In Vivo Expression of a Variant Human U6 RNA from a Unique, Internal Promoter†

Jay W. Tichelaar,‡,§ Eric D. Wieben,*,‡ Ram Reddy,|| Anne Vrabel,‡ and Patricia Camacho‡,⊥

Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905, and Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

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ABSTRACT: We previously isolated a variant of the human U6 small nuclear RNA gene (87U6) and demonstrated that transcription of this gene is controlled by a novel internal promoter. It has now been shown that two blocks of sequence within the coding region are both necessary and sufficient to direct expression of 87U6 in transcription assays performed in vitro. In addition, 87U6 is expressed in vivo and can assemble into snRNP complexes. Specific primer extension assays on total RNA from HeLa cells shows that 87U6 RNA is present in these cells. Also, microinjection of plasmid encoded 87U6 genes into *Xenopus laevis* oocyte nuclei results in the expression of this variant RNA. Immunoprecipitation with anti-Sm antibodies suggests that 87U6 RNA assembles into a snRNP particle with U4 snRNA. Finally, the variant snRNA is capped with the U6 specific γ-monomethyl phosphate cap when incubated in HeLa extracts. These data suggest that 87U6 RNA may function in the splicing process, in a manner similar to the wild-type U6 RNA. The recent observations of a minor class of mRNA introns that are spliced by a distinct collection of snRNP particles suggest an important role for variant snRNAs in the splicing of transcripts with alternative splice junctions.

The removal of introns from the primary transcripts of mRNAs is critical for the proper function of the eukaryotic cell. This process is carried out by the spliceosome, a large macromolecular complex composed of splicing factors and five small nuclear ribonucleoprotein particles (snRNPs or "snurps") assembled on a precursor mRNA. Each snRNP contains the unique RNA for which it is named (U1, U2, U4, U5, and U6), as well as common and specific snRNP proteins. The mechanisms involved in intron removal have now been described in some detail (for review see refs *1* and *2*). It is now thought that an RNA component of the spliceosome catalyzes the splicing reaction in the nucleus, and U6 RNA is thought to be a critical component of the active spliceosome (*3*−*6*).

In light of the central role that the snRNAs play in the splicing reaction, the observation that variant snRNAs are expressed in cells is of great interest. In humans, expressed variants of U1 (*7*, *8*) and U5 (*9*) have been described. Sequence variants of U2 and U4 snRNA, as well as U1 and U5, have also been described in other species (*10*−*12*), and in some cases the expression patterns have been well characterized (*13*−*16*). The role of these variant RNAs in cellular physiology remains unclear, but the possibility that they have roles in alternative splicing events has been noted (*7*, *9*). More recently variant U4 and U6 RNAs have been shown to be required for the splicing of rare AT-AC introns (*17*−*19*). U4 atac and U6 atac RNAs are present in HeLa cells at only 1/100 the quantity of U4 and U6 RNAs, and they exhibit only 40% sequence identity with their more abundant counterparts (*18*).

We have previously described a naturally occurring sequence variant of the human U6 gene (*20*) and shown that it is controlled by a unique promoter (*21*). This RNA differs from the consensus mammalian U6 RNA at 10 of 107 residues, with none of the changes occurring at positions that are perfectly conserved throughout evolution (*22*). In this study we present evidence that this variant gene is expressed in vivo, and further elucidate the internal sequences required for the transcription of this gene. We also show that 87U6 RNA is capable of assembling into Sm-precipitable snRNP complexes with an efficiency similar to the consensus U6 RNA. In addition, both naturally occurring alleles of this RNA (*21*) are capable of being capped with the unique γ-monomethyl phosphate cap of U6. These results suggest that 87U6 may participate in the splicing reaction in a manner similar to the consensus U6 RNA. These observations are especially intriguing in light of the likely catalytic function of U6 RNA and the fact that 87U6 is under the control of a distinct promoter.

MATERIALS AND METHODS

Plasmid Constructs and in Vitro Transcription Assay. The minimal promoter constructs were made by annealing mutually priming, overlapping oligonucleotides spanning the entire length of the coding region and filling in the overhangs with T4 DNA polymerase as described previously (*21*).

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* To whom correspondence should be addressed.

‡ Mayo Clinic.

§ Present address: Division of Pulmonary Biology, Children's Hospital Research Foundation, Cincinnati, OH 45215.

|| Baylor College of Medicine.

⊥ Present address: Department of Physiology, University of Texas Health Science Center, San Antonio, TX.

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Analysis of the transcription of these constructs was carried out in HeLa S100 extract as described previously (*20*). Synthetic transcripts for the control primer extensions were generated by standard in vitro transcription procedures on linearized plasmid templates with SP6 or T7 RNA polymerase. Densitometry was carried out on a Shimadzu CS9000U dual wavelength flying spot scanner in transmission/absorbance mode at 550 nm.

Isolation of HeLa Cell RNA and Primer Extensions. HeLa cell RNA was prepared as described previously (*7*). For primer extensions, 30 μg of input cellular RNA was annealed to an end labeled oligonucleotide ($\sim 2 \times 10^6$ cpm). In the 87U6 primer extensions, the 17-mer oligonucleotide was complementary to the sequence AT.AGCACAGTCCCCGCG. In the wtU6 primer extensions, the primer was a 17-mer complementary to the sequence TTAGCATGGCCCCTGCG. Annealing reactions in hybridization buffer (400 mM NaCl, 66 mM PIPES pH 6.6, and 1 mM EDTA in 80% formamide) were incubated at 43 °C for 6−12 h, followed by ethanol precipitation. First strand synthesis was carried out in extension buffer (100 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$, 12 mM KCl), 25 mM each dNTP mix, 15 mM DTT, 400 μg/mL actinomycin D, and 4 to 5 units of MMLV reverse transcriptase. The reverse transcriptase reaction was incubated at 45 °C for 1 h, terminated by the addition of EDTA pH 7.0 to 20 mM, and ethanol precipitated. Pellets were dried and dissolved in 5 μL of water, 20 μL of deionized formamide, and 1 μL of loading dye. The control primer extensions (Figure 3) were carried out similarly except that 50 ng of input synthetic RNA transcript and 5 μg of carrier tRNA were used. In the competition reactions, 20 pmol of unlabeled WtU6 primer was added during the annealing step. The extension products were resolved by gel electrophoresis on 10% polyacrylamide gels containing 8.3 M urea.

