found downstream of the NRS U11 site have been identified in a number of U2-dependent introns that are regulated by the hnRNP H family of proteins (42, 45, 48–51, 55–57). It was shown that all members of the hnRNP H/F family bind to GGGA but that only hnRNP H/H’ interact with GGGC, but other permutations for the last position were not analyzed (48). The functionally relevant sites in the NRS that are important for U11 binding contain GGGC, GGGA, and two GGGUs. The most obvious difference in protein binding between wild-type and nonfunctional NRS G-tracts was a ~50-kDa protein identified as hnRNP H, which was supported by Western blotting and cross-linking/immunoprecipitation. A doublet of proteins identified as the hnRNP A2/B1 and A1 proteins was also observed to be specific to the NRS G-tracts, but the binding of these proteins was unaffected by G-tract mutations. Whereas this rules out a role for their binding through the G elements, negative regulatory roles for hnRNP A1 in splicing in other systems warrant future work to estab-
lish a possible influence of hnRNP A1 in snRNP binding to the NRS. We did not detect by RNA affinity selection strong binding of any protein with a size consistent with hnRNP F, suggesting that this protein does not play a prominent role in U11 binding to the NRS. This observation contrasts with other systems where hnRNP H and F both associate with G elements and in some cases are functionally interchangeable (45, 50, 51, 58).

An hnRNP H binding site within the upstream SR protein-binding region of the NRS was previously described, but there was no clear impact of mutations in this site on NRS activity (34, 43). This is probably because of the additional strong hnRNP H sites described here. Mutations in the NRS G elements strongly reduced hnRNP H and U11 binding in vitro and splicing from the NRS in vivo. The observations that extracts depleted of hnRNP H show reduced U11 binding to the NRS, that add-back partially reverses this effect, and that artificially tethering hnRNP H to NRS-splicing substrates activates splicing in vivo collectively provide strong support for the conclusion that hnRNP H is the primary factor involved in G-tract-dependent U11 binding to the NRS. An augmenting role for the upstream hnRNP H site remains to be addressed. U11 does not bind well to SCN4A or P120 substrates in vitro (30), so direct U11 binding could not be assessed, but the strong correlation between hnRNP H binding and splicing, coupled with the tethering results, suggests that hnRNP H functions similarly in promoting U11 binding to these RNAs.

**FIGURE 9.** hnRNP H promotes splicing of the P120 U12-dependent intron. A, sequence of the P120 U11-type 5′ is (overlined) through 60 nt of intron, showing putative hnRNP H binding sites (underlined and labeled G1–G3). Mutations introduced into G1–G3 are shown above the sequence. B, schematic of wild-type and mutant P120 splicing cassettes. The lengths of exons and introns are indicated. X mutations in G1–G3 and stem-loop structures represent MS2 coat protein binding sites. C, hnRNP H cross-linking is sensitive to G-tract mutations. Full-length RNAs containing the indicated mutations were incubated in nuclear extract and subjected to UV cross-linking/immunoprecipitation as in Fig. 3. Total Xlink, lanes loaded with 20% of a total cross-linking reaction (lanes 1–7). HnRNP H IP, lanes containing immunoprecipitates. 3Z, reaction lacking nuclear extract; −XL, reaction with cross-linking step omitted. Pre, precipitations using preimmune serum. WT, wild type. The arrow at the left indicates the position of hnRNP H. D, G-tracts are required for optimal splicing. RT-PCR analysis of minigenes splicing. Minigenes depicted in A were transfected into 293 cells, and RNA was subjected to RT-PCR. Products were resolved on an agarose gel, and representative results are shown. The positions of bands representing unspliced and spliced RNA are indicated at the right. E, hnRNP H tethering rescues splicing. 293 cells were mock-transfected or transfected with the mG1–3 vector or cotransfected with the mG1–3/MS2 RNA plasmid and the indicated protein expression vectors. Total RNA was subjected to RT-PCR as in D. H, hnRNP H; F, hnRNP F. The positions of bands representing unspliced and spliced RNA are indicated at the right. The data are quantitated in F.

![hnRNP H and U12-dependent Splicing](image-url)
G-rich sequences and hnRNP H have been implicated in positive and negative splicing regulation in other settings. Cases of negative regulation include the alternative exclusion of the rat β-tropomyosin exon 7 in nonmuscle cells through hnRNP H binding to an exonic splicing silencer (ESS) (42), and hnRNP H binding correlates with the activity of an ESS in HIV (55). It was also reported that an hnRNP H site coincident with the 5′ss of NF-1 exon 3 exacerbates the effects of splice site mutations (59). Many short introns harbor G triplets that promote exon inclusion and enforce intron boundaries (60). One of the first demonstrations of positive roles for hnRNP H and F in splicing control was in the c-src gene where, in conjunction with G-rich binding sites, they are part of a multicomponent splicing enhancer that is required for inclusion of the NI exon in neuronal cells (45, 58). Also, hnRNP H is a functional component of an exonic splicing enhancer located in exon 6D in the Bcl-xs gene (51), whereas the G element abuts the U1 5′ss in the SCN4A 2 intron (49, 57). The position of the G-tracts in exons or introns and the existence of G-tract-dependent manner, the G elements are required for optimal binding to exonic UAGG and 5′ss-proximal GGGG motifs. The GGGG motifs themselves possess only mild CI suppression activity, and in general, exons with a GGGG motif in positions 3–10 are skipped, whereas skipping is not statistically significant when the motif is at nt 11–20 of the intron (and these sites may be helpful). The first G-tract in the NRS and SCN4A is at intron position 16 and 17, respectively, relative to the U11 5′ss. The first site in P120, however, is at position 12, and it was found that mutation of this site improved splicing. Thus, there may be a subtle distance constraint that determines positive or negative activity of hnRNP H. Consistent with this, rescue of splicing by MS2-hnRNP H was relatively inefficient for the NRS and SCN4A, where the MS2 binding sites were positioned quite close to the 5′ss (+13 and +15), but rescue was complete for P120, where the MS2 sites were at +67 in the intron. A number of other U12-dependent introns also have 5′ss-proximal G elements (Table 1). Thus, small differences in position of hnRNP H binding sites in both splicing pathways can have differential effects, and splicing of U12-dependent introns may be similarly repressed when the sites are proximal to the U11 5′ss.

Immunohistochemical analyses in normal and tumor cells showed differential expression patterns for hnRNP H (and F) in various tissues and differences in nuclear/cytoplasmic localization (61). There is also evidence that hnRNP H is post-translationally modified by phosphorylation, which could affect its function (62). These observations suggest the possibility that splicing of U12-dependent introns containing G-tracts might be subject to regulation by hnRNP proteins. This possibility and the mechanism by which hnRNP H influences U11 (and U1) snRNP binding and splicing await further experimentation.

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