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A Novel Spliceosome Containing U11, U12, and U5 snRNPs Excises a Minor Class (AT–AC) Intron In Vitro

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Summary

A minor class of introns with noncanonical splice (AT–AC) and branch site sequences exists in metazoan protein coding genes. We have established a HeLa cell in vitro system that accurately splices a pre-mRNA substrate containing such an intron from the human P120 gene. Splicing occurs via a lariat intermediate whose branch site A residue is predicted to bulge from a duplex formed with the low abundance U12 small nuclear ribonucleoprotein (snRNP), which we confirm by psoralen cross-linking. Native gel electrophoresis reveals that U11, U12, and U5 snRNPs assemble onto the P120 pre-mRNA to form splicing complexes. Inhibition of P120 splicing by 2′-O-methyl oligonucleotides complementary to U12 or U5 demonstrates that U12 and U5 snRNPs perform essential roles in the AT–AC spliceosome.

Introduction

Splicing of nuclear pre-mRNAs occurs in the spliceosome, a large and dynamic ribonucleoprotein complex. Spliceosome formation involves ordered assembly of the U1, U2, U4/U6, and U5 small nuclear ribonucleoproteins (snRNPs), together with a number of non-snRNP protein factors, onto the pre-mRNA. Biochemical and genetic data from a variety of eukaryotic systems have provided significant insight into snRNP–snRNP and snRNP–substrate interactions within the spliceosome (reviewed in Moore et al., 1993). To initiate spliceosome assembly, conserved sequences at the 5′ splice site and intron branch site direct association of U1 and U2 snRNPs, respectively, through base–pairing interactions. The U5-U4/U6 tri-snRNP then joins the pre-splicing complex, resulting in base-pairing between the 5′ end of U2 RNA and the 3′ end of U6 RNA (reviewed in Nilsen, 1994). Conformational changes subsequently occur to build an intricate network of RNA–RNA interactions, which juxtapose the intron splice sites. The association of U1 and U4 snRNPs becomes destabilized, allowing conserved sequences in U6 to interact with the 5′ splice site and with regions of U2 RNA adjacent to the intron branch site–U2 duplex (reviewed in Nilsen, 1994). Attack by the 2′-hydroxyl group of the branch nucleotide (usually adenosine) on the 5′ splice site initiates the splicing reaction, followed by a second transesterification reaction which yields the ligated exons and the intron as a lariat structure. Meanwhile, a phylogenetically invariant loop sequence of U5 RNA grips exon nucleotides adjacent to the 5′ splice site and aligns the two exons for the final ligation step (Newman and Norman, 1992; Sontheimer and Steitz, 1993).

The vast majority of pre-mRNA introns obey the so-called GT–AG rule. Several lines of evidence indicate that the guanosine residues at the 5′ and 3′ termini of the intron form a non-Watson-Crick interaction essential for the second step of splicing (Parker and Siliciano, 1993; Chanfreau et al., 1994; Scadden and Smith, 1995). Yet, substitution of A and C for the first and last intron nucleotides, respectively, can suppress the splicing defect caused by either individual mutation. A minor class of introns that possess AT and AC at their 5′ and 3′ ends, respectively, has recently been identified in both vertebrate and invertebrate genomes (Jackson, 1991; Hall and Padgett, 1994). These minor class (AT–AC) introns also exhibit longer, highly conserved but noncanonical sequences at their 5′ and 3′ splice sites. The 5′ splice site consensus is /ATATCCTT and the 3′ splice site is CCAC/, while a third conserved intron element (TCCTTAAC) appears upstream of the 3′ splice site. So far, the AT–AC intron has been found in only four genes: P120, characterized in several mammals including human, codes for a proliferation-associated nucleolar protein; CMP, in human and chicken, encodes a cartilage matrix protein; *Rep-3* in mouse is a homolog of the bacterial DNA mismatch repair protein MutS; and the Drosophila *prospero* gene encodes a homeobox protein involved in neurogenesis (Hall and Padgett, 1994, and references therein). In each case, the pre-mRNA contains other introns whose sequences conform to those of major class introns. Neither the size nor the position of the AT–AC intron within the host gene is conserved.

Hall and Padgett (1994) recently proposed that the 5′ splice site and the 3′ conserved element of AT–AC introns might be recognized by the low abundance U11 and U12 snRNPs. The U11 and U12 snRNPs are members of the Sm class, present in HeLa cells at about 1/100 the level of the U1 snRNP (Montzka and Steitz, 1988). In HeLa nuclear extracts, most U12 is found complexed with U11, while a fraction of U11 (which is more abundant than U12) behaves as a mono-snRNP (Wassarman and Steitz, 1992). Although the secondary structures proposed for U11 and U12 RNAs resemble those of U1 and U2 RNAs, respectively (Montzka and Steitz, 1988), definitive functional assignments have been lacking for these snRNPs. U12 RNA homologs have been characterized in two other vertebrates where genes containing AT–AC introns have been found (Tarn et al., 1995); invariant sequences at the 5′ end of U12 RNA, which include the predicted branch site–pairing region, are consistent with the involvement of the U12 snRNP in splicing of pre-mRNAs containing an AT–AC intron.

Here, we describe an in vitro system that splices a pre-mRNA substrate containing an AT–AC intron derived from the human P120 gene. Psoralen cross-linking confirms the base-pairing interaction predicted by Hall and Padgett (1994) between the branch site of the pre-mRNA substrate and U12 RNA. Probing the participation of Sm snRNPs reveals that, in addition to the low abundance U11 and U12 snRNPs, the U5 snRNP, but surprisingly not the U4 or U6 snRNP, becomes associated with

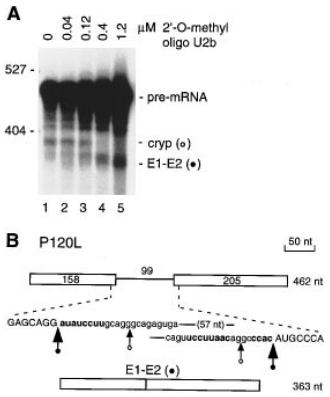

Figure 1. Activation of P120L Pre-mRNA Splicing by Debilitating U2 snRNPs

(A) Splicing was carried out in HeLa nuclear extract in the absence (lane 1) or presence (lanes 2–5) of different amounts of the 2′-O-methyl oligonucleotide U2b using uniformly labeled pre-mRNA (P120L) derived from the human P120 gene as substrate for 4 hr. The P120L precursor (pre-mRNA), the accurately spliced product (E1-E2), and a cryptic splicing product (cryp) are indicated at the right. The faster migrating splicing intermediates and excised intron product of accurate splicing could also be observed (not shown here) but were usually buried under a smear of degraded fragments. Size markers were ^{32}P-labeled pBR322 DNA digested with MspI, with fragment lengths in nucleotides given on the left.

(B) The schematic shows the P120L precursor, the accurately spliced product and the sequences surrounding the authentic (arrow with closed circle) and the cryptic (arrow with open circle) splice sites, determined by RT–PCR. Other possible cryptic splice sites are pointed out in the text. Upper and lower cases represent exon and intron regions, respectively. Bold nucleotides denote conserved elements (the 5′ and 3′ splice sites and the branch site) in AT–AC introns.

the spliceosome assembled on the P120 minor class intron. Our results suggest the existence of lower abundance U4 and U6 snRNP analogs and raise many questions concerning the AT–AC spliceosome.

