Initial recognition of U12-dependent introns requires both U11/5' splice-site and U12/branchpoint interactions

Mikko J. Frilander and Joan A. Steitz¹

Department of Molecular Biophysics and Biochemistry, Yale School of Medicine, Howard Hughes Medical Institute, New Haven, Connecticut 06536-0812 USA

We have investigated the formation of prespliceosomal complex A in HeLa nuclear extracts on a splicing substrate containing an AT-AC (U12-type) intron from the *P120* gene. Using an RNase H protection assay and specific blocking oligonucleotides, we find that recognition of the 5' splice-site (5'ss) and branchpoint sequence (BPS) elements by U11 and U12 snRNPs, respectively, displays strong cooperativity, requiring both sites in the pre-mRNA substrate for efficient complex formation. Deletion analysis indicates that beside the 5'ss and BPS, no additional elements in the pre-mRNA are necessary for A-complex formation, although 5' exon sequences provide stimulation. Cross-linking studies with pre-mRNAs containing the 5'ss or BPS alone indicate that recognition of the BPS by the U12 snRNP is stimulated at least 20- to 30-fold by the binding of the U11 snRNP to the 5'ss in the same pre-mRNA molecule, whereas recognition of the 5'ss by U11 is stimulated approximately fivefold by the U12/BPS interaction. These results argue that intron recognition in the U12-dependent splicing pathway is carried out by a single U11/U12 di-snRNP complex, suggesting greater rigidity in the intron recognition process than in the major spliceosome.

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The removal of introns from pre-mRNA molecules is carried out by the spliceosome, a large RNA-protein complex, which for most introns is composed of five small nuclear ribonucleoprotein (snRNP) particles (denoted U1, U2, U4, U5, and U6) and numerous protein factors. In vitro studies have indicated that the snRNP and protein components assemble sequentially onto each intron (Moore et al. 1993), yielding several distinct intermediates, complexes E, A, B, and C, for the mammalian system (Konarska and Sharp 1987; Michaud and Reed 1991; Jamison et al. 1992). Conserved sequence elements near the 5' and 3' ends of the intron are recognized during the formation of the earliest prespliceosomal complex E by the U1 snRNP and by the splicing factor U2AF, which bind to the 5' splice site (5'ss) and polypyrimidine tract (PPT), respectively, in an ATP-independent manner (Michaud and Reed 1991, 1993). The E complex contains several additional protein factors that contribute to its formation. For instance, SR proteins have been shown to promote 5'ss recognition by the U1 snRNP (Kohtz et al. 1994; Staknis and Reed 1994; Jamison et al. 1995), and both SR proteins and mBBP/SF1 are thought to bridge the 5' and the 3' portions of the

intron by simultaneously interacting with the U1 snRNP at the 5'ss and U2AF bound to the PPT, thus forming a functional link between the 5' and 3' ends of the intron (Michaud and Reed 1993; Wu and Maniatis 1993; Abovich and Rosbash 1997). Complex A is the first ATP-dependent spliceosome assembly intermediate and is formed when the U2 snRNP binds to the branchpoint sequence (BPS). The formation of A complex requires base-pairing between the U2 snRNA and the BPS (Zhuang and Weiner 1989; Query et al. 1994). In addition to this RNA-RNA interaction, the SF3 proteins present in the 17S form of the U2 snRNP and U2AF bound to the PPT are essential for A complex assembly (Ruskin et al. 1988; Gozani et al. 1996; Yu et al. 1998). Later spliceosomal complexes (B and C) are formed after the U4/U6.U5 tri-snRNP joins the nascent spliceosome. An extensive network of snRNA-snRNA and snRNA-pre-mRNA interactions then orchestrate dynamic changes that convert the spliceosome to a catalytically active conformation (for reviews, see Moore et al. 1993; Madhani and Guthrie 1994; Umen and Guthrie 1995).

In addition to the U2-dependent spliceosome described above, a parallel U12-dependent pre-mRNA splicing machinery exists in mammalian cells, as well as in other higher organisms, including insects and plants. U12-type introns contain divergent, but highly conserved, 5'ss and BPS signals (Hall and Padgett 1994; for

¹Corresponding author.

E-MAIL Steitzj@maspo2.mas.yale.edu; FAX (203) 624-8213.

reviews, see Sharp and Burge 1997; Tarn and Steitz 1997). Their removal is catalyzed by a novel, low-abundance spliceosome in which the U1, U2, U4, and U6 snRNPs are replaced by highly divergent counterparts, the U11, U12, U4atac, and U6atac snRNPs, whereas the U5 snRNP is apparently shared by the two spliceosomes (Hall and Padgett 1996; Tarn and Steitz 1996a,b). The assembly pathway of the U12-dependent spliceosome mimics that of the U2-dependent spliceosome, with characteristic prespliceosomal complex A, mature spliceosomal complex B, and catalytically active complex C (Tarn and Steitz 1996a,b). Moreover, the snRNA–snRNA and snRNA–pre-mRNA interactions formed during U12-dependent spliceosome assembly are highly similar to interactions detected during assembly of the U2-dependent spliceosome. Specifically, both biochemical and genetic evidence show that the 5'ss is recognized by the U11 snRNP, followed by U6atac, whereas the BPS binds the U12 snRNP. The base-pairing interactions between the U12 and U6atac snRNAs in the catalytically active spliceosome are also highly similar to interactions found for the U2-dependent spliceosome in both the mammalian and yeast systems (Hall and Padgett 1996; Tarn and Steitz 1996a,b; Kolosova and Padgett 1997; Yu and Steitz 1997; Incorvaia and Padgett 1998).

Despite their overall similarity, the two splicing systems display important differences. First, the 5'ss and the BPS elements in U12-type introns are highly conserved, with no apparent PPT between the BPS and 3' splice-site (3'ss) (Sharp and Burge 1997). In addition, some U11 and all of the U12 snRNPs exist in splicing extracts as di-snRNP complexes rather than as the discrete U1 and U2 snRNP particles characteristic of the U2-dependent system; free U11 mono-snRNP particle reflects the higher abundance of U11 compared with U12 (Montzka and Steitz 1988; K.M. Wassarman and Steitz 1992). Finally, the cellular abundance of all the snRNP components specific to the U12-dependent spliceosome is ~100-fold lower than their counterparts in the U2-dependent spliceosome (Montzka and Steitz 1988; Tarn and Steitz 1996b). These differences suggest that the early recognition steps in U12-dependent spliceosome formation might follow a different pathway, but it is equally possible that the 5'ss or BPS could initially be recognized independently (as in U2-dependent system) by the free U11 mono-snRNP or the U11/U12 complex. Here we show a requirement for both the 5'ss and BPS during early complex formation, suggesting recognition of both sites in a U12-dependent intron by a single U11/U12 di-snRNP particle.

