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Gel Electrophoretic Isolation of Splicing Complexes Containing U1 Small Nuclear Ribonucleoprotein Particles

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Assembly of splicing precursor RNAs into ribonucleoprotein particle (RNP) complexes during incubation in in vitro splicing extracts was monitored by a new system of RNP gel electrophoresis. The temporal pattern of assembly observed by our system was identical to that obtained by other gel and gradient methodologies. In contrast to the results obtained by other systems, however, we observed requirements of U1 small nuclear RNPs (snRNPs) and 5′ splice junction sequences for formation of specific complexes and retention of U1 snRNPs within gel-fractionated complexes. Single-intron substrate RNAs rapidly assembled into slow-migrating complexes. The first specific complex (A) appeared within a minute of incubation and required ATP, 5′ and 3′ precursor RNA consensus sequences, and intact U1 and U2 RNAs for formation. A second complex (B) containing precursor RNA appeared after 15 min of incubation. Lariat-exon 2 and exon 1 intermediates first appeared in this complex, operationally defining it as the active spliceosome. U4 RNA was required for appearance of complex B. Released lariat first appeared in a complex of intermediate mobility (A′) and subsequently in rapidly migrating diffuse complexes. Ligated product RNA was observed only in fast-migrating complexes. U1 snRNPs were detected as components of gel-isolated complexes. Radiolabeled RNA within the A and B complexes was immunoprecipitated by U1-specific antibodies under gel-loading conditions and from gel-isolated complexes. Therefore, the RNP antigen remained associated with assembled complexes during gel electrophoresis. In addition, 5′ splice junction sequences within gel-isolated A and B complexes were inaccessible to RNase H cleavage in the presence of a complementary oligonucleotide. Therefore, nuclear factors that bind 5′ splice junctions also remained associated with 5′ splice junctions under our gel conditions.

Understanding the mechanism of premessenger splicing will require delineation of the pathway of assembly and disassembly of the spliceosome during the splicing reaction. Single-intron substrates of 400 to 500 nucleotides rapidly assemble into large ribonucleoprotein particle (RNP) complexes when added to in vitro splicing extracts (2, 6, 8, 12–15, 17–19, 20a, 22, 23, 26–29, 31). Fractionation of splicing reactions by sucrose gradient sedimentation reveals several different complexes (2, 6, 8, 12, 13, 17, 26, 29; Kramer, in press). The largest complex of 50S contains precursor RNA and the reaction intermediates, exon 1 and lariat-exon 2. This complex contains small nuclear RNPs (snRNPs), including U1 snRNPs (2, 8, 12–14), and undergoes rapid splicing to products without a detectable lag when added back to extract (13). Furthermore, it does not form in the absence of ATP or with substrates that lack splicing consensus sequences (2, 8, 12, 13). On the basis of these characteristics, this complex can be considered as an active spliceosome. Smaller assemblies of 20S and 35S have also been noted which rapidly form with precursor RNA and which may be kinetic intermediates in the formation of the 50S complex.

Recently, several gel electrophoresis systems have been developed to resolve spliceosome assemblies (18, 19, 22, 27, 28). When splicing reactions were subjected to such analysis, a family of specific complexes were observed. The first of these, denoted the A preassembly complex in mammalian systems, required 3′ but not 5′ consensus sequences for formation (18). Northern (RNA) blot analysis of endogenous extract components suggested that this complex contained U2 snRNPs (18, 19, 22). A second complex, denoted B, appeared with the kinetics of 5′ cleavage and contained the intermediates of the reaction as well as U2, U4, U5, and U6 snRNPs. Interestingly, none of the gel-defined mammalian complexes appeared to contain U1 snRNPs. Use of affinity chromatography to isolate reconstituted spliceosomes also suggested that U1 snRNPs were not stable components of the spliceosome (14).