Xenopus Oocyte Nuclear Injections. Mature *Xenopus laevis* oocytes were obtained as previously described (*23*). Nuclei from the oocytes were isolated under oil as described (*24*, *25*). Equimolar amounts of plasmid (125 nM = 250 μg/mL for a 3 kb plasmid) were injected with 2 mCi/mL of [^{32}P]-GTP (650 Ci/mmol) in oocyte intracellular buffer (OIB; 10 mM NaCl, 125 mM KCl, 1.0 mM KH_2PO_4, 2.0 mM $NaHCO_3$, pH 7.2) and incubated 4 h at room temperature. RNA was harvested by adding 200 μL of homogenization buffer (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA 1.5% SDS, 200 μg/mL proteinase K) and incubating 15 min at room temperature. The samples were then phenol extracted and ethanol precipitated. RNA pellets were resuspended in 3 μL of H_2O per nucleus. Samples were analyzed on denaturing gels as described above. Hybridization selection of the RNA products to filter bound 87U6 DNA was carried out as described previously (*21*).

Electrophoretic Mobility Shift Assays. Pairs of single stranded oligonucleotides used in EMSA were labeled by annealing 1 μg of each complementary oligonucleotide in the presence of 1X T4 DNA polymerase buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9). The annealed oligos have four base overhangs on each end which were filled in with T4 DNA polymerase in the presence of 10 μCi of the appropriate [^{32}P]-dNTP. All reactions received 6 units of T4 DNA polymerase and were incubated at 30 °C for 1 h. Unlabeled competitor oligos were prepared in the same way, but reactions were set up without the radionucleotide. Aliquots of the cold reaction were removed and added to the appropriate [^{32}P]-dNTP to monitor the efficiency of the fill-ins. Double-stranded oligonucleotides were purified over Nensorb nucleic acid affinity columns (New England Nuclear, Boston, MA) or Quick-Spin G-25 columns (Boehringer-Mannheim, Indianapolis, IN) to remove unincorporated nucleotides and salts. The filled-in, double-stranded oligonucleotides correspond to nucleotides 35 to 68 for 87U6 (5′-ACAATACAGAGAAGATAGCA CACAGTCCCCGCGC-3′, sense strand), 35 to 68 for wtU6 (5′-ACGATACAGAGAAGATTAGCA TGGCCCCTGCGC-3′), and 40 to 73 for 5S rRNA (5′-TGATCTCGGAAGCTAAGCAGGGTCGG GCCTGGTT-3′). The sequence of the 23CTT oligomer from the snRNP E protein gene promoter has been published previously (*26*).

Labeled oligonucleotides (approximately 20 000 cpm) were incubated with 20 μg of total protein from HeLa S100 extract and 5 μg of poly(dI-dC) in a buffer containing 18.2 mM HEPES, 68.2 mM KCl, 2 mM MgCl2, 0.5 mM DTT, 0.6 mM ATP, 15% (v/v) glycerol, pH 7.9. The extract was preincubated with competitors (specific and nonspecific) for 10 min at room temperature before adding probe and incubating an additional 20 min at room temperature. Electrophoresis was on a 5%, nondenaturing polyacrylamide gel (30% acrylamide/0.8% bisacrylamide) in 0.5X TBE at 150 V for 2 h.

Reconstitution and Immunoprecipitation of U6-Containing snRNPs. Reconstitution reactions were carried out in 100 μL reactions according to the procedure developed by Pikielny et al. (*27*). Following reconstitution, reactions were adjusted to 10 mM Tris-HCl, pH 8.5, and 100 μg of purified anti-Sm or control IgG was added. Purification of immune complexes by chromatography on Protein A-Sepharose columns was performed according to Wieben et al. (*28*).

Capping of U6 RNAs. The plasmids indicated in the figure legend were transcribed in vitro to generate synthetic U6 transcripts. The RNAs were gel purified and analyzed for the presence of the γ-monomethyl phosphate cap as described previously (*29*).

RESULTS

A minimal promoter consisting of the A box-like element and the 5′ end of the coding region is sufficient to direct transcription of 87U6 in vitro. Mutational analysis of the 87U6 gene in vitro demonstrated that the promoter of this gene lies within the coding region and has an architecture distinct from previously described RNA polymerase III promoters (*21*). Substitution mutations have shown the boundaries of these elements to be the first 20 nucleotides of the coding region (5′ internal control region or 5′ ICR)[1] and nucleotides 47−60, a region that overlaps, but does not precisely correspond to, a sequence in 87U6 with homology to an A box (A box-like element or ABLE). To determine if these sequences alone are sufficient to direct transcription from the 87U6 internal promoter, we constructed a series of minimal promoter constructs. Four of these constructs were

[1] Abbreviations: snRNP, small nuclear ribonucleoprotein; ABLE, A-box-like element; ICR, internal control region.