Results

An ATP-independent commitment complex has been described as the earliest specific step in spliceosome assembly for both yeast and mammalian U2-dependent splicing systems. Such a complex can be formed in the absence of ATP and is resistant to subsequent challenge by a vast excess (50- to 1000-fold) of splicing substrate in the presence of ATP (Séraphin and Rosbash 1989; Mi-

chaud and Reed 1991; Jamison et al. 1992). We were not able to identify a stable ATP-independent commitment complex in the U12-dependent splicing system using the P120 splicing substrate (data not shown). Therefore, formation of the earliest detectable assembly intermediate, the A complex, was used to study intron recognition in the U12-dependent splicing system.

Use of 2'-O-methyl oligonucleotides complementary to U6atac or U6 snRNA to arrest spliceosome assembly

Previously, four specific complexes, A1, A2, B, and C, were observed during assembly of the U12-dependent spliceosome on the P120 splicing substrate (Tarn and Steitz 1996a). The appearance of these complexes required inhibition of the competing U2-dependent splicing machinery with a 2'-O-methyl oligonucleotide U2b (called U2₂₇₋₄₉ here), which prevents the U2/BPS interaction. Complex A2, containing only the U12 snRNP, was deduced to be a heparin-induced dissociation product of complex A1, which contains both the U11 and U12 snRNPs (Tarn and Steitz 1996a). We performed heparin titration experiments (not shown) and established that a single A complex band containing both the U11 and U12 snRNPs was obtained when the heparin concentration in the native gel loading buffer was decreased to 25 µg/ml (see below). This heparin concentration was therefore used in all subsequent native gel analyses of U12-dependent splicing.

To study initial recognition of the 5'ss and the BPS in a U12-type intron, it was necessary to prevent the displacement of the U11 snRNP by U6atac, which occurs during B complex formation (Tarn and Steitz 1996b; Incorvaia and Padgett 1998). Assembly of the U2-dependent spliceosome can be arrested at the A complex stage by preincubating the splicing reaction with a 2'-O-methyl oligonucleotide complementary to nucleotides 1–20 of U2 snRNA prior to the addition of pre-mRNA (Lamond et al. 1989; Furman and Glitz 1995). This oligonucleotide binds to the first stemloop of U2 snRNA and prevents critical U2/U6 snRNA interactions necessary for later steps in spliceosome assembly. The analogous region in U12 snRNA is adjacent to the region complementary to the BPS of U12-type introns. To avoid overlap with the anti-U12 oligonucleotide used later, a 2'-O-methyl oligonucleotide U6atac₁₋₂₀, complementary to the nucleotides 1–20 of the U6atac snRNA, was used instead to block the interaction of U6atac snRNA with the 5'ss of the P120 substrate (Fig. 1A).

On addition of a labeled P120 splicing substrate to a HeLa splicing reaction blocked for U2-dependent spliceosome assembly by preincubation with the 2'-O-methyl oligonucleotide U2₂₇₋₄₉, complexes A and B can be observed (Fig. 1B, lanes 1–6), as described previously (Tarn and Steitz 1996a). Complex C is not visible, as assembly of this complex requires ~2 hr of incubation. When the preincubation was performed in the presence of both the U2₂₇₋₄₉ and U6atac₁₋₂₀ 2'-O-methyl oligonucleotides, no B-complex formation was observed. Instead, spliceo-

U12-dependent spliceosome assembly

Figure 1. Arrest of spliceosome assembly at the A complex stage. (A) Secondary structure model of RNA–RNA interactions between the pre-mRNA and U6atac/U12 snRNAs in the U12-dependent spliceosome (modified from Tarn and Steitz 1996b). The region of U6atac complementary to the U6atac₁₋₂₀ 2'-O-methyl oligonucleotide is underlined. (B) Native gel analysis of splicing complexes formed on the U12-type P120 splicing substrate. (Left) Complex formation in a mock-treated splicing reaction; (right) complex formation in a reaction preincubated with 0.5 μ M U6atac₁₋₂₀ 2'-O-methyl oligonucleotide. (C) Northern analysis of the complexes detected in U12-dependent spliceosome assembly reactions. The reactions were preincubated either with or without the U6atac₁₋₂₀ 2'-O-methyl oligonucleotide, as indicated. Following preincubation, unlabeled P120 splicing substrate was added, incubation was continued for 60 min, and the reactions were separated in a native gel and transferred to a membrane. U11 and U12 snRNAs were detected using full-length complementary riboprobes as indicated. (D) Secondary structure model of RNA–RNA interactions between the pre-mRNA and U6/U2 snRNAs in the U2-dependent spliceosome. The structure is based on the model of Madhani and Guthrie (1992), modified to include helix III suggested by Sun and Manley (1995). The region of U6 snRNA complementary to the U6₂₇₋₄₆ 2'-O-methyl oligonucleotide is underlined. (E) Native gel analysis of splicing complexes formed on the U2-type adenovirus splicing substrate. (Left) Complex formation in a mock-treated splicing reaction; (right) complex formation in a reaction preincubated with 5 μ M U6₂₇₋₄₆ 2'-O-methyl oligonucleotide. The slowest migrating band present at later time points (80 and 100 min) has an intermediate mobility compared with complexes B and C (left).

some assembly was arrested at what appears to be the A complex stage (Fig. 1B, lanes 7–12). Northern analysis of reactions containing unlabeled P120 splicing substrate (Fig. 1C) confirmed the presence of U11 and U12 snRNAs in both normal and arrested A complexes, whereas no U11 snRNA was detected in the B complex, as described previously (Tarn and Steitz 1996a). The kinetics of A complex formation in untreated compared with U6atac₁₋₂₀-blocked assembly reactions was similar, both peaking after 60 min of incubation.

To confirm that arrest at the A complex stage by the U6atac₁₋₂₀ oligonucleotide is due to blockage of a specific region in U6atac snRNA, we examined the effect of an analogous 2'-O-methyl oligonucleotide on U2-dependent spliceosome assembly. Splicing reactions were preincubated either without any oligonucleotide or with a 2'-O-methyl oligonucleotide complementary to nucleo-

tides 27–46 of U6 (U6₂₇₋₄₆; Fig. 1D) prior to addition of the labeled adenovirus splicing substrate. Native gel analysis revealed that, similar to the U12-dependent system, U2-dependent spliceosome assembly was arrested at the A complex stage (Fig. 1E, lanes 9–16). We conclude that in both splicing systems, B complex formation can be prevented by specific 2'-O-methyl oligonucleotides that block the U6atac/5'ss or U6/5'ss interaction.