Results

Splicing of the P120 Pre-mRNA Is Activated by Debilitating the U2 snRNP in HeLa Nuclear Extracts

A splicing substrate (P120L) containing an AT–AC intron was constructed from intron F and flanking exons of the human P120 gene (Figure 1). This particular minor class intron was selected because of its small size (99 nt [nucleotides]); the other known mammalian AT–AC introns in the CMP and *Rep-3* genes are 0.64 and 1.4 kb, respectively (Hall and Padgett, 1994 and references therein). When the P120L substrate was incubated in a standard HeLa cell splicing extract, an aberrantly spliced product (labeled cryp in Figure 1A, lane 1) was generated apparently by the major spliceosome (see below). Changes in pH or in the concentration of monovalent or divalent salts failed to activate usage of the AT–AC splice sites. However, appearance of the accurately spliced product was induced by pretreatment of the nuclear extract with a 2′-O-methyl oligonucleotide complementary either to nt 27–49 (the branch site recognition region) of U2 RNA (U2b; Figure 1A) or to the 5′ end of U1 RNA ($U1_{1–14}$; data not shown). Concurrently, the aberrantly spliced product diminished with increasing amounts of the oligonucleotide U2b.

Sequences of bands labeled cryp and E1-E2 (Figure 1A) were determined by a reverse transcription–polymerase chain reaction (RT–PCR) procedure. The sequence across the splice junction of E1–E2 was as expected. Since the 5′ cleavage site was confirmed subsequently (see below), the 3′ splice site could be deduced (Figure 1B). Sequencing across the splice junction of the aberrantly spliced product (cryp) revealed that splicing occurred at sites within the P120 intron (Figure 1B) in the absence of the oligonucleotide U2b. The bona fide cryptic splice sites remain uncertain because splicing could occur one nucleotide upstream or one or two nucleotides downstream of each indicated splice site (Figure 1B) and yield the same sequence. Figure 1B indicates the most likely splice sites (5′ /GC and 3′ AG/), which are probably recognized by the highly abundant snRNPs to assemble a major spliceosome that competes with authentic splicing.

A pre-mRNA with a truncated 3′ exon (P120) was next constructed to optimize the sizes of splicing intermediates and products and to remove nuclease-susceptible regions in the 3′ exon. Figure 2A shows splicing of the P120 substrate in nuclear extract pretreated with the 2′-O-methyl oligonucleotide U2b. Splicing products, including the ligated exons and the lariat intron, accumulated with incubation time; ~6% of the pre-mRNA became spliced after 4 hr at 30°C.

Identification of splicing intermediates and products was confirmed by debranching assays and by use of an oligonucleotide complementary to the predicted branch site recognition region of U12 to block splicing (Figure 2B). When the P120 substrate was uniformly labeled, the lariat intron–exon 2 intermediate could not be distinguished from a 3′-end-degraded substrate fragment (asterisks in Figures 2A and 2B, lanes 1 and 2), which was also visualized in U12-blocked extract (Figure 2B, lane 2 and below). However, debranching of RNAs generated in a splicing reaction containing 3′-end-labeled P120 substrate (Figure 2B, lane 3) showed conversion of the band indicated as lariat intron–exon 2 intermediate to a faster migrating band of the expected size (199 nt; Figure 2B, lane 5, and Figure 2C). Likewise, when RNAs isolated from a reaction containing uniformly labeled P120 were subjected to the debranching assay, the lariat intron visualized in lane 1 (Figure 2B) was converted to a size of 99 nt (data not shown).

The branch nucleotide of the P120 intron was determined by primer extension on gel-purified lariat intron–exon 2. Figure 2D shows specific blockage of extension at C^{+91} using a primer complementary to a region of the 3′ exon (lane 6). This result suggests that the penultimate nucleotide, an A residue, of the conserved 3′ element (Figure 1C) serves as the nucleophile in the first step of the splicing reaction. Other minor terminated fragments probably derive from degraded substrate fragments or aborted extension.

Extension on the lariat intron was also performed to determine the exact 5′ splice site utilized for intron excision in vitro. Using an intron primer, the major termination site was at A^{+1}, the predicted first nucleotide of the

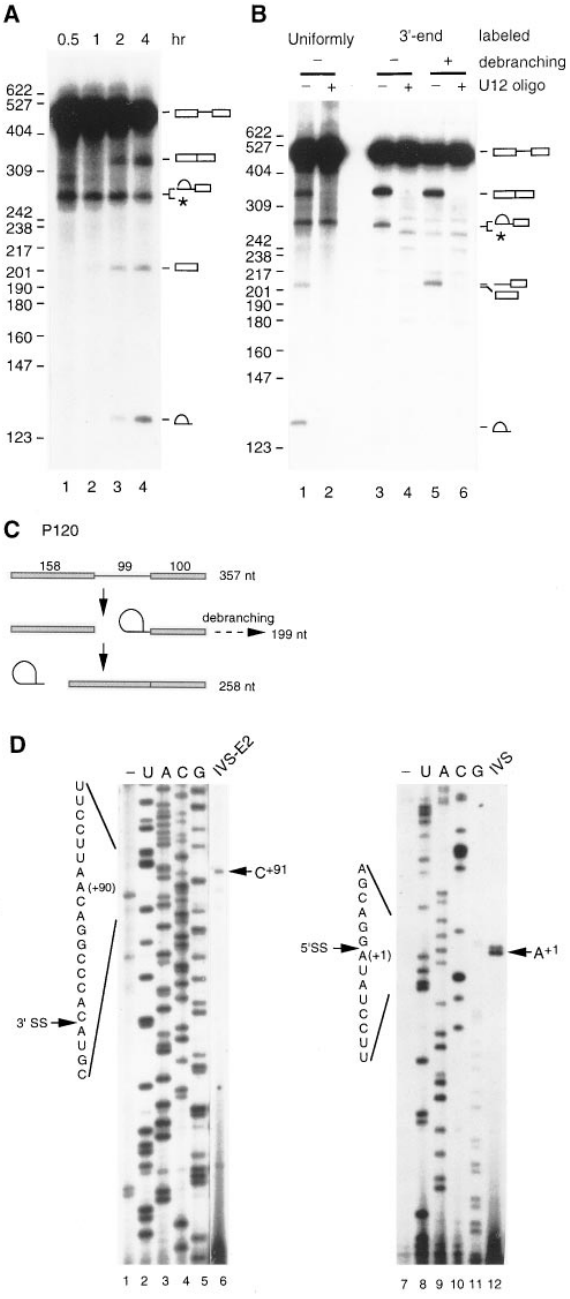

Figure 2. Splicing of the P120 Pre-mRNA and Characterization of the Lariat Splicing Intermediate

(A) Splicing of uniformly labeled P120 pre-mRNA was carried out in a U2-blocked (1.2 μM U2b) nuclear extract at 30°C for various times as indicated at the top. Symbols on the right indicate the P120 precursor, splicing intermediates, and products. The aberrant mobility of linear RNA species containing the 5′ exon in the 8% polyacrylamide gel is probably due to its several stretches of purine-rich sequence. The asterisk represents a degraded substrate fragment comigrating with the lariat intron–exon 2 intermediate. The numbers at the left are the lengths of ^{32}P-labeled fragments of MspI-digested pBR322.

(B) Splicing of uniformly labeled (lanes 1 and 2) or 3′-end labeled (lanes 3–6) P120 pre-mRNA was performed in U2-blocked extracts not containing (lanes 1, 3, and 5) or containing (lanes 2, 4, and 6) an additional 2′-O-methyl oligonucleotide, $U12_{11–28}$, for 4 hr. RNAs recovered from splicing reactions containing the 3′-end-labeled P120 substrate were aliquoted into two fractions: one was incubated further in S100 extract for debranching (lanes 5 and 6). Note that although the excised 5′ exon and linearized intron–exon 2 intermediate comigrate, only the former is visualized in lane 1 (nondebranched) and only the latter in lane 5 (3′-end labeled).

(C) The schematic shows the pathway of P120 pre-mRNA splicing.