We have recently investigated the involvement of U1 snRNPs in spliceosome assembly (33). We observed that U1 snRNPs participate early during assembly and are required for the stable association of other snRNPs with precursor RNA. This requirement and the presence of U1 snRNPs in gradient-defined but not gel-defined complexes suggested that U1 snRNPs might be stable components of isolated spliceosomes if appropriate fractionation conditions could be discovered. To address this question, we developed an alternative gel electrophoresis system for resolution of RNP assemblies. This system detected an initial assembled RNP complex A and an active complex B containing U1 snRNPs bound to 5′ splice junctions and dependent on intact U1 RNA and 5′ splice junction sequences for formation. Therefore, we suggest that U1 snRNPs are stable components of the spliceosome and that fractionation systems can be designed to maintain this association.

MATERIALS AND METHODS

Substrate RNAs. The template DNAs used to generate deleted substrate RNAs were derivatives of pIVS (25). The structures of the RNAs produced by SP6 transcription of these template DNAs are diagrammed in Fig. 3. IVS ΔΔ5′, a deletion of the 5′ splice junction, was produced by subcloning an *Rsa*I fragment of the parental IVS plasmid into pSP65. DNA truncated with *Bam*HI directed the production of an SP6 transcription product of 289 nucleotides terminating within intron 2. Substrate RNAs lacking a branch point,

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polypyrimidine track, and 3' splice junction were produced by truncating IVS DNA with *Hin*dIII before SP6 transcription. The resulting substrate RNA, IVS Δ3', was 255 nucleotides. The miniature wild-type substrate (MINX) was subcloned from a construct containing a duplicated exon 2. An *Rsa*I fragment originating and terminating within the duplication was subcloned into the *Sma*I site of pSP65. This plasmid was linearized with *Bam*HI to give an RNA of 220 nucleotides upon transcription, the same approximate size as the deletion substrates. All transcripts were capped during transcription as described previously (1).

In vitro splicing. Nuclear extract preparation and standard splicing reactions were done as described previously (1). Individual U RNAs were cleaved through the use of oligonucleotide-directed RNase cleavage as described previously (1). RNA products of splicing were analyzed on denaturing (8 M urea) 10 to 13% polyacrylamide gels as indicated in the figure legends.

Native RNP gels. Splicing reaction mixtures (25 μl) to be analyzed on RNP gels were terminated by the addition of 5 μl of stop solution which brought the sample to a final concentration 1.5 to 2 mg of heparin per ml and 10 mM EDTA. The samples were then quick frozen in liquid nitrogen until needed. Before loading, the samples were thawed at room temperature. At the end of the incubation, 5 μl of glycerol containing 0.2% each of bromphenol blue, xylene cyanole, and phenol red was added. Samples (5 μl) were loaded onto a 3.5% polyacrylamide-bisacrylamide (80:1, vol/vol)–0.5% low-melting-temperature agarose (Bio-Rad Laboratories, Richmond, Calif.) gel cast in 50 mM Tris–50 mM glycine–10 mM EDTA (pH 8.8). Electrophoresis was conducted at 4°C at 10 V/cm for 4 to 5 h. Gels were fixed in 50% methanol–15% acetic acid for 1 min before autoradiography.

Two-dimensional gels. Lanes were excised from RNP gels after electrophoresis and treated for 10 min at 37°C in a solution of 0.5-mg/ml predigested protease K and 1% sodium dodecyl sulfate. This solution was removed and replaced with formamide loading dye (90% deionized formamide, 10 mM EDTA, [pH 8.0], 0.2% each of xylene cyanole and phenol red). The dye solution was allowed to soak into the gel slice for 5 min, the excess was then removed, and the gel slice was loaded into a prewarmed 10 to 13% polyacrylamide denaturing gel.