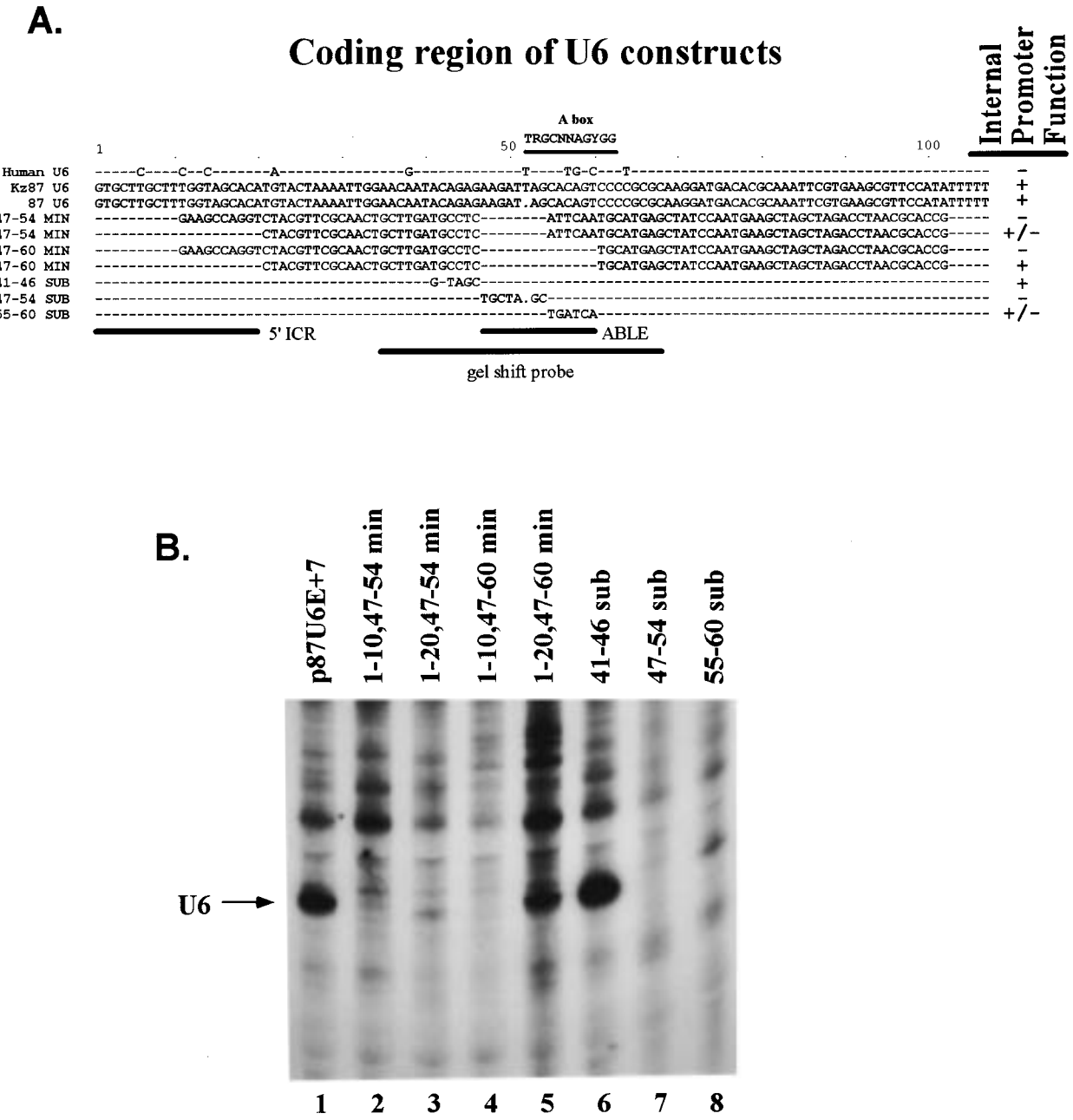

FIGURE 1: Two regions within the coding region of 87U6 are sufficient to support transcription from the internal promoter. (A) The minimal promoter constructs tested for transcriptional activity in HeLa cell S100 extracts are shown. Dashes indicate identity with the 87U6 sequence. The sequence of wtU6 is shown for comparison. The RNA polymerase III termination signal has been incorporated into these constructs at the correct site for production of full length transcripts. (B) The constructs diagrammed in part A were tested for transcriptional activity in HeLa cell extract as described previously (*21*). The position of the U6 band is indicated with an arrow. All of the clones are inserted into the vector pBluescript and lack any native flanking sequences. The identity of each U6 product has been confirmed by hybridization to filter bound 87U6 DNA.

made consisting of 87U6 residues 1−10 or 1−20 in the 5′ ICR and residues 47−54 or 47−60 in the ABLE (Figure 1A). Spacer sequences maintain the proper positioning of these elements, and an RNA polymerase III termination signal (TTTTT) was placed downstream at the same relative location as in the wild-type gene (nucleotides 102−107).

The transcription of these constructs was analyzed in a HeLa cell S100 extract, and the results obtained are shown in Figure 1B. The presence of all 20 residues of the 5′ ICR appears to be required for transcription from the internal promoter (compare lane 3 (clones p87U6(1−20,47−54 min)) and lane 5 (p87U6(1−20,47−60 min)) to lanes 2 and 4 (clones p87U6(1−10,47−54 min) and p87U6(1−10,47−60 min)). In agreement with our previously published results, the presence of nucleotides 55−60 is also required for efficient transcription from the internal promoter (compare lane 5, construct p87U6(1−20,47−60 min) with lane 3, p87U6(1−20,47−54 min)). The construct p87U6(1−20,-47−60 min) (lane 5) results in transcript accumulation at approximately 50% the level of p87U6E+7. Substitution of the 5′ ICR (*21*) or the ABLE (Figure 1B, lanes 7 and 8) eliminates or strongly inhibits transcription from the internal promoter, while substitution of nucleotides 41−46 (which lie just 5′ of the ABLE, lane 6) has no effect on transcription