(D) The lariat intron–exon 2 (IVS-E2) and the lariat intron (IVS) were gel purified from a splicing reaction containing P120L. The branch point was determined by primer extension on the lariat intermediate using P107 as primer (lane 6). The 5′ cleavage site was determined by primer extension on the lariat intron using P110 as primer (lane 12). Dideoxy sequencing reactions used P107 (lanes 1–5) or P110 (lanes 7–11) as primer.

intron (Figure 2D, lane 12). The weaker upper band of the doublet could result from nonspecific termination of reverse transcription. These results confirm the accuracy of splicing and also indicate that the branch forms between two A residues during splicing of the P120 pre-mRNA.

Psoralen Cross-linking of U12 snRNA to the Branch Site of the P120 Intron

To identify RNA–RNA interactions, the P120L substrate was incubated under conditions that allow accurate splicing for 15 min and then irradiated on ice with 365 nm light in the presence of AMT (4′-aminomethyl-4,5′, 8-trimethyl) psoralen (Wassarman, 1993). No specific crosslinked products were detected in the absence of nuclear extract (data not shown), whereas three retarded bands appear in lane 1, Figure 3A. The snRNA species contained in the crosslinked products were identified by oligonucleotide-directed RNase H cleavage. The bands labeled U12x1 and U12x2 each decreased to <20% their original level (lane 2) when cleavage was induced by an oligonucleotide complementary to nt 53–71 of U12 snRNA (lane 6), arguing that they both contain U12. The intensity of the U12x1 cross-linked band was ~3.5 fold that of U12x2 in several experiments. The slower migrating product (labeled udx) was unchanged in the presence of oligonucleotides $U2_{79–98}$, $U1_{64–75}$, $U11_{52–71}$, $U12_{53–71}$ (lanes 3–6), or $U11_{11–27}$ (data not shown), each of which had previously been demonstrated to target its respective RNA (Montzka and Steitz, 1988; Tarn and Steitz, 1994).

Without addition of the 2′-O-methyl oligonucleotide U2b, U2 snRNA cross-linking to the P120 substrate was detected in the same extract. With increasing amounts of U2b, all three cross-links visualized in Figure 3A, lane 1, increased (data not shown). This suggests that the highly abundant U2 snRNP competes directly with U12 for assembly onto the P120 pre-mRNA, activating cryptic splice sites and preventing authentic AT–AC splicing.

Primer extension analyses were next performed to determine the sites of psoralen cross-linking on both U12 snRNA and P120L. When total RNA isolated from the cross-linked splicing reaction was analyzed by primer extension from an oligonucleotide complementary to the P120L 3′ exon, several stops were detected (Figure 3B, lane 11), suggesting abundant psoralen monoadducts or intramolecular cross-linking. Use of the same primer with either of the gel-purified U12 cross-linked species detected a primary stop at U^{+84}, which

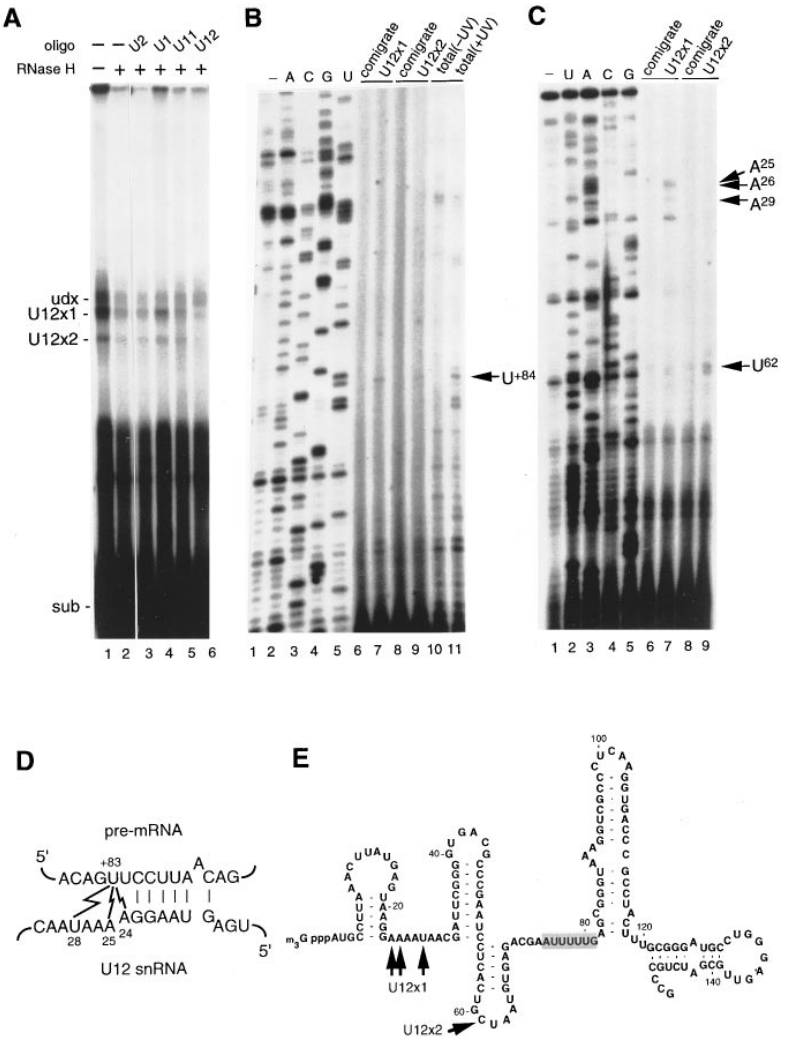

Figure 3. U12 snRNA Base Pairs with the Conserved Branch Site Sequence of the P120 Intron

(A) Splicing reactions were performed in a U2-blocked nuclear extract as in Figure 2 for 15 min. AMT psoralen was added and irradiation with 365 nm light was carried out on ice for 10 min. Recovered RNAs were aliquoted into six fractions, followed by addition of RNase H alone (lane 2) or RNase H plus 1 μg $U2_{79–98}$ (lane 3), $U1_{64–75}$ (lane 4), 10L (anti-U11; lane 5) or 9C (anti-U12; lane 6). The P120L substrate (sub), two U12/pre-mRNA cross-linked species (U12x1 and U12x2) and an as-yet-unidentified cross-linked band (udx) are indicated on the left.

(B) U12x1, U12x2, and comigrating RNAs from a reaction not containing substrate were gel purified. Primer extension blockage using oligonucleotide P118 as primer was performed on gel-purified RNAs (lanes 6–9) or on total RNA isolated from a splicing reaction without (lane 10) or with (lane 11) UV irradiation. The same primer was used in dideoxy sequencing reactions (lanes 1–5).

(C) The cross-linked sites of U12x1, U12x2, and their comigrating RNAs were mapped on U12 snRNA using oligonucleotide 9P as primer (lanes 6–9). Dideoxy sequencing reactions also used oligonucleotide 9P as primer and HeLa nuclear RNA as template (lanes 1–5). Note that the stop at G^{32} for U12x1 (lane 7) also appears in lane 1 and therefore may result from aborted extension or a possible additional cross-link at C^{31} (not shown here nor in D and E).

(D) Potential base-pairs between the 3′ conserved element of the P120 intron and U12 snRNA are indicated by straight lines, with cross-links indicated by lightning bolts. The branch point adenosine is illustrated as bulged from the U12/pre-mRNA helix.

(E) Cross-linked nucleotides of human U12 snRNA (Tarn et al., 1995) identified for U12x1 and U12x2 are indicated. The Sm site is shaded.

is adjacent to the predicted intron branch site (lanes 7 and 9).