The 5'ss and BPS of the P120 splicing substrate are recognized cooperatively

The existence of a U11/U12 di-snRNP complex, as opposed to U1 and U2 mono-snRNPs, suggested that the 5'ss and the BPS might be recognized concurrently during the earliest stage of U12-dependent spliceosome assembly. To test this possibility, a modification of the

RNase H protection assay developed to study 5'ss choice (Eperon et al. 1993) was used to measure the level of occupancy at specific sites along the P120 splicing substrate. After spliceosome assembly for 40 min in reactions arrested at the A complex stage, five specific sites located in the 5' exon, at the 5'ss, in the intron, at the BPS, and in the 3' exon were targeted for RNase H cleavage by incubating the reactions in the presence of one or two 14-mer DNA oligonucleotides (Fig. 2A) for 20 min. Gel analysis then revealed whether any of these sites was bound by factors that prevent the binding of the DNA oligonucleotide(s) and consequently protect the splicing substrate from RNase H hydrolysis. During the 60 min required to carry out the assay, no dissociation of the arrested A complex was detected (see Fig. 1B).

When protection at the five individual sites diagrammed in Figure 2A was examined with a single DNA oligonucleotide (Fig. 2B, lanes 1–5), only the 5'ss and BPS were found to be protected from RNase H hydrolysis (lanes 2 and 4), as indicated by the recovery of full-length

Figure 2. Interactions of the P120 substrate analyzed by RNase H protection. (A) Schematic diagram showing the sites on the P120 splicing substrate targeted for RNase H cleavage by five DNA oligonucleotides. (B) Protection of the P120 splicing substrate from RNase H hydrolysis after A complex assembly for 40 min. Either an individual site or two sites were targeted as indicated. The autoradiogram of a denaturing polyacrylamide gel shows an example of the RNase H hydrolysis pattern observed for the A complex. The graph (top) presents the mean values obtained in four independent experiments, which were normalized against the level of 5'ss protection, set at 100%. Standard deviation is indicated by error bars.

P120 splicing substrate from those reactions. Quantitation revealed that ~10%–15% of the input pre-mRNA was protected in reactions targeted with either the 5'ss- or BPS-specific DNA oligonucleotide, with the BPS protection usually being somewhat higher (10%–20%) than protection at the 5'ss. Protection of the other three regions (Fig. 2B, lanes 1,3,5) was ~10- to 20-fold lower, the same as the background level observed when DNA oligonucleotides and the P120 splicing substrate were added to the reaction simultaneously (see below). These data indicate that the 5'ss and BPS are selectively recognized, presumably by U11 and U12 snRNPs, respectively, during A complex assembly.

To ask whether these two sites are occupied simultaneously in a single substrate molecule, the 5'ss and BPS were targeted in the same reaction with two DNA oligonucleotides. The recovery of full-length P120 (in which both sites must be protected in a single pre-mRNA molecule) was ~70% of the 5'ss value (Fig. 2B, lane 6). Because competition experiments with unlabeled full-length substrates indicated that the amount of labeled P120 used in the assay is saturating (data not shown), this result suggests that the 5'ss and BPS in a single substrate molecule are being recognized concurrently, suggesting cooperativity between U11 and U12 binding. As the DNA oligonucleotides used in the RNase H protection assay can compete with snRNPs for binding to the pre-mRNA, the decreased RNase H protection of the full-length substrate in reactions containing two DNA oligonucleotides instead of one can be explained by an additive competitive effect.

Cooperativity between U11 and U12 binding was tested further by examining substrate protection after blocking either the U11/5'ss or U12/BPS interaction by use of antisense oligonucleotides targeted to critical regions of the U11 or U12 snRNA. Whereas 2'-O-methyl oligonucleotide U12_{11–28} (see Fig. 3A) was reported previously to block the U12/BPS interaction (Tarn and Steitz 1996a,b), attempts to use 2'-O-methyl oligonucleotides targeted to the 5' end of U11 snRNA to prevent 5'ss recognition or to select U11 snRNPs had failed (K.M. Wassarman and Steitz 1992; Tarn and Steitz 1996a). We therefore designed two peptide nucleic acid (PNA) oligomers that should form an RNA-PNA₂ triplex (Knudsen and Nielsen 1996) with nucleotides 1–8 of U11 snRNA (Fig. 3A). Preincubation with these PNA oligomers inhibited splicing of the U12-type P120 substrate (Fig. 3B) but did not affect splicing of the U2-type adenovirus substrate (Fig. 3C). Partial RNase T₁ hydrolysis of nuclear extracts and subsequent primer extension analysis revealed that the PNA oligomers bind to the single-stranded 5' end of U11 snRNA (see Fig. 3A) and protect the G residues from cleavage (data not shown).

The effect of the U11 and U12-specific antisense oligonucleotides on recognition of the 5'ss and BPS was tested by performing three separate reactions (Fig. 3D,E), which were analyzed by the RNase H assay (see Fig. 2). The first was arrested A complex formation on the P120 splicing substrate. The second was the same reaction but preincubated with either the U11- or U12-specific anti-

mediate and product are indicated at *right*. (D) Effect of the 2'-O-methyl antisense oligonucleotide U12₁₁₋₂₈ (0.5 μM) on the RNase H protection profile of the P120 splicing substrate. After arrested A complex formation, individual sites were targeted as in Fig. 2A. (Bottom) Gel slices show recovery of the full-length P120 splicing substrate in the RNase H protection profile (see Fig. 2B). (Top) The graph shows mean values obtained from four independent experiments with the standard deviation indicated by error bars. (Solid bars) Protection observed on a mock-treated reaction; (open bars) reactions containing the U12₁₁₋₂₈ oligonucleotide; (shaded bars) control reactions in which splicing substrate was added at the time of the RNase H assay. (E) Effect of PNA oligonucleotides U11-2P and U11-3P (5 μM each) on the RNase H protection profile of the P120 splicing substrate. (Bottom) Gel slices show recovery of the full-length P120 splicing substrate in the RNase H protection profile (see Fig. 2B). (Top) The graph shows mean values obtained from three independent experiments, with the standard deviation indicated by error bars. The color coding is the same as in D.

sense oligonucleotide(s). The third was an RNase H control reaction, identical to the second reaction except that after the preincubation, the reaction was kept on ice until the simultaneous addition of splicing substrate and DNA oligonucleotides immediately prior to the RNase H hydrolysis step. Reactions containing the U12-specific 2'-O-methyl oligonucleotide, revealed a dramatic decrease in the protection of both the 5'ss and BPS regions: Protection of the BPS dropped 10-fold, whereas protection of the 5'ss decreased 5-fold compared with the arrested A complex (open bars compared with solid bars in Fig. 3D). This result indicates a strong cooperative interaction between U11 binding to the 5'ss and U12 binding to the BPS. Interestingly, residual protection at the 5'ss after blocking the U12/BPS interaction was consistently approximately twofold higher than background (gray bars), suggesting some U11 binding to the 5'ss independent of U12 binding to the BPS (see below).