Immunoprecipitation of whole extract and gel-purified complexes. Immunoprecipitation from whole extracts and the antisera used have both been described previously (33). Briefly, the anti-RNP antibody and the control antibody used were from serum from patients and have been previously characterized and described by this laboratory. The anti-Sm antibody used was the Sm Y12 monoclonal antibody. Complexes were eluted from native gel slices after visualization by brief autoradiography at 4°C by masticating the gel matrix in 4 volumes of ice-cold Roeder buffer D (11). Extraction was allowed to proceed on ice for 30 min, after which time the gel matrix was cleared by a 5-min centrifugation in a microcentrifuge. Unlabeled precursor (roughly 40 ng) was added per 100 μl of eluted complex, and this mixture was left on ice for 10 min to allow the binding of free factors to the added RNA. After this time, 3 μl of antiserum and 2 μl of RNAsin (Promega Biotec, Madison, Wis.) were added. Antibody was allowed to bind for 30 min before the addition of 10 μl of prewashed Pansorbin. After 15 min of further incubation, the Pansorbin was washed three times in NETG (50 mM Tris [pH 7.4], 150 mM NaCl, 0.05% Nonidet P-40, 0.5 mM dithiothreitol, 10% glycerol). The washed pellets were suspended in 200 μl of urea buffer (175 mM NaCl, 5 mM Tris hydrochloride [pH 7.4], 5 mM EDTA, 0.5% sodium dodecyl sulfate). After a 5-min incubation at 80°C, the Pansorbin was removed by centrifugation and the supernatant was extracted with phenol-chloroform and isopropanol precipitated. RNA released from the Pansorbin served as the carrier. RNA was analyzed by denaturing electrophoresis. The gel was silver stained upon completion of electrophoresis and dried before autoradiography.

Oligonucleotide accessibility of gel-eluted complexes. A 90-min preparative splicing reaction containing saturating amounts of radiolabeled precursor RNA was fractionated on RNP gels. Complexes were detected by brief autoradiography and by counting individual gel slices. Gel slices containing desired complexes were crushed in 5 volumes of Roeder buffer D (11) by passage through successively smaller hypodermic needles, terminating with a $22\frac{1}{2}$-gauge needle. The resulting suspension was incubated on ice for 30 min, and the gel matrix was removed by centrifugation. The supernatant was removed and divided into four aliquots. RNA was prepared directly from one aliquot. $MgCl_2$ was added to the other three aliquots to a final concentration of 2.5 mM. Oligonucleotide was added at a concentration required to direct 100% cleavage of isolated RNA (from 0.4 to 4 μg), and cleavage was initiated by the addition of 0.8 U of RNase H and incubation for 20 min at room temperature. Three oligonucleotides were used. An oligonucleotide complementary to 5' splice junction sequences cleaved MINX RNA to produce fragments of 55 and 153 nucleotides. An internal intron oligonucleotide produced fragments of 96 and 112 nucleotides. An oligonucleotide complementary to the branch point produced cleavage bands of 149 and 59 nucleotides. Isolated RNA was analyzed on 13% denaturing acrylamide gels and exposed for autoradiography.

RESULTS

Detection of multiple complexes during in vitro splicing. We developed a gel electrophoresis system for the analysis of RNP complexes. This system is a modification of an existing system developed for analysis of ribosomes (10). The gel and electrophoresis buffer contains 10 mM EDTA. Inclusion of EDTA permitted differentiation of complexes that frequently had similar migration in systems lacking EDTA. We suspect that inclusion of EDTA causes this gel system to be more responsive to exposed RNA charge in complexes of similar size, thus permitting conformational changes to be discerned. Gel analysis of the temporal pattern of assembly of single-intron precursor RNAs revealed multiple specific gel-visualized bands (Fig. 1). After incubation at 30°C, slow-migrating complexes appeared (Fig. 1A, bands A, A', and B). The A complex was observed considerably before reaction intermediates could be detected (15 min; Fig. 1B). The A' and B complexes migrated more slowly than complex A and appeared concurrently with activity. Late in the reaction when splicing product RNAs had accumulated, rapidly migrating, heterogeneous complexes appeared that were only obvious upon overexposure of the gels (data not shown). The observed pattern of assembly is quite similar to that observed by using different gel systems to analyze both mammalian and yeast assemblies (18, 19, 22, 27, 28).