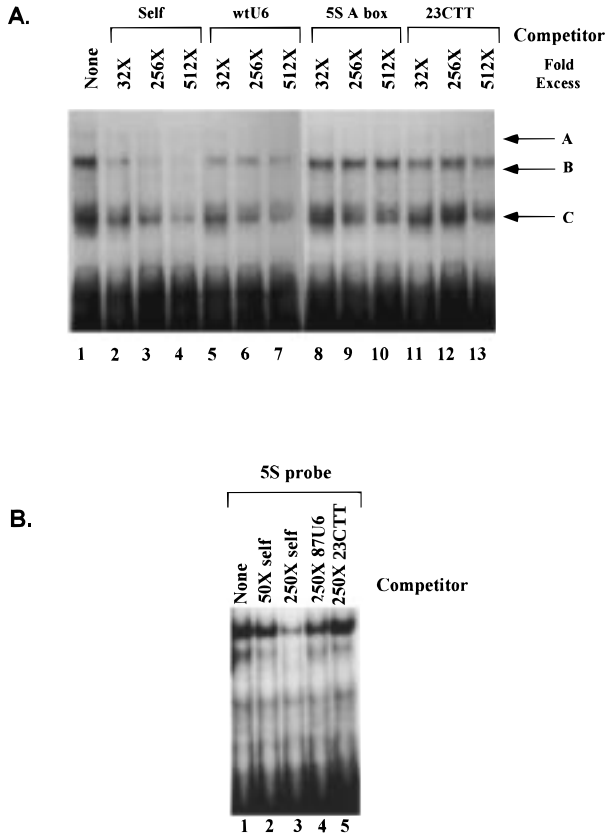

FIGURE 2: The A-box region of 87U6 binds to different factors than bind the 5S A box. Electrophoretic mobility shift assays were carried out in the presence of total HeLa cell S100 extract proteins. (A) Shifted bands produced with an oligonucleotide probe corresponding to the 87U6 ABLE (nucleotides 35−68) are marked with arrows. Competitions were conducted with the indicated molar excess of unlabeled self-oligonucleotide, the corresponding region of wtU6 (nucleotides 35−68), human 5S A box (nucleotides 40−73 of the human 5S gene), or an unrelated promoter element, 23CTT, from the human snRNP E protein gene (*26*). (B) Mobility shift using the 5S A box (nucleotides 40−73l). The identity and molar excess of competitor oligonucleotides is indicated above each lane as described in A).

in this system. Thus, these two elements are both necessary and sufficient to direct transcription from the internal promoter.

Gel Shift Analysis of the 87U6 A Box-Like Element Reveals a Unique Interaction. Given the unique nature of the 87U6 gene promoter, the interaction of factors with 87U6 promoter elements was investigated. Electrophoretic mobility shift assays using the central A-box-like element (ABLE) of 87U6 were carried out (see Figure 1 for relative location of gel shift probe). This region has been shown to be critical for transcription of 87U6 (*21* and Figure 1, this manuscript). When the radiolabeled 87U6 ABLE probe is incubated with total protein from HeLa S100 extracts two main bands (Figure 2A, arrows B and C) and one faint band (Figure 2A, arrow A) are observed.

When increasing amounts of excess unlabeled self-competitor are included in the reaction, all three shifted bands can be competed (Figure 2A, lanes 2−4). Using an excess of wtU6 competitor results in a lesser degree of competition (Figure 2A, lanes 5−7). The 87U6 and wtU6 33mers differ by only six nucleotides, yet these changes result in a difference in the wtU6 oligomers' ability to compete for factor binding. We have previously shown that the 87U6 ABLE can substitute for the A box of the human 5S gene (*21*). However, when the human 5S A box region is used as a competitor (5S nucleotides 41 to 73), very little competition is seen (Figure 2A, lanes 8−10). This suggests that the factor(s) binding to this region of 87U6 are distinct from those binding the 5S gene's A box. As expected, using a competitor from the snRNP E protein gene promoter, an RNA polymerase II transcription unit, results in no competition of the shifting pattern (Figure 2A, lanes 11−13).

The A-box region of the human 5S gene (residues 41 to 73) was also labeled and used as a probe in EMSA (Figure 2B, lanes 1−5). In agreement with the results in Figure 2A, the 5S A-box shift pattern can be competed with an excess of unlabeled self-competitor (Figure 2B, lanes 2 and 3), but not with an excess of unlabeled 87U6 ABLE (Figure 2B, lane 4).

Detection of 87U6 RNA Expression in Human Cells by Primer Extension. Since 87U6 is regulated by a unique RNA polymerase III promoter, we wanted to determine if this gene was expressed in vivo. We first monitored this in established cell lines by primer extension reactions using primers specific for the variant U6. To demonstrate the specificity of our primers for the appropriate template, we performed primer extensions on pure, synthetic RNA transcripts. Half of the changes between 87U6 and wtU6 are clustered between +51 and +64, so primers were designed which are complementary to this region (for primer sequences see Materials and Methods). As shown in Figure 3, the 87U6 primer only yields a product from the 87U6 template (Figure 3A; compare lanes 1 and 2 to lanes 3 and 4), and the wtU6 primer only yields a product from the wild-type template (Figure 3B; compare lanes 1 and 2 to lanes 3 and 4). No cross priming was observed (Figure 3A,B, lanes 3 and 4). Specificity was further confirmed by preincubating the RNA templates with an excess of cold wtU6 primers (Figure 3A,B, lanes 2 and 4; labeled "+"). This treatment failed to compete the binding of 87U6 primer to the SP6−87U6 template (Figure 3A, lane 2) but did compete the binding of wtU6 primer to its template (Figure 3B, lane 2).

When extensions with these primers were performed on total RNA samples from HeLa cells, products of the expected size were detected with both the 87U6 (Figure 3C, lanes 1 and 2, lower arrow) and the wtU6 primers (Figure 3C, lanes 3 and 4, lower arrow). As a further confirmation that the product detected with the 87U6 primer did not result from cross-priming on a wtU6 template, the extension reactions shown in lanes 5−8 of Figure 4 were supplemented with unlabeled T7-MU6−23 synthetic transcripts. Since the synthetic transcript contains extra 5′ nucleotides derived from 5′ flanking and vector sequences, it yields a longer extension product and can be used as an internal control for specificity (Figure 3C, upper arrow). In agreement with the control experiments, extension products from the T7-MU6−23 transcripts were detected only when the wtU6 primer was used (Figure 3C, lanes 7 and 8). Furthermore, the amount of 87U6 extension product, although low, was not decreased by the prior addition of an excess of unlabeled wtU6 primer to the extension reaction (Figure 3C, even numbered lanes). Densitometry of timed exposures from our primer extensions suggests that 87U6-like transcripts constitute approximately 1−3% of the total U6 present in HeLa cells. Similar results were obtained using template RNA samples isolated from