In the reciprocal experiment (Fig. 3E), the two PNA oligonucleotides targeted to the 5' end of U11 snRNA decreased protection at the 5'ss 10-fold, close to the background level, whereas protection at the BPS was reduced to ~40%. These results again show significant cooperativity between U11 and U12 binding. Apparent independent U12 binding to the BPS was ruled out by subsequent RNase H experiments in which both the 5'ss and the U12/BPS interactions were simultaneously blocked; these revealed that the PNA oligonucleotides were partially inhibiting RNase H hydrolysis at the BPS (data not shown). For unknown reasons, this inhibitory effect requires 40 min incubation at +30°C and is therefore not observed in the RNase H control reaction shown. Similar interference can be observed in the RNase H protection profile of the 5' exon (Fig. 3E). Consistent with this interpretation, no A complexes were detected by native gel analysis in reactions containing

Figure 3. RNase H protection analyses in the presence of U11- or U12-specific blocking oligonucleotides. (A) Oligonucleotides designed to inhibit U11/5'ss and U12/BPS binding. (Top) Schematic diagram showing the base-pairing interaction between the U11 snRNA and the 5'ss of the P120 splicing substrate. The two PNA oligonucleotides used to inhibit the U11/5'ss interaction by forming a PNA₂-RNA triplex are indicated as lines above and below the U11 snRNA sequence. (Bottom) Schematic diagram showing the base-pairing interaction between the U12 snRNA and the BPS of the P120 splicing substrate. The U12₁₁₋₂₈ 2'-O-methyl oligonucleotide used to inhibit the U12/BPS interaction is indicated as a line below the U12 snRNA sequence. The conserved nucleotides at the 5'ss and BPS are boxed (Dietrich et al. 1997). (B) Inhibition of P120 splicing by the U11-2P and U11-3P PNA oligonucleotides. Splicing reactions without substrate were preincubated in the presence of increasing concentrations of the two PNA oligonucleotides and the splicing activity was assayed 4 hr after addition of the P120 substrate by electrophoresis on a 5% denaturing polyacrylamide gel. Splicing intermediates and products are at *right*. (Asterisk) Degraded substrate fragment. (C) Effect of the U11-2P and U11-3P PNA oligonucleotides on U2-dependent splicing. Splicing reactions without substrate were preincubated in the presence of increasing concentrations of the two PNA oligonucleotides and splicing activity was assayed 1 hr after the addition of adenovirus substrate by electrophoresis on an 8% denaturing polyacrylamide gel. The lariat intermediate and product are indicated at *right*. (D) Effect of the 2'-O-methyl antisense oligonucleotide U12₁₁₋₂₈ (0.5 μM) on the RNase H protection profile of the P120 splicing substrate. After arrested A complex formation, individual sites were targeted as in Fig. 2A. (Bottom) Gel slices show recovery of the full-length P120 splicing substrate in the RNase H protection profile (see Fig. 2B). (Top) The graph shows mean values obtained from four independent experiments with the standard deviation indicated by error bars. (Solid bars) Protection observed on a mock-treated reaction; (open bars) reactions containing the U12₁₁₋₂₈ oligonucleotide; (shaded bars) control reactions in which splicing substrate was added at the time of the RNase H assay. (E) Effect of PNA oligonucleotides U11-2P and U11-3P (5 μM each) on the RNase H protection profile of the P120 splicing substrate. (Bottom) Gel slices show recovery of the full-length P120 splicing substrate in the RNase H protection profile (see Fig. 2B). (Top) The graph shows mean values obtained from three independent experiments, with the standard deviation indicated by error bars. The color coding is the same as in D.

either U11- or U12-specific antisense oligonucleotides (data not shown).

The most straightforward interpretation of the strong cooperativity seen between U11 and U12 binding to the 5'ss and BPS, respectively, in RNase H protection experiments is that a U11/U12 di-snRNP complex recognizes these important intron regions simultaneously. To insure that the di-snRNP structure was not being directly perturbed by antisense oligonucleotide pretreatment, glycerol gradient analysis of nuclear extracts blocked either with U11- or U12-specific antisense oligonucleotides were performed. No dissociation of the di-snRNP was detected (data not shown).

Both the 5'ss and BPS are required for efficient snRNP recognition of a U12-type intron

In the U2-dependent splicing system, the U1 or U2 snRNP can bind independently to a 3' or 5' half-substrate containing only its respective binding site (Barabino et al. 1990; Michaud and Reed 1993; Tarn and Steitz 1995). We therefore examined the binding of U11 or U12 snRNPs to 5' or 3' P120 half-substrates using native gel analyses. No A complex formation was detected with either half-substrate or even when the two half-substrates were used together (data not shown).

More sensitive cross-linking assays were then used to compare the relative binding of U11 and U12 snRNPs

with the 5' and 3' half-substrates and full-length P120 substrate. Psoralen was used to probe U12/BPS interactions (Tarn and Steitz 1996a,b), whereas detection of U11/5'ss interactions required the construction of splicing substrates containing a single ^{45}U residue close to the 5'ss (Yu and Steitz 1997). We placed ^{45}dU in position +7 relative to the 5'ss, as ^{45}U at this position had been shown previously to cross-link to U11 snRNA (Yu and Steitz 1997). ^{45}dU was used because it is commercially available as the phosphoramidite monomer and therefore could be readily incorporated into RNA oligonucleotides. Splicing substrates containing ^{45}dU exhibited normal splicing activity as monitored by the appearance of the lariat product (Fig. 4C).

Unlabeled splicing substrates (for psoralen cross-linking) or trace-labeled substrates (for ^{45}dU cross-linking) were cross-linked in arrested A complex assembly reactions, followed by Northern analysis (Fig. 4A,B). The identities of the cross-links were confirmed by RNase H targeting (see Materials and Methods) and reprobing the filters successively with probes complementary to different snRNAs. As seen in Figure 4A, lane 3, and Figure 4B, lane 4, both U12/P120 and U11/P120 cross-links formed with the full-length P120 splicing substrate. The U12/P120 cross-links were completely abolished by preincubation with either the U11- or U12-specific antisense oligonucleotide (Fig. 4A, lanes 4,5). In contrast, the U11/P120 cross-links were completely inhibited only by the

Figure 4. Northern blot analysis of cross-linked products containing U12 or U11 and the full-length P120 splicing substrate or 3' or 5' half-substrate. Cross-linking conditions ($\pm$ UV), RNA substrates, and antisense oligonucleotides used during preincubation are indicated (α U11—5 μM U11-2P and U11-3P; α U12—0.5 μM U12₁₁₋₂₈). The identities of the cross-linked bands are shown at right. (Asterisk) Cross-reacting bands detected in the absence of UV irradiation or splicing substrate. The sizes of DNA markers (M), a ^{32}P -labeled pBR322 digest, are at left. (A) Psoralen cross-linking of the unlabeled full-length P120 splicing substrate (lanes 1–5) or 3' half-substrate (lanes 6–10). Cross-links were detected by hybridization with a U12-specific riboprobe. (Lanes 6–10) Exposure was eight times longer than that for lanes 1–5. (B) Cross-linking of a trace-labeled full-length P120 splicing substrate (lanes 1–6) or 5' half-substrate (lanes 7–10) containing ^{45}dU at position +7 of the intron. The full-length P120 control lacking the ^{45}dU (lane 1) was not trace labeled. Cross-linked products were detected by hybridization with a U11-specific riboprobe. (C) Time course appearance of the lariat product in a standard splicing reaction containing either the full-length P120 splicing substrate (P120) or a substrate containing ^{45}dU at intron position +7 (P120- ^{45}dU).