To determine cross-linked positions on U12 snRNA, an oligonucleotide complementary to nt 122–147 was used as primer. In the major U12 cross-link, U12x1 (Figure 3C, lane 7), stops were observed downstream of A^{24}, A^{25}, and U^{28}, adjacent to the predicted branch site pairing sequence (Figures 3D and 3E). The unusual cross-linking of A nucleotides, previously observed in tRNA (reviewed in Cimino et al., 1985), was confirmed by primer extension blockage using another oligonucleotide, $U12_{53–71}$, as primer (data not shown). Analysis of the minor U12 cross-link, U12x2, showed a primary stop prior to nucleotide C^{61} in the third loop of U12 snRNA (Figure 3C, lane 9, and Figure 3E). Additional studies will be required to determine whether or not the U12x2 cross-link derives from a functional U12–branch site interaction.

Deduced base-pairing interactions adjacent to the major cross-linked nucleotides in U12 snRNA and the branch site sequence of the P120 intron are shown in Figure 3D. These results indicate that the U12 snRNP interacts with the branch site of AT–AC introns and executes a function homologous to that characterized for the U2 snRNP in the splicing of major class introns in pre-mRNAs.

Assembly of Splicing Complexes on the P120 Pre-mRNA

Complexes assembled on the AT–AC intron were examined by incubating the P120 pre-mRNA in U2-inactivated nuclear extract for various times, quenching with 0.25 mg/ml heparin, and electrophoresis on native polyacrylamide gels (Figure 4A, lanes 5–13). Splicing reactions containing an adenovirus pre-mRNA substrate were fractionated in parallel (lanes 1–4) to compare the mobility and kinetics of appearance of splicing complexes assembled on a major class versus an AT–AC intron.

With the P120 pre-mRNA, no specific complexes except for a highly retarded band labeled ¥ were observed in the absence of ATP (Figure 4A, lane 5). In addition to band ¥, specific complexes A-1 and A-2 appeared under

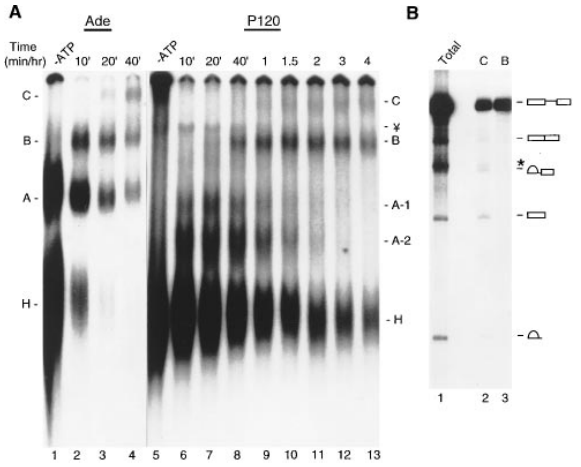

Figure 4. Native Gel Electrophoresis of Splicing Complexes

(A) Splicing reactions using adenovirus substrate were carried out in untreated nuclear extracts in the absence (lane 1) or presence (lanes 2–4) of ATP for various times (min′) as indicated at the top (40 min for the minus ATP reaction). Splicing reactions using uniformly labeled P120 substrate were performed in U2-blocked (1.2 μM U2b) nuclear extracts in the absence (lane 5) or presence (lanes 6–13) of ATP for the indicated times (min′ or hr) (4 hr for the minus ATP reaction). Complexes H, A, B, and C assembled on the adenovirus substrate are labeled on the left. Complexes H, A-1, A-2, B, and C assembled on the P120 substrate and an unidentified band (¥) are indicated on the right.

(B) A splicing reaction containing uniformly labeled P120 substrate was performed in U2b-treated extract for 4 hr. Complexes were fractionated on a native gel as shown in (A). RNAs recovered from complexes C and B, respectively, were fractionated on a denaturing polyacrylamide gel (lanes 2 and 3). Lane 1 shows an aliquot of the reaction before fractionation on the native gel.

splicing conditions after 10 min (lane 6). Complex A-1, which comigrates with complex A assembled on the adenovirus substrate (lanes 2–4), disappeared when the heparin concentration was increased to 1 mg/ml (data not shown), whereas complex A-2 was more resistant to heparin. Because both A-1 and A-2 complexes have identical kinetics of appearance, it seems likely that complex A-2 is a heparin-generated breakdown product of complex A-1 (see below). The nature of band ¥ remains obscure, since it does not hybridize to any anti-snRNA probes (see below) and appears in reactions lacking ATP or functional U12 snRNPs (lane 5 and data not shown). Complex B was observed after 40 min of incubation and continued to accumulate at later times (lanes 8–13), whereas the level of complexes A-1 and A-2 and nonspecific complex H declined as complex B increased. After 1 hr of incubation, the slowest migrating complex C appeared and accumulated (lanes 9–13). Complexes B and C assembled on the P120 pre-mRNA have electrophoretic mobilities similar to complexes B and C previously characterized for the adenovirus substrate (Konarska, 1990).

Analysis of radioactive RNAs recovered from complexes A-1, A-2, and B formed on the P120 substrate revealed only unspliced precursor and a few degraded fragments (Figure 4B, lane 3, and data not shown). Complex C contained splicing intermediates and products, as well as precursor RNA (Figure 4B, lane 2). Indeed, the time of appearance of intermediates and products in the splicing reaction (Figure 2A) correlates well with the formation of complex C in Figure 4A, arguing that complex C represents the catalytic spliceosome. The slow kinetics of formation of P120 splicing complexes suggest that some of the multiple factors that assemble on the P120 pre-mRNA may be even more limiting in the nuclear extract than those assembling on the adenovirus substrate.

The snRNP Content of AT–AC Splicing Complexes

snRNPs present in splicing complexes assembled on the P120 pre-mRNA were next examined. Splicing reactions containing optimal concentrations of nonradioactive substrate (20 nM P120 versus 75 nM adenovirus) were separated on nondenaturing polyacrylamide gels as in Figure 4A, followed by Northern blot analyses (Figure 5). U12 snRNA was readily detected in all specific complexes (A-1, A-2, B, and C) assembled on the P120 intron (lanes 7–14). The reaction lacking ATP also showed U12 comigrating with A-1 and A-2 (lane 15), perhaps because of residual ATP in the extract or representing an ATP-independent binding of U12 to the pre-mRNA.

Hybridization with a U11 snRNA probe gave a signal, albeit faint, in complex A-1, but not in any of the other splicing complexes (lanes 17–21); reactivity near the bottom of the gel represents a U11 snRNP complex not containing U12 (compare to lanes 4–15). When the heparin concentration was increased to 1 mg/ml, no significant signal whatsoever was observed with the U11 probe and U12 snRNA was detected in complexes A-2, B, and C, but not in the position of complex A-1 (data not shown). We hypothesize that complex A-1 is a functional pre-spliceosome containing both the U11 and U12 snRNPs, and that heparin treatment strips the U11 snRNP from complex A-1, reducing it to complex A-2. Accordingly, affinity chromatography examining association of snRNPs with the P120 pre-mRNA showed approximately equal signals for U11 and U12 snRNPs at both early (30 min) and later (4 hr) times in the splicing reaction (data not shown). These results hint that the U11 snRNP is present not only in presplicing complex A but also in the functional spliceosome.

The U2 probe reacted with complexes assembled on the adenovirus pre-mRNA (lane 22), but not on the P120 pre-mRNA (lanes 23–27). This confirms our earlier indications (Figures 1–3) that the U12 snRNP, but not the U2 snRNP, is a component of the spliceosome that catalyzes excision of AT–AC introns.