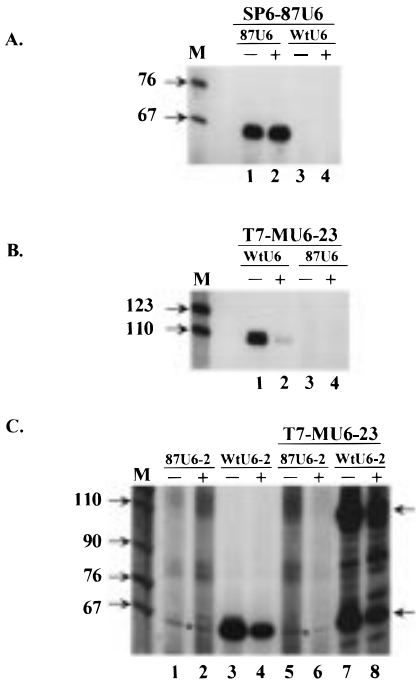

FIGURE 3: Primer extension analysis of 87U6 expression. (A) Specificity of the primers for the appropriate template demonstrated using synthetic 87U6 RNA (SP6−87U6) with either 87U6 (lanes 1 and 2) or WtU6 primers (lanes 3 and 4; for primer sequence, see Materials and Methods). The + or − over the lanes indicates preincubation of template RNA with or without an excess of unlabeled WtU6 primer, respectively. (B) Specificity of the primers for the appropriate template demonstrated using synthetic wtU6 RNA (T7-MU6−23) with either WtU6 (lanes 1 and 2) or 87U6 primers (lanes 3 and 4). + and − designations are as in 3A. The size of the extension products is larger in panel B due to the plasmid template used (see Materials and Methods for description of plasmids). In both panels M indicates the sizes in bp of ^{32}P end-labeled pB*R*322/HpaII digested DNA used as a size marker. (C) Primer extensions on total HeLa RNA (30 μg) using either the 87U6 specific primer (87U6−2) or the wtU6 specific primer (WtU6−2). Template RNA consisted of of total HeLa RNA (lanes 1 to 4) or HeLa RNA plus synthetic wtU6 transcript (T7-MU6−23; lanes 5 to 8) as an internal control for specificity. The asterisks mark the position of the specific 87U6 extension products. Due to the presence of 23 nucleotides of 5′ flanking sequence, the extension product from the synthetic wtU6 transcript (top arrow) is distinguishable from that produced from the endogenous U6 RNA (bottom arrow). The + and − indicators and molecular weight marker, M, are as in A.

human K562 and kidney 293 cell lines (data not shown), suggesting that the expression of minor U6 species is not limited to HeLa cells.

Analysis of 87U6 Expression in Xenopus Oocyte Nuclei. The expression of 87U6 was also assayed in *Xenopus laevis* oocyte nuclei. The Xenopus system has long been used to study the transcription of snRNAs (*30*−*32*) as well as other RNA polymerase III products (*33*, *34*). We first manually dissected the nuclei from the oocytes under oil. These oil isolated nuclei retain transcriptional activity (*24*, *35*), as well as other in vivo characteristics such as proper nuclear import (*25*, *36*). Isolation of the nuclei under oil prevents the loss of ions and small molecules by diffusion that would occur if isolation of nuclei was performed in an aqueous environment. Plasmid encoded U6 genes and [^{32}P]GTP were co-injected into the oil isolated nuclei, and the accumulation of newly synthesized RNA was monitored by denaturing gel electrophoresis. No U6-sized products are detected on

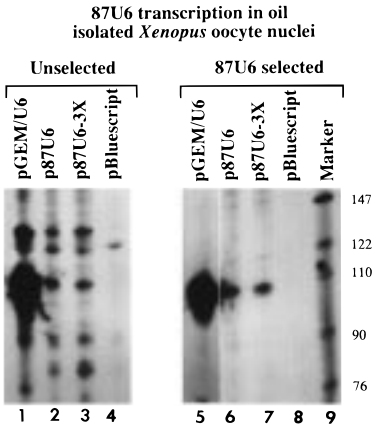

FIGURE 4: 87U6 is expressed when microinjected into *Xenopus laevis* oocyte nuclei. Nuclei from mature *Xenopus laevis* oocytes were manually dissected from the oocytes under paraffin oil as described in Materials and Methods. The plasmids indicated above each lane were microinjected into nuclei with [^{32}P]GTP. After incubation the RNA was harvested and analyzed on denaturing polyacrylamide gels. pGEM/U6 contains the consensus human U6 gene with all of its flanking promoter elements. p87U6 contains 87U6 without native flanking sequence. p87U6−3X has three tandem repeats of 87U6 without flanking sequence inserted into the vector. pBluescript indicates pBluescriptSK+ used as a vector control. Both p87U6 and p87U6−3X are inserted in this vector. The left panel is total radiolabeled RNA harvested from oocytes (2 nuclear equivalents per lane). The right panel has been selected by hybridizing harvested RNA to filter bound 87U6 DNA as described previously (*21*). pBluescript and pGEM/U6 lanes contain 5 nuclear equivalents, p87U6 lane contains 4 nuclear equivalents, and p87U6−3X lane contains 3 nuclear equivalents). The pGEM/U6 lane in the right panel has been exposed for one-fourth the time of the other lanes.

denaturing gels in the absence of exogenous template in these nuclei if cells are harvested 4 h after injection.

When plasmid-encoded 87U6 genes without any native flanking sequences are injected into the nuclei, U6-sized products accumulate (Figure 4, lane p87U6). No U6-sized transcripts accumulate when plasmid without an insert is injected (Figure 4, lane pBluescript). Hybridization selection has confirmed the identity of these bands as U6 RNA (Figure 4, right panel). The level of transcription seen from the p87U6 construct is much lower than that seen when the consensus human U6 gene is injected (Figure 4, compare lane pGEM/U6 to p87U6). Increasing the 87U6 gene dosage by injecting a plasmid containing three tandem repeats of 87U6 does not increase transcription (Figure 4, lane p87U6−3X).