U11-specific PNA oligonucleotides (Fig. 4B, lane 5); inhibition of the U12/P120 interaction produced a fivefold decrease in U11/P120 cross-link formation (Fig. 4B, lane 6). These results confirm strong cooperativity between the U12/BPS and U11/5'ss interactions seen in Figure 2, D and E, and again suggest that the U11 snRNP can bind weakly to the 5'ss in the absence of the U12/BPS interaction (see Fig. 2D).

Cross-linking was also performed with P120 half-substrates (Fig. 4A,B, right). With the 3' half-substrate, faint U12/P120-3' cross-linked bands, ~60-fold weaker than with the full-length P120 substrate, were detected on longer (8×) exposures (Fig. 4A, lane 8; see also Fig. 6, below). Preincubation with either U11- or U12-specific antisense oligonucleotides led to the disappearance of these cross-links (Fig. 4A, lanes 9,10). With the 5' half-substrate, only the U11-specific antisense oligonucleotide inhibited formation of the U11/P120-5' cross-link (Fig. 4B, lane 8 vs. lane 9), demonstrating that the 5' end of U11 snRNA is necessary for cross-link formation; antisense inhibition of the U12/BPS interaction did not change the level of the U11/P120-5' cross-link (Fig. 4B, lane 10). The efficiency of 5' half-substrate cross-linking to the U11 snRNA was approximately fivefold lower than with the full-length splicing substrate (Fig 4B, lane 4 vs. 8), consistent with the fivefold dependence on U12 for cross-linking to the 5'ss of the full-length P120 substrate (U11/P120) in the presence of the U12-specific antisense oligonucleotide (Fig. 4B, lanes 4,6).

A U12/U6atac cross-linked species with identical mo-

bility to a previously mapped U12/U6atac helix Ia cross-link (Tarn and Steitz 1996b) was also detected in reactions arrested at the A complex stage. This cross-link is visible in long exposures of autoradiograms of reactions that did not receive any splicing substrate (Fig. 4A, lane 7), but is greatly stimulated in full splicing reactions (Fig. 4A, lane 3; see also Fig. 6, below). The stimulation of U12/U6atac cross-link formation in reactions arrested at the A complex stage is consistent with recent observations of U2/U6 interactions in the absence of B complex formation in both the mammalian and yeast U2-dependent systems (see Discussion for details) and provides an additional internal control for spliceosome assembly. Note that the reactions treated with either U11- or U12-specific antisense oligonucleotides (Fig. 4A, lanes 4,5) do not show enhanced levels of this U12/U6atac cross-link, consistent with the lack of stable A complex formation in these reactions.

Parallel cross-linking experiments were performed with the well-studied U2-dependent splicing system arrested at the A complex stage (Fig. 5) to ensure that the results in Figure 4 are not due to some peculiarity of our assay. We used the adenovirus splicing substrate (Solnick 1985), which contains a consensus 5'ss and strong BPS followed by a PPT—features that promote efficient spliceosome assembly even in the absence of other stimulatory signals (e.g., exonic enhancers). Psoralen cross-linking (D.A. Wassarman and Steitz 1992) was conducted with or without preincubation with antisense 2'-O-methyl oligonucleotides reported to block U1/5'ss

Figure 5. Northern blot analysis of cross-linked products containing U1 or U2 and the full-length adenovirus splicing substrate or 3' or 5' half-substrates. The cross-linking conditions ($\pm$ UV), the RNA substrates, and antisense oligonucleotides used during preincubation are indicated (α U1—15 μ M U1₁₋₁₄; α U2—4.8 μ M U2₂₇₋₄₉; α U11—5 μ M each U11-2P and U11-3P; α U12—0.5 μ M U12₁₁₋₂₈). The identities of cross-linked bands are shown at right and the sizes of DNA markers, a ³²P-labeled pBR322 digest, at left. (A) Psoralen cross-linking of the unlabeled full-length adenovirus splicing substrate (lanes 1–7) or 5' half-substrate (lanes 8–14). Cross-linked products were detected by hybridization with a U1-specific riboprobe. (B) Psoralen cross-linking of the unlabeled full-length adenovirus splicing substrate (lanes 1–7) or 3' half-substrate (lanes 8–14). Cross-linked products were detected by hybridization with a U2-specific riboprobe. (Asterisk) Cross-reacting bands detected in the absence of UV irradiation or splicing substrate.

and U2/BPS interactions (Lamond et al. 1989; Barabino et al. 1990); the U11- and U12-specific antisense oligonucleotides described above provided negative controls. The results (Fig. 5) agree with previous reports on the early assembly of the U2-dependent spliceosome (Ruby and Abelson 1988; Séraphin and Rosbash 1991; Hall and Konarska 1992; Chiara and Reed 1995). The U1/5'ss interaction (Fig. 5A, lane 3) is completely independent of the U2/BPS interaction: Neither inhibition of U2/BPS binding by the 2'-O-methyl oligonucleotide (lane 5) nor use of the 5' half-substrate (lanes 10,12) altered the level of U1/5'ss cross-linking. Also in agreement with previous results (Michaud and Reed 1993; Chiara and Reed 1995; Tarn and Steitz 1995), the 3' half-substrate cross-linked to U2 snRNA in the absence of the 5'ss (Fig. 5B, lane 10), whereas blocking the 5' end of the U1 snRNA with the antisense 2'-O-methyl oligonucleotide U1₁₋₁₄ abolished the U2/BPS interaction with both the full-length adenovirus substrate (lane 4) and the 3' half-substrate (lane 11) (Seiwert and Steitz 1993; Tarn and Steitz 1994, 1995). The U11- and U12-specific antisense oligonucleotides had no effect on adenovirus cross-link formation (Fig. 5A,B, lanes 6,7,13,14). Therefore, we conclude that our assay using reactions blocked at the A complex stage is valid.