Hybridization with anti-sense U5, U4, and U6 RNA probes (lanes 28–51) detected all three snRNAs in splicing complexes B and C assembled on the control adenovirus substrate (lanes 29, 40, and 46); the smear near the bottom of the gel presumably corresponds to the U5–U4/U6 tri-snRNP in the nuclear extract (lanes 28–51). Surprisingly, only the U5 probe yielded a distinct signal in complex B (and perhaps also in complex C) assembled on the P120 pre-mRNA (compare lanes 33–38 with 41–45 [anti-U4] and 47–51 [anti-U6]). snRNP aggregates appeared in many of the wells of the gel whether or not the reactions contained splicing substrates and may be

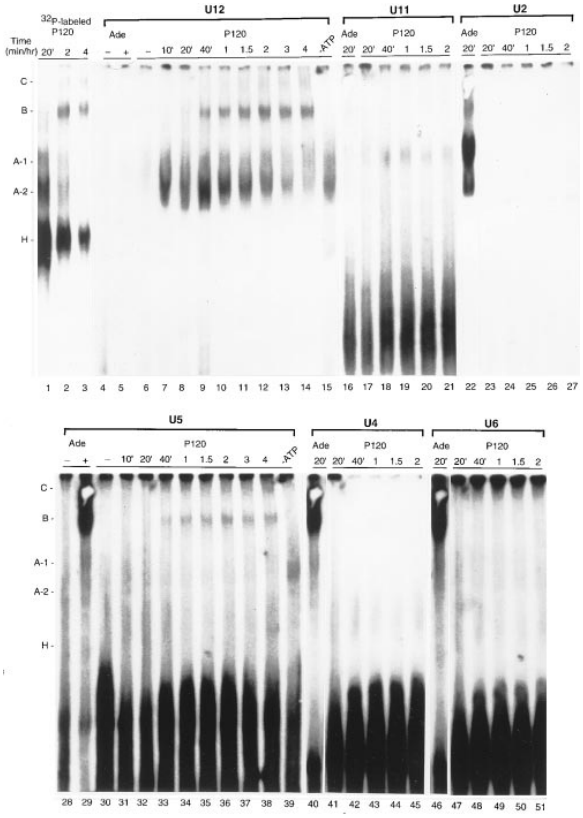

Figure 5. Northern Blot Analyses of Splicing Complexes

Splicing reactions were performed in untreated nuclear extract for 20 min in the absence (lane 4) or presence (lane 5) of adenovirus pre-mRNA, or in a U2-blocked (1.2 μM U2b) extract for the indicated times (min′ or hr) in the absence (lane 6) or presence (lanes 7–15) of P120 pre-mRNA. ATP was not included in lane 15; 4 hr incubations were performed for the minus substrate (lane 6) and minus ATP (lane 15) reactions. Complexes were separated on a native gel and transferred onto a nylon membrane. The blot was successively hybridized with the following probes: anti-U12 (lanes 4–15; 20-hr exposure), -U2 (lanes 22–27; 5 hr), -U6 (lanes 46–51; 20 hr), -U5 (lanes 28–39; 12 hr), -U4 (lanes 40–45; 20 hr), -$U5_{59-117}$ (data not shown), and -U11 (lanes 16–21; 40 hr). Selected lanes are shown for the anti-U11, -U2, -U4 and -U6 hybridized blots. Lanes 1–3 show splicing reactions containing ^{32}P-labeled P120 substrate carried out in U2b-treated extract for various times. The identities of P120 complexes as determined in Figure 4 are indicated at the left.

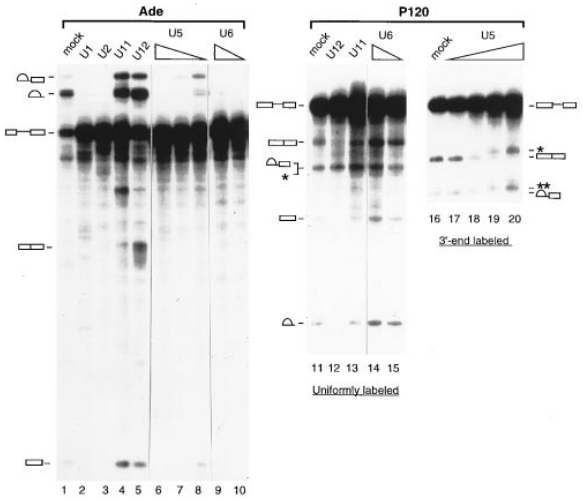

Figure 6. Effects of 2′-O-methyl Oligonucleotides on Splicing

Splicing reactions were carried out in nuclear extracts using adenovirus pre-mRNA substrate for 1.5 hr in the absence (lane 1) or presence (lanes 2–10) of 2′-O-methyl oligonucleotides as follows: 15 μM $U1_{1-14}$ (lane 2); 4 μM U2b (lane 3); 15 μM $U11_{1-20}$ (lane 4); 0.5 μM $U12_{11-28}$ (lane 5); 15, 10, or 5 μM U5L (lanes 6–8), 15 or 5 μM $U6_{27-45}$ (lanes 9–10). Uniformly labeled (lanes 11–15) or 3′-end labeled (lanes 16–20) P120 substrate was incubated in splicing reactions for 4 hr in the presence of the 2′-O-methyl oligonucleotide U2b (at 1.2 μM) alone (lanes 11 and 16) or U2b plus an additional oligonucleotide as follows: 0.5 μM $U12_{11-28}$ (lane 12); 15 μM $U1_{11-20}$ (lane 13); 15 or 5 μM $U6_{27-45}$ (lanes 14–15); 2, 5, 10, or 15 μM U5L (lanes 17–20). Symbols are as in Figure 2A. Asterisks represent degraded P120 substrate fragments. Note that lane 1 was underloaded.

especially prominent because of the high concentration of extract used in our splicing reactions (see Experimental Procedures).

We conclude that the U11 and U12 snRNPs initially and the U5 snRNP subsequently assemble on the P120 pre-mRNA. Both the U12 and U5 snRNPs remain stably associated during splicing of the AT–AC intron.

P120 Splicing Is Inhibited by Blocking the U12 and U5 snRNPs

We next investigated whether the snRNPs identified in association with the P120 pre-mRNA are essential components of the AT–AC spliceosome by using 2′-O-methyl oligonucleotides complementary to various snRNAs (Figure 6). As expected, splicing of the control adenovirus substrate was inhibited by 2′-O-methyl oligonucleotides $U1_{1-14}$ and U2b at concentrations of 15 and 4 μM, respectively, whereas oligonucleotides complementary to nt 1–20 of U11 or 11–28 of U12 had no effect (lanes 1–5). With the P120 pre-mRNA, titration of $U1_{1-14}$ at 5–15 μM or U2b at 0.4–4 μM instead increased correct splicing (Figure 1 and data not shown). In contrast, the 2′-O-methyl oligonucleotide $U12_{11-28}$, which sequesters the branch site pairing sequence of U12, effectively blocked splicing of the P120 substrate (lane 12; 97% inhibition), confirming data shown in Figure 2B.

Because the 5′ end of U11 snRNA has been proposed to pair with the 5′ splice site of AT–AC introns (Hall and Padgett, 1994), a 2′-O-methyl oligonucleotide designed to sequester its first 20 nucleotides was titrated into the extract. No effect on the splicing of the P120 pre-mRNA was detected even at an oligonucleotide concentration of 15 μM (3000-fold more than the calculated concentration of U11 snRNPs in the extract; lane 13). The binding of a biotinylated oligonucleotide complementary to a similar region of U11 snRNA had been assessed previously; the selection of only 15% of the U11 snRNPs in nuclear extract suggested limited accessibility of this region (Wassarman and Steitz, 1992). At present, whether U11 directly contacts the P120 pre-mRNA is not clear.