Given the fact that flanking sequences can play a slight modulatory role on transcription from the internal promoter in vitro (*21*), we decided to investigate the effects of flanking sequence on transcription in the Xenopus oocyte system. While there appears to be a stimulatory effect of flanking sequences on transcription, hybridization selection has indicated that the U6-sized transcripts generated from constructs containing 87U6 flanking sequences are not U6 RNA (data not shown). Although we have not investigated this further, the clones which yielded these transcripts contain an Alu repeat downstream of the 87U6 coding region and this sequence may be responsible for directing the transcription of these U6-sized RNAs.

87U6 Assembles into Sm-Precipitable Ribonucleoprotein Complexes. While expression levels of 87U6 are low as

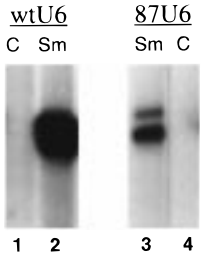

FIGURE 5: Immunoprecipitation of in vitro assembled snRNPs. WtU6 and 87U6 RNAs were transcribed in vitro from an SP6 promoter (plasmids SP6−87U6 and SP6-wtU6) in the presence of [^{32}P]-GTP, and the synthetic transcripts were incubated in HeLa S100 extract according to the method of Pikielny et al. (*27*). Anti-Sm (Sm) or normal human serum antibodies as a control (C) were used to select snRNP complexes as described previously (*28*). Lanes 1−2: WtU6 SP6 transcripts. Lanes 3−4: 87U6 SP6 transcripts. The lower band in lanes 2 and 3 results from premature termination of the SP6 transcription at the oligo(T) region of U6 rather than by runoff at the plasmid cleavage site. Replicates of this experiment have shown that the two RNAs typically immunoprecipitate with similar efficiencies.

determined by primer extension analysis, they do not exclude a possible role for this variant in RNA processing reactions. Most of the sequence differences between 87U6 and wtU6 occur at positions that are not highly conserved during evolution, and the four sequence differences from wtU6 in the U4/U6 interaction domain would have little effect on base-pairing interactions with wild type human U4 (Figure 7A). To test the ability of 87U6 to associate with U4 experimentally, in vitro transcripts of 87U6 were incubated in HeLa cell S100 extracts under conditions shown previously to permit assembly of wtU6 RNA into native U4/U6 complexes (*27*). As shown in Figure 5, no labeled RNAs are recovered when control human sera are used in the immunoprecipitations (Figure 5, lanes 1 and 4). However, both 87U6 and wtU6 transcripts are also recovered in immunoselection experiments using anti-Sm sera (lanes 2 and 3). Multiple replications of this experiment have demonstrated that 87U6 and wtU6 RNAs typically assemble with similar efficiencies. Since neither 87U6 nor wtU6 contains a consensus binding site for the Sm proteins, this result suggests that 87U6 can base pair with U4 to form an snRNP complex similar to that formed with wtU6. However, the possibility that 87U6 binds directly to an Sm reactive protein cannot be excluded.

87U6 Is Capped with the U6 Specific γ-Monomethyl Phosphate Cap. U6 RNA possesses a unique γ-monomethyl phosphate cap structure that is distinct from the 2,2,7-trimethyl guanosine cap found on the other spliceosomal snRNAs (*37*). The cap structure is thought to play a role in the stability of the mature RNA within the nucleus (*38*), and it has been demonstrated that sequences in the 5′ end of the RNA are necessary and sufficient for capping. Specifically, base-pairing in the 5′ stem-loop and the presence of the hexanucleotide sequence, AUAUAC, of the U6 RNA are important for efficient capping (*29*). 87U6 RNA has three changes that fall within the 5′ stem-loop, although only one of them is predicted to affect base-pairing in the stem (nucleotide 14, see Figure 7A). The other two changes are either at a position that is proposed to be bulged in the stem (nucleotide 6) or in the loop itself (nucleotide 11). Nucleotide 22 is changed from an A to a G in 87U6 and lies within the sequence AUAUAC immediately downstream of the

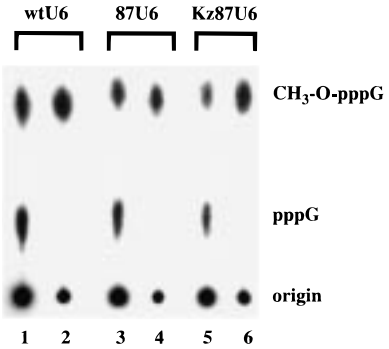

FIGURE 6: 87U6 RNA can be capped by the U6-specific capping enzyme. Plasmid DNAs containing different U6 genes were transcribed in vitro using HeLa cell extract and [α-^{32}P]-GTP as the radiolabeled precursor (*29*). The wild-type gene corresponds to the mouse U6 gene −315/+286 described in Singh et al. (*29*). The 87U6 gene is the variant U6 gene with no native flanking sequences, and Kz87U6 is an allelic variant of this gene containing flanking sequences from −286 to +336 (*21*). The transcribed RNAs were fractionated on polyacrylamide gels and the purified U6 snRNAs were digested with nuclease P1 (lanes 1, 3, and 5) or with nuclease P1 followed by alkaline phosphatase (lanes 2, 4, and 6), subjected to electrophoresis on DEAE−cellulose paper, dried and subjected to autoradiography.

stem-loop. Deletion or substitution of this entire hexanucleotide in U6 impairs the ability of the RNA to serve as a substrate for capping.

We have previously reported that there are at least two alleles of the 87U6 locus (*21*). These two alleles differ by only a single nucleotide, the one base deletion at position 52 of the coding region. The capping of both alleles of 87U6 RNA was investigated and compared to that of the wtU6 RNA. U6 RNAs transcribed in HeLa cell extracts were gel purified and treated with nuclease P1 (Figure 6, lanes 1, 3, and 5) or with nuclease P1 followed by alkaline phosphatase (Figure 6, lanes 2, 4, and 6) as described previously (*29*). Both 87U6 alleles possess a cap structure that is resistant to alkaline phosphatase treatment and migrates during electrophoresis on a DEAE−cellulose paper at a position consistent with the presence of a γ-monomethyl phosphate cap (Figure 6, compare lanes 4 and 6 with lane 2).