The 5'ss and BPS suffice for A complex formation on the P120 substrate

To investigate whether substrate sequences beside the 5'ss and BPS are required for stable U11/U12 complex binding, we carried out a deletion analysis of the P120 splicing substrate (Fig. 6A). Both psoralen cross-linking of U12 to the BPS and native gel analysis were used to test A complex formation (Fig. 6B,C). The data in Figure 6B (gel probed with U12 antisense) define a minimal P120 splicing substrate that can efficiently cross-link to U12 snRNA. Substrate c with a deletion of virtually all sequences downstream from the BPS (including the 3'ss) had nearly the same efficiency of U12 cross-linking as

the full-length substrate (Fig. 6B, lane 4 vs. lane 2). The other two deletions, which either removed the majority of the 5' exon (substrate b) or combined deletions b and c and, in addition, contained an intron deletion (substrate d) that moved the BPS closer to 5'ss (the distance between 5'ss and the branch site decreased from 89 to 39 nucleotides), displayed somewhat reduced U12 cross-linking efficiencies (68% for b and 48% for d of the level observed with full-length substrate; Fig. 6B, cf. lanes 3 and 5 with lane 2). In contrast, removal of the 5'ss reduced U12 cross-linking efficiency to background levels (Fig. 6B, lane 6) as observed in Figure 4A.

These results were confirmed by native gel analysis (Fig. 6C). The efficiency of A complex formation for deletion substrate c lacking the 3' exon and 3'ss was nearly the same as for the full-length P120 splicing substrate (Fig. 6C, lane 3, note that the P120 3' exon contains one-third of the labeled U residues in the substrate). In contrast, the other two deletion substrates displayed reduced levels of complex formation (Fig. 6C, lanes 2,4; typically 30%–60% relative to substrates containing the intact 5' exon), suggesting that the 5' exon may contain an element that stimulates A complex formation. Northern analysis confirmed the presence of U11 and U12 snRNPs in these complexes (data not shown) except for the smallest deletion (d), which could not be assessed because its A complex exhibited nearly identical mobility to the U11/U12 complex in native gels. We conclude that the 5'ss and BPS comprise necessary and sufficient signals for stable U11 and U12 binding to the P120 pre-mRNA substrate.

Discussion

We present the first detailed analysis of the early assembly stages of the U12-dependent spliceosome. Interactions were monitored by native gel electrophoresis, RNase H protection assays and RNA–RNA cross-linking using the full-length P120 splicing substrate, as well as 5' and 3' half-substrates, under conditions which arrest

Figure 6. Psoralen cross-linking and arrested A complex formation on P120 deletion substrates. (A) Schematic diagram of the P120 deletion mutants. The sizes in nucleotides of the intron and exon portions are indicated. (B) Psoralen cross-linking of the unlabeled P120 splicing substrate and its deletion mutants after A complex assembly. Cross-linked products were detected by hybridization with a U12-specific riboprobe. The substrate used in each reaction is indicated at top. The identity of the cross-linked bands are indicated at right. (Asterisk) Cross-reacting bands detected in the absence of UV irradiation or splicing substrate. The multiple cross-linked species detected in lanes 4 and 5 are probably due to the multiple U12/BPS cross-links described by Tarn and Steitz (1996a). (C) Native gel analysis of A complex formation on the full-length P120 splicing substrate and its deletion mutants.

formation of the U12-dependent spliceosome at the A complex stage. We conclude that binding to the 5'ss and BPS is a cooperative process, in which U11/5'ss and U12/BPS interactions together mediate efficient intron recognition leading to A complex formation. Our observation that prevention of one interaction, by either an antisense oligonucleotide or use of a half-substrate, inhibits the other interaction, strongly supports a model in which a single U11/U12 di-snRNP recognizes both the 5'ss and BPS as opposed to a model in which these two sites are recognized by separate snRNPs. Deletion analysis of the P120 splicing substrate further indicates that beside the 5'ss and BPS, no other sequence elements are required for A complex formation, although some sequence(s) located in the 5' exon may provide stimulation.

Our experiments were carried out with a model splicing substrate derived from intron F and the flanking exons of the human *P120* gene (Hall and Padgett 1996; Tarn and Steitz 1996a). This intron belongs to a subclass of U12-type introns that contains A and C at the 5' and 3' termini, respectively. The other subclass of U12-type introns has Gs at both the 5' and 3' ends of the intron (Dietrich et al. 1997). There is no indication that the terminal nucleotides form base-pairing interactions with either the U11 or U12 snRNA and the three nucleotides at the 3' end of the P120 intron are dispensable for A complex formation (see Fig. 6). Because of the extraordinarily high conservation of 5'ss and BPS signals from plants to mammals (Sharp and Burge 1997; Burge et al. 1998), we believe that cooperative recognition of the 5'ss and BPS is a common feature of spliceosome assembly on U12-type introns.

Differences in the early assembly of U12- and U2-dependent spliceosomes

Comparison of A complex assembly in the U12- and U2-dependent systems reveals a major difference. In yeast and mammalian cells, the 5'ss is recognized by the U1 mono-snRNP independent of U2 (Ruby and Abelson 1988; Séraphin and Rosbash 1991; Michaud and Reed 1993; Chiara and Reed 1995), whereas in the U12-dependent system we find that the U11/5'ss interaction is markedly stimulated by the U12/BPS interaction (see Figs. 3 and 4). Such cooperative recognition and the high conservation of the 5'ss and BPS in U12-type introns suggest that this splicing system is more rigid, with intron definition occurring in a single binding event. Thus, in the U12-dependent system, the bridge that connects the 5' and the 3' ends of the introns appears already built in to the U11/U12 di-snRNP structure, whereas in the U2-dependent system, separate components are required (see Wu and Maniatis 1993; Abovich and Rosbash 1997). Several studies have indicated that in addition to signals present within U12-type introns, exon definition interactions and exonic enhancers can stimulate the splicing of U12-type introns (Wu and Krainer 1996, 1998). Cooperative recognition of the highly conserved 5'ss and BPS signals argues that stimulatory elements in the vicinity of U12-type introns may simply enhance the basal level

of splicing rather than promoting elaborate alternative splicing patterns characteristic of the U2-dependent system.

Another major difference between the two systems is the apparent lack of an ATP-independent commitment complex in the U12-dependent system. Both U11/5'ss and U12/BPS interactions have been detected previously by cross-linking (^{45}S or psoralen, respectively) in the absence of ATP (Tarn and Steitz 1996b; Yu and Steitz 1997), and similar results were obtained in this study (M. Frilander and J. Steitz, unpubl.). However, we were unable to document the formation of a stable ATP-independent commitment complex that could be chased into functional spliceosomes in the presence of excess competitor RNA, as for the mammalian and yeast U2-type introns (Séraphin and Rosbash 1989; Michaud and Reed 1991; Jamison et al. 1992). In the U2-dependent system, U1 and U2AF can form stable ATP-independent complexes with the 5'ss and PPT, respectively, but ATP is needed to stabilize the U2/BPS interaction in complex A (Bindereif and Green 1987; Liao et al. 1992). Therefore, if ATP is also required to stabilize the U12/BPS interaction, and the 5'ss and BPS are recognized in a single binding event, then the failure to detect an ATP-independent commitment activity in the U12-dependent system is expected.