To correlate bands observed on the RNP gel system with substrate RNA content, we analyzed the RNA intermediates present in each of the aforementioned complexes by two-dimensional electrophoresis (Fig. 1C). Initial complexes in the I region contain only precursor RNA (data not shown).

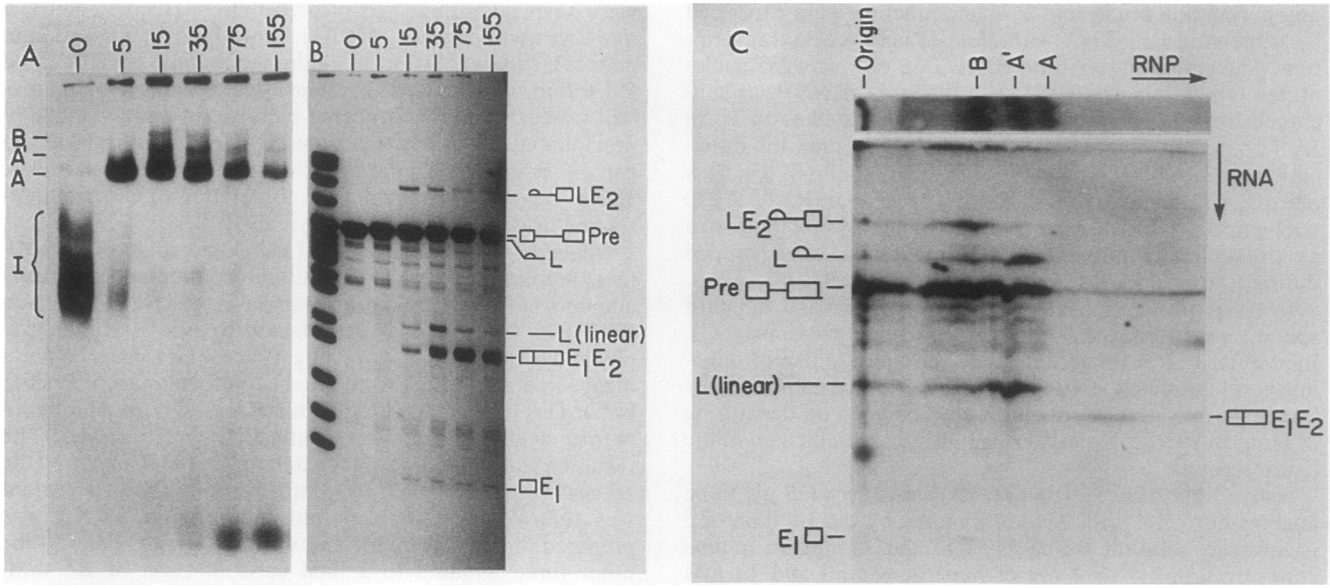

FIG. 1. Multiple RNP complexes assemble on exogenous splicing precursor RNA in in vitro splicing extracts. Splicing reactions with MINX precursor RNA were stopped at the indicated times (minutes) by the addition of heparin and EDTA to 1.5 mg/ml and 10 mM, respectively, and quick freezing in liquid nitrogen. Samples were analyzed for assembled complexes by electrophoresis on native RNP gels (A) or for RNA intermediates and products by electrophoresis of isolated RNA on denaturing gels (B). The intermediate and product RNAs produced upon splicing were identified schematically: LE_2, lariat-exon 2; L, lariat; E, exon. Individual complex bands on the RNP gels are also identified by letters. (C) A 35-min assembly reaction containing radiolabeled MINX RNA was subjected to RNP gel electrophoresis as the first dimension and RNA gel electrophoresis as the second dimension. The RNP gel is shown horizontally with labeled A, A', and B complexes. Precursor (Pre) and product RNAs are indicated for the second dimension. The bulk of the ligated product RNA migrated off the bottom of the first-dimension RNP gel.