Although there exist at least seven U5 variants in HeLa cells, all contain an invariant 9 nt sequence in their first loop (Sontheimer and Steitz, 1992) that is implicated in exon interactions during splicing of major class introns

(Newman and Norman, 1992; Wyatt et al., 1992; Sontheimer and Steitz, 1993). When a 2′-O-methyl oligonucleotide (also containing 2,6-diaminopurine; Lamm et al., 1991) complementary to loop 1 of U5 was used to pretreat nuclear extracts, inhibition of adenovirus splicing resulted (lanes 6–8), as expected. Lanes 16–20 show that P120 splicing likewise became inactivated with increasing amounts of the U5L oligonucleotide (94% inhibition at 15 μM U5L). For unknown reasons, high concentrations of U5L markedly enhanced degradation of the P120 pre-mRNA; therefore, 3′-end-labeled rather than uniformly labeled P120 substrate was used for this experiment and electrophoresis was carried out for twice as long as usual to resolve the spliced product and lariat intermediate from prominent degraded fragments (asterisk and double asterisk; lanes 16–20). U5 participation in P120 splicing was confirmed by the observation that formation of complexes B and C on the P120 substrate was impaired by pretreatment of the splicing extract with 10–15 μM U5L; control experiments also ruled out specific degradation of splicing product and lariat intermediate by the U5L oligonucleotide (data not shown). Note that the concentrations of U5L required to inactivate splicing of the adenovirus and P120 substrates are similar (lanes 6–8 and 17–20), arguing that the same conserved 9 nt loop sequence is shared by the U5 variant(s) involved in P120 splicing and those in the major spliceosome (Sontheimer and Steitz, 1992).

Finally, 2′-O-methyl oligonucleotides complementary to U4 and U6 snRNAs were evaluated for their effect on P120 pre-mRNA splicing. In control experiments, splicing of the adenovirus substrate was completely abolished by oligonucleotide $U6_{27–46}$, which masks the phylogenetically conserved ACAGAG box and its upstream region in U6 snRNA (lanes 9–10). Strikingly, splicing of the P120 pre-mRNA in the $U6_{27–46}$-pretreated extract was not diminished (lanes 14–15). This result is consistent with the lack of detectable U6 in Northern blot analyses of splicing complexes formed on the P120 substrate (Figure 5), but alternative explanations are possible (see Discussion). Unfortunately, under our splicing conditions, a 2′-O-methyl oligonucleotide complementary to nt 1–20 of U4 snRNA did not inhibit adenovirus splicing as previously reported (Blencowe et al., 1989). Hence, the negative result we obtained with this oligonucleotide for the P120 pre-mRNA (data not shown) cannot be interpreted.

Discussion

An active in vitro system for the splicing of a pre-mRNA containing an AT–AC intron has provided the first mechanistic insights into the functioning of a novel minor spliceosome present in vertebrate (and perhaps all metazoan) cells. We have established that the low abundance U12 snRNP and the U2 snRNP act analogously with respect to branch site recognition in their respective spliceosomes. Additionally, we have characterized a splicing intermediate, the intron–3′ exon, as a lariat structure in which an adenosine within the conserved element upstream of the 3′ splice site serves as the branch point, forming an A–A linkage. Thus, excision of AT–AC introns follows a two-step pathway akin to that characterized for self-splicing of group II introns and for splicing of the major class of pre-mRNA introns. Finally, we show that the AT–AC spliceosome contains three known Sm snRNPs (U5, U11, and U12) and demonstrate that two of these (U5 and U12) are essential for the splicing reaction.

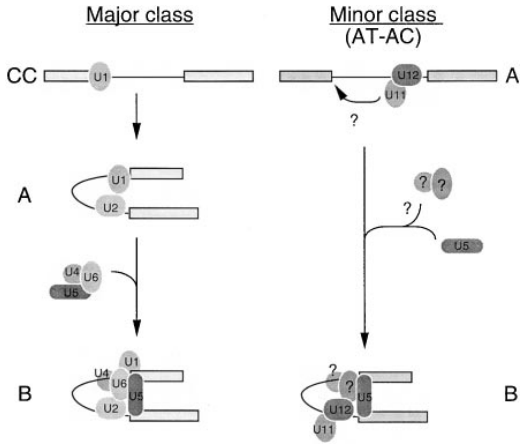

Figure 7. Schematic Pathway of Splicing Complex Assembly

The left diagram shows splicing complexes assembled on a pre-mRNA containing a major class intron, modified from Moore et al. (1993). U1 binds to the 5′ splice site, together with non-snRNP factors (not shown here), to form a commitment complex (CC). U2 subsequently binds to the branch site to form presplicing complex A. Complex B is formed by recruiting the U5-U4/U6 tri-snRNP into the spliceosome. The right diagram shows assembly of splicing complexes on an AT–AC intron. Association of U11, U12, and U5 snRNPs with the pre-mRNA was observed by native gel electrophoresis. The pre-spliceosome, complex A, contains both U11 and U12 snRNPs; the binding of U12 to the intron branch site was demonstrated by psoralen cross-linking. Complexes A-1 and A-2 are not discriminated here but are discussed in the text. The U5 snRNP then joins the prespliceosome to form complex B, which may contain other as-yet-unidentified snRNPs. Whether the 5′ splice site is recognized by U11 and whether U5 associates with other snRNP(s) before entering the splicing complex remain to be established.

In Figure 7, a proposed pathway for assembly of the AT–AC spliceosome is compared with that of the well-studied spliceosome formed on the majority of pre-mRNA introns. Under splicing conditions, the U11 and U12 snRNPs associate with the precursor RNA to form a presplicing complex, detected by both native gel electrophoresis (Figure 5) and affinity chromatography (data not shown). The binding of the U12 snRNP involves a base-pairing interaction with the intron branch site, yielding a diagnostic psoralen cross-link. Presplicing complexes A-1 and A-2 are distinguished by the presence of the U11 snRNP: U11 appears only in the A-1 complex on native gels, suggesting that complex A-2 results from stripping of the U11 snRNP by heparin. The U5 snRNP subsequently joins complex A to form a slower migrating complex B, whose mobility hints that other as-yet-unidentified snRNPs may be present. Complex B then undergoes conversion to the catalytically active C complex, which generates splicing intermediates and products (not shown in Figure 7). The parallels with the major spliceosome are striking, but much remains to be learned about the AT–AC spliceosome.

The U12 snRNP Replaces U2 in the AT–AC Spliceosome

Accurate splicing of the P120 pre-mRNA was activated by debilitating either the U2 snRNP (Figure 1) or the U1 snRNP (data not shown) in vitro, suggesting that formation of the major spliceosome effectively competes with the binding of low abundance snRNPs to this particular AT–AC intron. Indeed, when the U2 snRNP remained unblocked in the extract, aberrant splicing at cryptic sites that are close to the authentic AT–AC splice sites (Figure 1) and cross-linking of U2 snRNA to the P120 pre-mRNA (data not shown) were observed. Although we do not yet know whether such cryptic splicing characterizes all AT–AC introns, how the low abundance U11 and U12 snRNPs manage to associate competitively with U1 and U2 on the P120 intron in vivo remains mysterious.

Our cross-linking evidence argues that U12 snRNA, like U2, base-pairs with a conserved sequence near the 3′ end of the P120 intron, probably bulging out an adenosine from the intermolecular helix (Figure 3). Accordingly, sequestering the recognition sequence in U12 snRNA with a 2′-O-methyl oligonucleotide blocks formation of both U12/pre-mRNA cross-links (see Figure 3), as well as the appearance of splicing complexes, intermediates, and products (Figures 2 and 6 and data not shown). Very recently, Hall and Padgett (1996) have demonstrated a requirement for U12 snRNA in in vivo splicing of an AT–AC intron by showing that mutations in the conserved 3′ element of the P120 intron can be suppressed by compensatory changes in the predicted branch site pairing sequence of U12 snRNA. Leaving an adenosine unpaired for nucleophilic attack is a mechanism similar to that established for the self-splicing of group II introns and for spliceosome-mediated removal of the major class of introns from nuclear pre-mRNAs. Thus, U12 snRNA, like U2 snRNA and sequences within domain VI of group II introns, is a likely contributor to activation of the branch site 2′-OH group during the first step of splicing.