In contrast to differences observed in early complex formation, complex B formation appears similar for U12- and U2-dependent spliceosomes. Stable B complex is not observed in either system in the presence of a 2'-O-methyl oligonucleotide that blocks the U6atac/5'ss or U6/5'ss interaction (see Fig. 1). But the resulting arrested A complexes apparently interact with the tri-snRNP particle, as indicated by the presence of U12/U6atac (Figs. 4A and 6B) and U2/U6 cross-links (Fig. 5B). The U12/U6atac cross-linked band (Fig. 4A) has identical mobility to a previously mapped cross-link in helix Ia (Tarn and Steitz 1996b), whereas the U2/U6 cross-link is located most probably in helix II (Hausner et al. 1990). A previous study similarly reported a U2/U6/3' half-substrate triple cross-link that occurred in the absence of a functional U6/5'ss interaction (Tarn and Steitz 1995). Using yeast U1 snRNA mutants that prevent the unwinding of U1/5'ss helix, Staley and Guthrie (1999) recently reported association of the tri-snRNP particle with the pre-spliceosome in the absence of U6/5'ss interactions. There, the catalytic core (U2/U6 base-pairing) was not formed, as no unwinding of U4/U6 base-pairing was observed, whereas in our case, detectable U12/U6atac cross-links suggest catalytic core formation. Possibly, the 2'-O-methyl oligonucleotide we used to arrest spliceosome assembly binds to the 5' end of U6atac and mimics functional 5'ss recognition, subsequently triggering the U4atac/U6atac unwinding process.

U11/U12 snRNP binding in a nonspliceosomal context

In addition to its role in the splicing of U12-type introns, the U11/U12 complex has also been ascribed a regula-

tory function during the life cycle of Rous sarcoma virus (RSV) (Gontarek et al. 1993). To produce genomic RNA that can also function as mRNA encoding the Gag and Gag-Pol proteins, an RNA element called the negative regulator of splicing (NRS) suppresses most of the viral (U2-dependent) splicing. The NRS contains a consensus U11-binding site (5'-GUAUCCUU-3') and has been shown to bind the U11/U12 di-snRNP, generating stable large snRNP complexes that also contain the U1 snRNP. Surprisingly, no U12-type BPS has been identified downstream of the NRS U11-binding site, suggesting that a U12/BPS interaction is not needed to stabilize this particular U11/5'ss interaction (Gontarek et al. 1993). The apparent conflict with our findings using the P120 U12-type intron may be resolved by the observation that a second element necessary for the function of the RSV NRS resides upstream of the U11-binding site. This purine-rich region binds SF2/ASF, stabilizing the binding not only of U11/U12 but possibly also of U1 to the NRS (McNally and McNally 1998). Therefore, it is conceivable that the virus has evolved a system in which the stimulatory function of the BPS is replaced by a purine-rich element, thereby avoiding activation of splicing by the U12/BPS interaction.

Implications for the evolutionary fate of U12-type introns

A puzzling feature of the U12-dependent splicing system is its distribution in the eukaryotic lineage (Sharp and Burge 1997; Burge et al. 1998). U12-type introns have been identified in vertebrate, insect, and plant genes, but they are absent in nematodes and lower eukaryotes, for example, yeast. A fission/fusion model for the origin of U12-type introns, on the basis of sequence comparisons, has been proposed recently to account for their absence from certain branches of the evolutionary tree and for their overall low abundance compared with U2-type introns (Burge et al. 1998). According to this model, the two splicing systems evolved in two separate lineages, which then fused, followed by conversion of most U12-type introns into U2-type introns.

Intron conversion from U12 to U2 type has experimental support (Dietrich et al. 1997; Kolossova and Padgett 1997). These studies indicate that the U12-type splicing system is highly sensitive to mutations that disrupt the binding of U11 to the 5'ss. Because of the cooperative recognition we have documented, such mutations would prevent BPS recognition in addition to 5'ss recognition, leading to effective inhibition of U12-dependent spliceosome assembly. The absence of competition from the U12-dependent system would then allow utilization of even weak U2-dependent splice sites, facilitating the conversion of U12- to U2-type introns.

Materials and methods

Oligonucleotides

DNA, RNA, and 2'-O-methyl oligonucleotides were purchased from the Keck Oligonucleotide Synthesis Facility, Yale Univer-

sity. The peptide nucleic acid (PNA) oligonucleotides were purchased from Perceptive Biosystems (Framingham, MA). The 2'-O-methyl oligonucleotides used for the pretreatment of the nuclear extracts were U1₁₋₁₄, used to block U1/5'ss interactions (Tarn and Steitz 1994); U2₂₇₋₄₉, used to block U2-dependent splicing (Lamond et al. 1989; Tarn and Steitz 1996a); U6atac₁₋₂₀ (Tarn and Steitz 1996b), used to arrest U12-dependent spliceosome assembly at the A complex stage; U12₁₁₋₂₈ (Tarn and Steitz 1996a), used to block the BPS/U12 interaction; U6₂₇₋₄₆, used to arrest U2-dependent spliceosome assembly at the A complex stage. The PNA oligonucleotides used for nuclear extract pretreatment were U11-2P [H-TTTTTT//Lys-NH₂; in which / stands for pseudoisocytosine (Knudsen and Nielsen 1996)]; U11-3P (H-CCCTTTTT-Lys-NH₂), which together form a RNA-PNA₂ triplex with the 5' end of U11 snRNA. RNA oligonucleotide P120-49 (5'-UAUCC⁴⁵dUUGCA-3') was used to construct P120 splicing substrates containing a ⁴⁵dU residue position +7 in intron. The ⁴⁵dU phosphoramidite monomers were purchased from Glen Research (Sterling, VA). DNA oligonucleotides P120-23 (5'-GGGCAGCACAAATG-3'), P120-13 (5'-GCAAGGATATCCTG-3'), P120-26 (5'-CCTAAGGCCTG-GAA-3'), P120-17 (5'-GTAAAGGAAGTGTG-3'), and P120-20 (5'-GATCCGCTTGTGAA-3') are complementary to the P120 5' exon (nucleotides 123-136), 5'ss (nucleotides 155-168), intron (nucleotides 213-226), BPS (nucleotides 236-249), and 3' exon (nucleotides 285-298), respectively, and were used in the RNase H assay. DNA oligonucleotides complementary to U1 (nucleotides 64-75), U2 (nucleotides 78-98), U11 (nucleotides 52-71), U12 (nucleotides 53-71), U4atac (nucleotides 63-83), U6atac (nucleotides 61-79), adenovirus splicing substrate (nucleotides 101-144 or 325-338), or the P120 splicing substrate (oligonucleotide P120-23, P120-26, or P120-20) were used for RNase H analysis of cross-linked bands.