Bands A, A', and B contained precursor RNA at all times during the reaction. The reaction intermediates lariat-exon 2 and exon 1 were found exclusively in the B complex. Released lariat initially appeared in the A' region. At later times, considerable lariat was observed in rapidly migrating complexes (these complexes migrated off the RNP gel shown in Fig. 1C). Ligated exons migrated very rapidly. The above complex assignments are identical to those observed with other gel systems (18, 19, 22, 27, 28).

ATP and splicing consensus sequences required for formation of complexes A, A', and B. ATP is required for appearance of the active spliceosome. Reactions depleted of ATP accumulate a 35S complex upon gradient analysis and I complexes upon gel analysis (2, 8, 12, 13, 17, 18, 26). Our gel system showed a similar dependence (Fig. 2). No A, A', or B complexes were observed after a 30-min preincubation to remove endogenous ATP before addition of substrate.

When assemblies containing substrate RNAs lacking 5' splice junctions were analyzed by sucrose gradient centrifugation, 35S complexes accumulated (12), suggesting a requirement for 5' splice junctions early during assembly. Gel electrophoresis indicated normal formation of complex A with similar 5' deletion substrate RNAs (18). In our system, 5' deletion substrate RNA assembled into a unique complex migrating faster than A, denoted α in Fig. 2. The wild-type substrate RNA used in Fig. 2 was 220 nucleotides; the 5' deletion substrate RNA was 289 nucleotides (Fig. 2B). The faster migration of complex α, therefore, is not caused by use of a shorter deletion substrate RNA. Complex α can sometimes be seen in wild-type reactions, especially if the splicing reactions have undergone several rounds of freeze-thawing before electrophoretic analysis. Therefore, we suggest that complex α lacks factors present in the A complex. These factors are presumably those that normally recognize 5' splice site sequences. One of these factors would be U1 snRNPs.

Our gel system differs from that reported by Konarska and Sharp (18) in the differentiation of complexes A and α with 5' deletion substrate RNAs. In accordance with their results, we cannot distinguish A and α when we run a gel equivalent to that in Fig. 2 in which EDTA was omitted from both the sample buffer and from the gel (data not shown). Thus, EDTA alters either association of factors with the gel-resolved assemblies or resolution of those assemblies within gels.

Sequences within the 3' portion of the intron were also required for assembly of complex A (Fig. 2A). 3' deletion substrate RNAs formed only I complexes; the pattern of assembly with 3' deletion substrate RNAs was indistinguishable from that observed with substrates containing no consensus sequences. Therefore, sequences at both the 5' and 3' ends of the intron are required for formation of complex A.

U snRNPs are required for formation of complexes A, A', and B. To address the requirement for individual snRNPs in the assembly of complexes A, A', and B, we subjected U RNAs to oligonucleotide-directed cleavage (1, 3, 4, 7–9, 20, 20a, 21) before the addition of substrate and initiation of assembly (Fig. 3). Oligonucleotides complementary to U1, U2, U4, and U5 RNAs were used as well as a control oligonucleotide not complementary to any U RNA sequences. U1-, U2-, and U4-specific oligonucleotides directed cleavage of over 95% of their targeted U RNAs and completely inhibited splicing activity (data not shown). The U5 and control oligonucleotides directed no observable U RNA cleavage and did not inhibit splicing activity.