What Recognizes the 5′ Splice Site?

We have not been able to confirm a base-pairing interaction between the 5′ splice site of the AT–AC intron and the 5′ end of U11 snRNA, as proposed by Hall and Padgett (1994). First, a 2′-O-methyl oligonucleotide complementary to the 5′ end of U11 RNA did not inhibit splicing of the P120 substrate (Figure 6). Second, even though an ideal cross-linking site with U11 is predicted near the 5′ end of the P120 intron, an unidentified psoralen cross-linked species could not be targeted by RNase H in the presence of two different U11 oligonucleotides (Figure 3 and data not shown). Nonetheless, U11's structural similarities to U1 (Montzka and Steitz, 1988), as well as its association with U12 (Wassarman and Steitz, 1992) and with the pre-mRNA during splicing (Figure 5), suggest an active role for the U11 snRNP in splice site selection or in assisting U12 association, as characterized for the U1 snRNP in addition to its base-pairing function (Tarn and Steitz, 1995).

An alternative possibility is that a splicing component other than U11 recognizes the 5′ splice site and recruits an snRNP that functions analogously to U6 (Konforti et al., 1993; Crispino and Sharp, 1995) to the AT–AC spliceosome. Whereas the 5′ splice site of major class introns is sequentially recognized by SR proteins, U1 and U6 snRNPs (Kandels-Lewis and Seraphin, 1993; Konforti et al., 1993; Lesser and Guthrie, 1993; Kohtz et al., 1994), AT–AC introns may bypass this sequential recognition mechanism by containing a stretch of absolutely conserved nucleotides at the 5′ splice site (Hall and Padgett, 1994). The 5′ end of the intron could be defined by the U5 snRNP alone, just as U5 interactions can determine the 5′ cleavage site in major class introns under special circumstances (Newman and Norman, 1992; Cortes et al., 1993). Or, a U6 analog could be recruited directly to the 5′ splice site. Previously, we showed that splicing of the adenovirus pre-mRNA could be reactivated by excess amounts of SR proteins in nuclear extracts pretreated with a 2′-O-methyl oligonucleotide $U1_{1–14}$ (Tarn and Steitz, 1994), suggesting direct U6 binding. In contrast, proper splicing of the P120 pre-mRNA in U1- or U2-debilitated extracts does not require excess SR proteins; supplementation of U2-blocked S100 extract with SR proteins showed that endogenous levels of SR proteins are essential (Tarn and Steitz, unpublished data). This indicates that at least some SR proteins are shared by the major and the AT–AC spliceosome and could contribute to 5′ splice site recognition in AT–AC introns.

U5: a Common Component of Two Kinds of Spliceosomes

Our results implicate a U5 snRNP in the splicing of the P120 pre-mRNA (Figures 5 and 6). Seven characterized U5 variants can be detected in affinity-purified spliceosomes assembled on a major class intron (Sontheimer and Steitz, 1992); it is not yet known which one (or more) of these, or even an as-yet-unidentified U5 variant, is used by an AT–AC intron. Rehybridization of a Northern blot containing P120 splicing complexes with an antisense U5 probe lacking the invariant loop 1 sequence (data not shown) argues against the possibility that only this conserved sequence, imbedded in a novel snRNA, was detected in Figure 5.

Why is U5 apparently the only snRNP that is shared by two different kinds of spliceosomes? Both genetic (Newman and Norman, 1992) and site-specific cross-linking (Wyatt et al., 1992; Sontheimer and Steitz, 1993) studies have revealed intimate contacts of the invariant first loop of U5 RNA with both the 5′ and 3′ exons during the splicing reaction. Thus, this U5 loop has been viewed as the spliceosomal counterpart of the internal guide sequence of self-splicing group I introns and of the exon binding site 1 of group II introns (Sontheimer and Steitz, 1993, references therein); its U-rich character has been hypothesized to confer extensive flexibility in interacting with divergent exon sequences. The highly conserved very large U5 snRNP protein, p220, may stabilize or act to facilitate these interactions (Wyatt et al., 1992). U5 is not known to be involved in specific base-pairing interactions with any other spliceosomal snRNP and may therefore be uniquely suited to join a distinct set of snRNPs in the low abundance AT–AC spliceosome.

Definitive analysis of the U5 sequence(s) present in the AT–AC spliceosome is now required.

What Substitutes for U4 and U6 in the AT–AC Spliceosome?

U6, the most conserved spliceosomal snRNA, plays critical roles in pre-mRNA splicing, including proofreading of the 5′ splice site (Kandels-Lewis and Seraphin, 1993; Lesser and Guthrie, 1993) and possibly catalysis of the splicing reaction itself (reviewed in Guthrie, 1991; Weiner, 1993). In the major class spliceosome, U6 engages in several distinct base-pairing interactions with U2, which serve to juxtapose the two reactants (the 5′ splice site and the bulged branch nucleotide) prior to the first covalent reaction (Madhani and Guthrie, 1992; Sun and Manley, 1995). Two alternative U2/U6 structures, similar either to domain V of group II introns or to a hairpin ribozyme, have been postulated. We previously pointed out that U12 snRNA might be able to substitute for U2 in each of these RNA–RNA interactions with U6 (Tarn et al., 1995); however, our current results from Northern blot analyses (Figure 5) and splicing inhibition experiments (Figure 6) do not support this proposal. Failure to detect U6 snRNA in the P120 spliceosome by Northern blot analysis could be due to sequestration of U6 sequences by other splicing components. Yet, the apparent absence of U4 as well argues that this well-characterized two-snRNP complex may not participate in the removal of AT–AC introns from nuclear pre-mRNAs.

If the U4/U6 snRNP is not essential for splicing of the P120 pre-mRNA, what snRNP(s) or protein factor(s) substitute? And, does it (they) preassociate with the U5 snRNP to form a multi-snRNP particle prior to AT–AC spliceosome assembly? We have investigated the possible participation of the 7SK snRNP, whose RNA (like U6) is γ-methyl capped and highly conserved, in P120 splicing, but no positive evidence has emerged so far. It will be interesting to examine whether one of the additional HeLa cell snRNAs precipitated with antibodies against γ-methyl guanosine is a U6 counterpart in the AT–AC spliceosome (Gupta et al., 1990). Characterization of such an RNA will provide important new information to test and hone current ideas (Weiner, 1993) concerning the functional relationship between U6 snRNA and domain V (the most highly conserved) of group II self-splicing introns.

Other Questions Raised by the AT–AC Spliceosome

Presently, it appears that only a few splicing components are shared by the major and minor class human spliceosomes. These include the U5 snRNP, the Sm (core) proteins of the U11 and U12 snRNPs, and some member(s) of the SR protein family. It is not clear if the U11 particle contains any U1-specific proteins or if U12 associates with polypeptides specific to the 17S U2 snRNP (reviewed in Hodges and Beggs, 1994). Although two proteins that seem to be unique to the U11 snRNP have been identified as targets of rare autoantibodies (Gilliam and Steitz, 1993), whether they are associated with the AT–AC spliceosome remains to be seen. In contrast to the major class of introns, the region between the branch site and the 3′ splice site of AT–AC introns is relatively short (9–12 nt) and lacks a long pyrimidine stretch. This and the high conservation of the branch site sequence suggest that binding by non-snRNP proteins (such as U2 auxiliary factor) to the region between the branch point and the 3′ splice site (reviewed in Lamm and Lamond, 1993) may not be required for splicing of AT–AC introns. But, if the orderly assembly of spliceosomes on a multi-intron pre-mRNA is achieved by U1/U2 contacts across exons (reviewed in Berget, 1995), can U1 communicate with U12 and U11 with U2 as well?