DISCUSSION

Transcriptional Regulation. The transcriptional regulation of an expressed, variant U6 RNA is of interest because of the potentially unique role this RNA may play in the catalysis of splicing. The presence of multiple shifted complexes present when the 87U6 probe is incubated in HeLa extracts is consistent with the possibility that a multi-subunit complex assembles on the ABLE. The importance of this region for transcription of 87U6 is supported by the minimal promoter constructs described here, which demonstrate that this ABLE, in conjunction with the 5′ internal control element, is sufficient to direct 87U6 transcription from the internal promoter.

The observation that a human 5S gene A box cannot efficiently compete for the same factors binding to the 87U6 A-box-like element is intriguing. This implies that distinct transcription factors are binding to the 87U6 A box domain compared to the 5S A box. This is not entirely surprising in light of previous results showing that the boundaries of

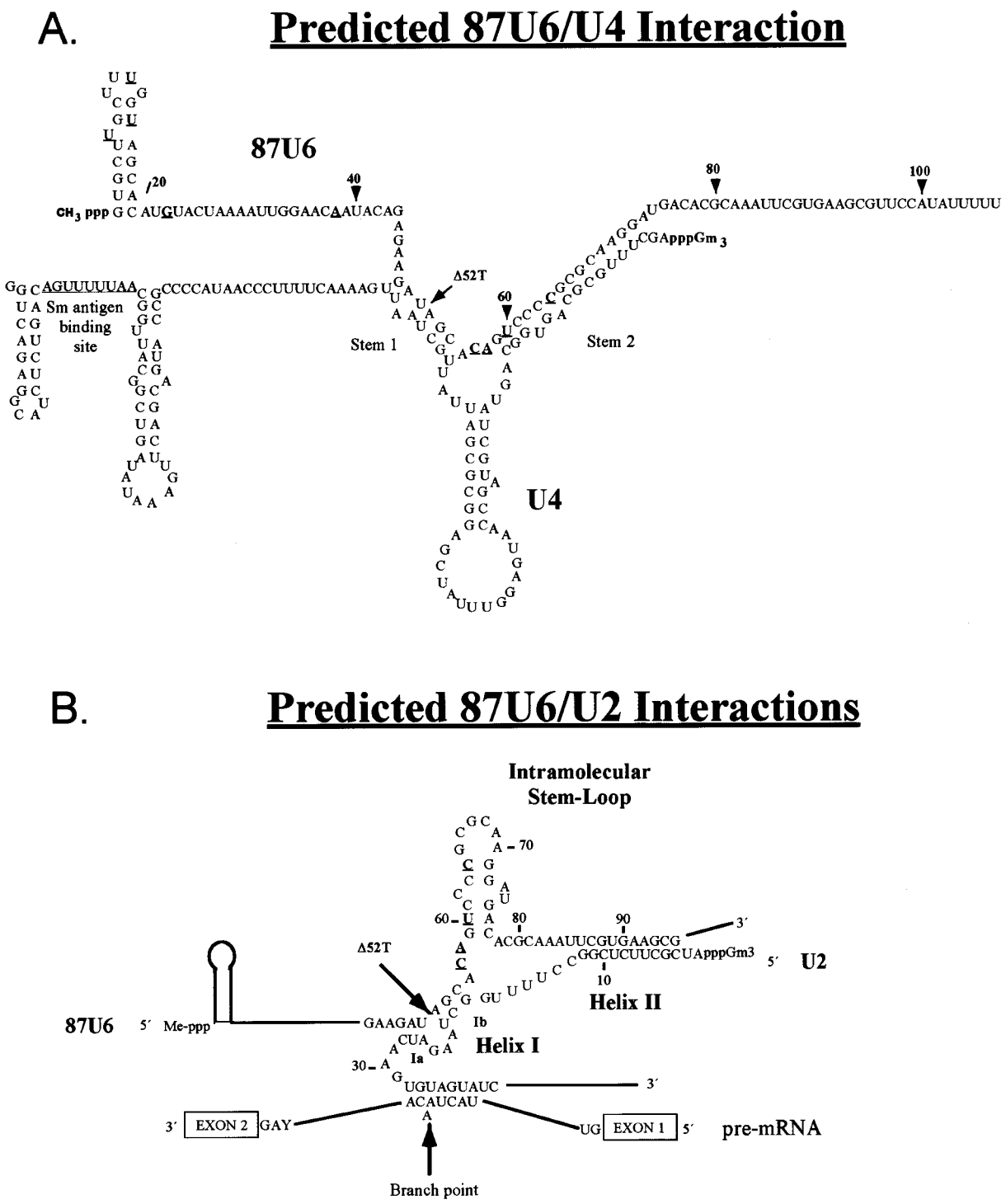

FIGURE 7: Predicted RNA−RNA interactions involving 87U6. (A) Interaction between the variant snRNA 87U6 and U4 snRNA following the structure proposed by Guthrie and Patterson (*57*). Sequence differences between 87U6 and wtU6 are underlined and in bold type. The deletion at position 52 is indicated with an arrow. The Sm antigen binding site on U4 snRNA is underlined. (B) Predicted base-pairing between 87U6 snRNA and U2 snRNA according to the model proposed by Madhani and Guthrie (*49*) with modification proposed by Sun and Manley (*3*). The interaction between U2 RNA and the branch point region of the pre-mRNA intron is also shown. U6 and U2 are proposed to base pair in two separate domains. The sequence differences between 87U6 and wtU6 are in bold print and underlined. The deletion at position 52 is indicated with an arrow.

this internal promoter element in 87U6 do not precisely overlap the region of A-box homology and that this region of 87U6 can only partially restore transcription to a human 5S gene when it is substituted in place of the native 5S gene A box (*21*).