Splicing substrates

Capped full-length P120 and adenovirus splicing substrates were produced by in vitro transcription (Tarn and Steitz 1994, 1996a) with either T7 (P120) or SP6 (adenovirus) RNA polymerase (Pharmacia) and plasmid pP120 linearized with *Hind*III (Tarn and Steitz 1996a) or plasmid pSPAd linearized with *Hinc*II (Solnick 1985) as a template. Templates for the P120 and adenovirus half-substrates were generated by PCR from plasmids pP120 and pSPAd. The P120 half-substrates contained nucleotides 1-209 (5' half-substrate) or 209-357 (3' half-substrate). The adenovirus half-substrates contained nucleotides 1-188 (5' half-substrate) or 245-407 (3' half-substrate). Each 3' half-substrate contains a sufficient region upstream of the branchpoint residue to facilitate the potential sequence-independent binding of the SF3 complex (39 nucleotides for P120 and 37 for adenovirus substrate; see Gozani et al. 1996). In addition to the indicated sequences, 3' half-substrates contained 10 nucleotides at the 3' end with a site for *Hind*III restriction enzyme. All splicing substrates were purified in denaturing polyacrylamide gels before use.

Full-length P120 splicing substrates and 5' half-substrates containing a ⁴⁵dU residue at the +7 position of the intron were constructed by RNA ligation technology (Moore and Sharp 1992; Query et al. 1994). The ⁴⁵dU residue was included in a chemically synthesized RNA oligonucleotide P120-49 (nucleotides 160-169 of the P120 splicing substrate), which was joined to in vitro-transcribed 5' (nucleotides 1-159) and 3' fragments (for full-length P120: nucleotides 170-357; for the 5' half-substrate: nucleotides 170-229). DNA templates for the 5' and 3' fragments were generated by PCR with 5' primers containing the T7 RNA polymerase promoter region. The three RNA frag-

ments and a bridging DNA oligonucleotide complementary to residues 137–192 of the P120 splicing substrate were mixed together, denatured at +95°C for 3 min and slowly cooled to room temperature. The ligation reaction was carried out at +20°C for 12–14 hr in reaction buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 20 mM DTT, 1 mM ATP, 1% polyvinyl alcohol, 1 U/μl RNasin, and 3 U/μl T4 DNA ligase (United States Biochemical). The final concentrations of the DNA bridging oligonucleotide and the RNA fragments were 1–3 μM. Trace-labeled 5' or 3' fragments were included in the ligation reaction to facilitate detection of ligated products by autoradiography. The splicing substrates containing ⁴⁵SdU residues were purified in 5% denaturing polyacrylamide gels.

Splicing and A complex assembly reactions

The P120 and adenovirus in vitro splicing reaction conditions were essentially as described (Tarn and Steitz 1996a), except that 4.8 μM U2b 2'-O-methyl oligonucleotide (same as U2_{27–49}) was used in P120-containing reactions. To arrest U12-dependent spliceosome assembly at the A complex stage, a standard P120 splicing mixture lacking pre-mRNA was preincubated in the presence of 0.5 μM U6atac_{1–20} 2'-O-methyl oligonucleotide at +30°C for 10 min, after which the splicing substrate was added and the incubation was continued for an additional 40 min. Similarly, U2-dependent spliceosome assembly was arrested at the A complex stage by preincubation with the U6_{26–47} 2'-O-methyl oligonucleotide for 10 min at +30°C, followed by 20 min incubation at +30°C in the presence of the adenovirus splicing substrate.

RNase H assay

A modification of the RNase H protection assays described previously (Barabino et al. 1990; Eperon et al. 1993) was used to examine the binding of U11 and U12 snRNPs to pre-mRNA. Briefly, U12-dependent A complexes were formed as described above in reactions containing 2 nM ³²P-labeled P120 splicing substrate and 0.05 U/μl RNase H (Pharmacia). Aliquots of the assembly reactions were subsequently divided into tubes containing DNA oligonucleotides (20 μM final concentration; sufficient for >99% cleavage of the substrate in nuclear extract) complementary to specific regions of the P120 splicing substrate, and incubation was continued for 20 min. The reactions were stopped by adding 3 volumes of proteinase K mixture (5 mg/ml proteinase K, 50 mM EDTA, 0.5% SDS), incubated at +55°C for 30–60 min, extracted with phenol/chloroform/isoamyl alcohol (PCA), and precipitated with ethanol. Reactions were separated in 6% denaturing polyacrylamide gels and the level of protection of full-length P120 splicing substrate in each lane was analyzed by PhosphorImager (Molecular Dynamics).

Native gel electrophoresis

Spliceosomal complexes were resolved on 4% native gels according to Konarska (1989). To prevent the dissociation of the A complex into two bands (see Tarn and Steitz 1996a), the amount of heparin added to P120 reactions prior to loading was lowered to 25 μg/ml (final concentration). Heparin (1 mg/ml) was used when loading reactions containing the adenovirus splicing substrate.

Cross-linking

Psoralen cross-linking was used to detect the U12/BPS, U1/5'ss, and U2/BPS interactions in A complexes. The concentra-

tions of the unlabeled full-length and half-substrates were 20 nM for U12/P120 cross-linking and 5 nM for U1/adenol and U2/adenol cross-linking. After A complex formation, the samples were transferred to ice, AMT (4'-aminomethyl-4,5',8-trimethyl) psoralen (HRI Associates, Concord, CA) was added to a final concentration of 20 μg/ml, and the samples were irradiated with 365 nm UV light for 10 min. To detect U11/P120 interactions, 4 nM trace-labeled P120 ⁴⁵SdU splicing substrate or P120 ⁴⁵SdU 5' half-substrate was used for A complex assembly. Subsequently, the samples were transferred to ice and irradiated with 365 nm UV light for 10 min. Cross-linked samples were subsequently treated with proteinase K as described above, PCA extracted and precipitated with ethanol. Cross-linked RNAs were separated in 5% denaturing polyacrylamide gels and transferred to Zeta-Probe membranes (Bio-Rad). Cross-links were detected with ³²P-labeled riboprobes as described previously (Tarn and Steitz 1996a). Both RNase H targeting by use of DNA oligonucleotides complementary to snRNAs or splicing substrates, as described previously (Tarn and Steitz 1996a), and reprobing of the filters successively with probes for different snRNAs were used to confirm the identities of the cross-linked bands.

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Initial recognition of U12-dependent introns requires both U11/5' splice-site and U12/branchpoint interactions

Mikko J. Frilander and Joan A. Steitz

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