Previously, Gontarek et al. (1993) proposed a role for the U11/U12 snRNP in negative control of splicing of an NRS (negative regulator of splicing)-containing intron. The NRS within the *gag* gene of RSV modulates the ratio of unspliced to spliced retroviral RNA. Although a base-pairing interaction between loop 3 of U11 snRNA and the NRS was suggested, exactly how binding of the U11/U12 snRNP might inhibit splicing of the NRS-containing pre-mRNA remained unclear. Perhaps, the U11/U12 snRNP plays opposing roles in the removal of major class versus AT–AC introns from pre-mRNAs.

The few genes known to contain an AT–AC intron appear to be quite diverse; however, commonalities may emerge as more AT–AC introns are identified. Perhaps the discovery of additional AT–AC introns will reveal a tissue specificity or other regulatory role for this novel intron class. The distinctive components of the AT–AC spliceosome, the U11 and U12 snRNPs, need to be investigated for their relative abundance in various tissues and their presence in other species. The fundamental question of why metazoan cells maintain a second, low abundance pre-mRNA splicing machinery provides an impetus for many future studies of the AT–AC spliceosome.

Experimental Procedures

Oligonucleotides

Both deoxyoligonucleotides and 2′-O-methyl oligoribonucleotides were synthesized by John Flory at Yale University on an Applied Biosystems DNA synthesizer. The following 2′-O-methyl oligoribonucleotides were used for pretreatment of nuclear extracts: $U1_{1–14}$; U2b, complementary to nt 27–49 of U2 snRNA (both described by Tarn and Steitz, 1994); U5L (5′-UUZGUZZZZGGCGAAA-3′; Z represents diaminopurine), complementary to nt 33–48 of U5 snRNA; $U6_{27–46}$ (5′-CUCUGUAUCGUUCCAAUUUU-3′); $U11_{1–20}$ (5′-UCACGAC AGAAGCCCUUUUU-3′); and $U12_{11–28}$ (5′-AUUUUCCUUACUCAU AAG-3′). Biotinylated 2′-O-methyl oligoribonucleotide P120bio (5′-BBBBGCUUGCCAAGCAGGAAGUCUCC-3′; B represents biotin), complementary to nt 153–174 of exon 2 of the P120L pre-mRNA substrate, was used for affinity selection of splicing complexes. Deoxyoligonucleotides used for primer extension were P107 (5′-CAGAACGAGACCGCCCTTCC-3′), complementary to nt 86–105 of the 3′ exon of P120L; P110 (5′-ACTGTGTCATGATGTCCTAA-3′), complementary to nt 64–83 of the P120 intron; P118 (5′-CCCACAAT ATCCTGGATCCG-3′), complementary to nt 36–55 of the 3′ exon of P120; and 9P, complementary to nt 122–147 of U12 snRNA (Montzka and Steitz, 1988). Antisense deoxyoligonucleotides used for RNase H cleavage were $U1_{64–75}$, $U2_{79–98}$ (both described by Tarn and Steitz, 1994), 10L and 9C, complementary to nt 52–71 of U11 and 53–71 of U12 snRNAs, respectively (Montzka and Steitz, 1988).

Plasmid Construction

For construction of pP120L, a PCR-amplified DNA fragment was first generated using pCB6P120m (a gift from Richard Padgett,

Cleveland Clinic) as template; this fragment containing the T7 promoter and the entire sequence of exon 6-intron F-exon 7 of the human P120 gene (Larson et al., 1990; Hall and Padgett, 1994) was then inserted into pSP64. Likewise, the pP120 plasmid was constructed by insertion of the fragment described above but lacking 105 bp from the 3′ end of exon 7 into pSP64. Both plasmids were linearized with HindIII for use as template in transcription reactions.

Splicing Substrates

Pre-mRNA substrates for in vitro splicing were prepared by in vitro transcription; reactions were performed according to Tarn and Steitz (1994), except for using T7 RNA polymerase to produce P120L and P120 substrates. 3′-end labeling of RNA was according to Lingner and Keller (1993) except that the concentration of [^{32}P]cordycepin (5000 Ci/mmol) was 5-fold higher than that of the RNA substrate and E. coli poly(A) polymerase (Pharmacia) was used. Nonradioactive transcripts were prepared in the absence of [^{32}P]UTP in reactions with 10-fold the usual volume. All transcripts were gel purified.

In Vitro Splicing Assay

In vitro splicing assays using 60% HeLa nuclear extract were essentially as described by Tarn and Steitz (1994). For splicing of the P120 substrates, 40 mM KCl and 1.2 μM 2′-O-methyl oligonucleotide U2b were used, and the incubation time was extended to 4 hr. For the minus ATP reaction, ATP and creatine phosphate were omitted from the reaction, and the extract was preincubated at 30°C for 15 min to hydrolyze endogenous ATP. Pretreatment of the nuclear extract with 2′-O-methyl oligoribonucleotides was performed by incubating the extract with oligonucleotides under splicing conditions for 10 min before adding the splicing substrate. Each microliter of reaction contained 1×10^3 cpm (~0.3 fmol) ^{32}P-UTP-labeled adenovirus substrate, 3×10^3 cpm (~2 fmol) uniformly labeled P120 pre-mRNA, or 2×10^3 cpm (~2 fmol) 3′-end-labeled P120 pre-mRNA. For 2′-O-methyl oligonucleotide U5L-pretreated extracts, 1 mM dithiothreitol was included to maintain the activity of RNasin (Boehringer Mannheim), an RNase inhibitor. Debranching reactions were carried out as described by Ruskin and Green (1990). RNAs isolated from splicing reactions were fractionated on 8% denaturing polyacrylamide gels, except for Figure 1 where a 5% gel was used.

Psoralen Cross-Linking, RNase H Digestion and Primer Extension

For psoralen cross-linking experiments, splicing reactions contained 1.5×10^4 cpm high specific-activity substrates (per microliter of reaction), which were prepared as described by Tarn and Steitz (1994), and 3% PEG8000, which increases cross-linking efficiency. To gel purify lariat intermediate and psoralen cross-linked RNAs, the volume of splicing reactions was enlarged to 100–200 μl and 0.5–1 ng/μl unlabeled pre-mRNA was included. Psoralen cross-linking, RNase H digestion, and primer extension were performed as described by Tarn and Steitz (1994).

Native Gels and Northern Blot Analysis

Splicing reactions were carried out as described above and terminated by addition of 0.25 mg/ml heparin at the indicated time. The gel system used was described by Konarska (1990), with electrophoresis performed at 300 V for 30 min and then at 250 V for 4 hr. For Northern blot analysis, optimal concentrations of nonradioactive splicing substrates (10 ng/μl adenovirus substrate or 2.5 ng/μl P120 pre-mRNA) were included in the reactions. Electrophoretic transfer was performed at 20 volts in 0.5 × Tris–borate–EDTA overnight. Prehybridization and hybridization were performed as described by Tarn et al. (1995) except that 0.1 mg/ml yeast RNA and 5×10^6 cpm anti-sense RNA probe were included. Anti-sense RNA probes for small RNAs with similar specific activities were produced by in vitro transcription as described by Wassarman and Steitz (1992). Plasmids used to synthesize anti-sense U snRNA probes were described by Black and Pinto (1989) (U2, U4, U5 and U6) and Wassarman and Steitz (1992) (U11 and U12). The membrane was washed sequentially in 2 × SSC plus 0.1% SDS, 0.5 × SSC plus 0.1% SDS, and 0.1 × SSC plus 0.1% SDS at room temperature for 15 min at each step and finally at 55°C for 15 min in 0.1 × SSC plus 0.1% SDS. The blot was stripped by incubation in 0.1 × SSC plus 0.5% SDS at 95°C for 15 min.

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