The ability of the wtU6 oligonucleotide to compete weakly for binding with the 87U6 probe is somewhat unusual in light of the fact that this region of wtU6 (nucleotides 35−68) is known not to be required for transcription of the human wtU6 gene, which utilizes an entirely external promoter (*39*−*41*). However, the two 33mers differ by only six nucleotides and it is not surprising that the factor which binds this region of 87U6 binds the comparable region of wtU6 with a lower affinity.

Expression of 87U6 in Vivo. Evidence presented here strongly suggests that 87U6 RNA is expressed in human cells. Specific primer extension analyses on three human cell lines demonstrate low, but detectable levels of 87U6-like RNA in these cells. Densitometry of timed exposures from our primer extensions suggests that 87U6-like transcripts constitute approximately 1−3% of the total U6 present in HeLa cells. This level of expression is similar to the levels of other variant snRNA species expressed in human cells (including U6atac RNA; *42*), and provides an explanation why this species could have been overlooked in earlier studies of U6 expression in human cells.

Rigorously interpreted, our primer extension experiments demonstrate that transcripts initiating the expected distance upstream from the primer for an 87U6 transcript, and containing the 87U6 central region, are expressed in three human cell lines. Since the human genome contains approximately 200 copies of U6 sequences (*43*), it is possible that the primer extension experiments reflect the expression of related U6 variants rather than 87U6 per se. It is also possible that the higher levels of wtU6 RNA compared to 87U6 RNA detected in the primer extension assays reflect the number of functional copies of each type of gene, and not relative promoter strength.

The expression of 87U6 in oil isolated *Xenopus laevis* oocyte nuclei demonstrates that this particular U6 variant can be expressed in eukaryotic cells. Injection of the clone p87U6 confirmed that transcription can occur from the 87U6 gene without the need for any flanking sequences. This agrees with the results we obtained in vitro in HeLa cell S100 extracts (*21*).

The difference in the relative transcription levels of 87U6 and wtU6 may be due to the relative abundance of a necessary factor for transcription from the 87U6 internal promoter in amphibian cells. The internal promoter of 87U6 is distinct from previously described internal promoters (*21*), and the gel shift data suggest that the A box-like element of 87U6 binds at least one distinct factor from the human 5S gene A box. It will be interesting to examine the evolutionary conservation of the 87U6 genomic locus, as this may offer an explanation for the relatively low levels of transcription seen in Xenopus. A report on the cloning of the hamster Gi3-α gene (*44*) revealed that the 87U6 gene in the seventh intron of this G protein is not conserved. While this demonstrates that the location of 87U6 within the intron of Gi3-α is not conserved, it does not preclude the presence of 87U6, or a similar U6 gene, in the hamster genome.

Functional Implications. The immunoprecipitation results (Figure 6) suggest that 87U6 assembles into an snRNP particle, presumably with U4 (*45*). This suggests a role for 87U6 in the splicing pathway. The differences between wtU6 and 87U6 include three substitutions and a one base deletion within the U4/U6 interaction domain (Figure 7A). However, these changes do not have a major destabilizing effect on the U4/U6 stem. It is also possible that human cells produce a variant U4 RNA that base pairs more specifically with 87U6, as found for U6atac RNA (*18*). The assembly of 87U6 into an Sm-precipitable particle appears to be approximately equivalent to the efficiency of assembly seen with wtU6 in this in vitro system.

U6 RNA was originally proposed to interact with U2 snRNA in two domains (*46*−*49*). 87U6 and wtU6 are identical in the region of the molecule that interacts with U2 in the complex termed helix II (Figure 7B). This region has been shown to be necessary for spliceosome assembly (*50*). There is one difference between the two U6 RNAs in the helix I U2/U6 interaction domain (*49*). This is the single base deletion at position 52 and causes the bulge in U2 RNA between helix Ia and Ib to increase from two to three nucleotides (Figure 7B). Although the U2 residues in the bulged region have been implicated as being important in splicing (*5*), no mutagenesis studies have looked at the single base deletion corresponding to position 51 or 52 (*6*, *49*−*54*).

The changes in 87U6 also cause a significant change in the structure of the intramolecular stem-loop that has been proposed to play a critical role in the first step of splicing (*55*, *56*). While several of the changes present in 87U6 disrupt the base-pairing that has been proposed, alternative stem-loop structures with similar stabilities can be proposed for this region of the molecule in 87U6 (Figure 7B).

The finding that 87U6 can be capped with the unique gamma-monomethyl phosphate cap found on wtU6 demonstrates that the sequence changes in the 5′ end of 87U6 do not disrupt structures necessary for the capping reaction. In particular, the ability of 87U6 to be capped suggests that the 5′ stem-loop is formed in the variant despite three sequence differences within this region (*29*). The capping of wtU6 is known to improve its stability in vivo (*38*), and a similar benefit may be realized by 87U6.

Significance. The possibility that variant U6 snRNAs may function in alternative splicing has now been confirmed experimentally. Recent results describing a rare class of introns that utilize a distinct set of snRNP particles (*17*, *18*, *42*) confirm that low-abundance, variant snRNAs can have important roles in the splicing of minor classes of introns. Taken together, our results demonstrate that 87U6 accumulates in human cells and can be capped and assembled into an immunoprecipitable ribonucleoprotein complex. Given these findings, there is no reason to believe that this variant snRNA does not have a role in mRNA splicing.

An alternative hypothesis for 87U6 function is suggested by the location of the 87U6 gene within an intron of the Giα-3 gene (*28*). If these two genes are transcribed at the same time they will necessarily produce antisense RNA to one another. Thus, it is possible that 87U6 could be serving as an antisense regulator by inhibiting the splicing of the Giα-3 transcript or by causing the targeted degradation of Giα-3 pre-mRNA.

Future functional studies will be required to test these hypotheses and to determine whether the sequence differences between 87U6 and wtU6 might influence splicing specificity and/or efficiency.

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