Control and U5-specific oligonucleotides had no effect on assembly. Cleavage of U1, U2, or U4 RNA inhibited normal assembly. Each of the cleavages resulted in a different

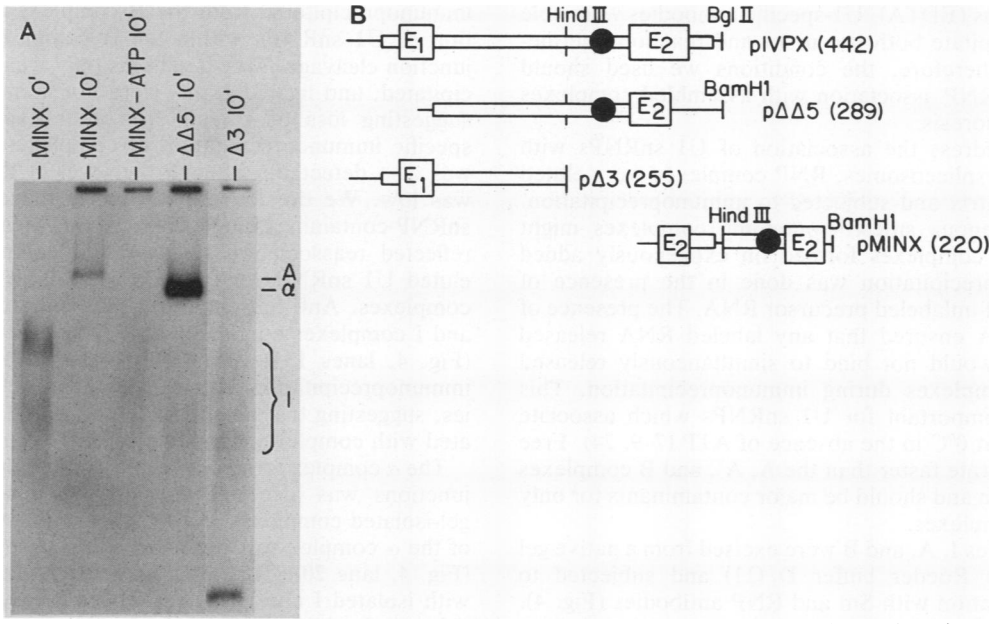

FIG. 2. Assembly of RNP complexes A and B requires splicing consensus sequences. RNP gel electrophoretic analysis of splicing reactions containing wild-type and mutant substrates is shown in panel A. Time of incubation is indicated for each substrate. The −ATP lane was preincubated for 30 min at 30°C to deplete endogenous ATP pools before the addition of wild-type substrate. (B) Structure of utilized precursor RNAs. Large boxes indicate exons (E), small boxes indicate adenovirus type 2 sequence, thin lines indicate vector sequence, and dots indicate branch points.

assembly phenotype. No specific complexes were observed after cleavage of U1 or U2 RNAs with oligonucleotides complementary to the 5′-proximal 16 bases in either RNA (denoted $U1_0$ and $U2_5$ in Fig. 3). Complexes migrating in the I region were the primary products of assembly under these conditions. Therefore, both U1 and U2 snRNPs are required for assembly of the A complex. Formation of the α complex with substrates deleted for 5′ splice junctions also required U1 and U2 snRNPs. Only I complexes were observed with a 5′ deletion substrate after removal of the 5′ terminus of U1 or U2 RNA (data not shown). When U2 snRNA was cleaved to remove nucleotides 28 to 42 (denoted $U2_L$ in Fig. 3), complex A was formed in significant amounts. No A′ or B complex was observed under these conditions, suggesting that internal regions of U2 RNA participate in the conversion of complex A into complexes A′ and B. Cleavage with an oligonucleotide specific for U4 RNA resulted in detection of A and A′ complexes, but not B. Therefore, U4 snRNPs are required for complex B formation.

U1-specific antibodies immunoprecipitate assemblies under gel conditions. The requirement of U1 snRNPs for the appearance of complex A, coupled with the change in complex migration observed upon deletion of the 5′ splice junction, suggested that U1 snRNPs remained associated with RNP complexes A, A′, and B formed on wild-type RNA in our system. To address this question more directly, assembly reactions were diluted into buffers resembling gel electrophoresis conditions and subsequently subjected to immunoprecipitation with U1-specific antibodies. When assembly was terminated with 5 mg of heparin per ml under the conditions described by Konarska and Sharp (18), little precursor RNA was immunoprecipitable with U1-specific antibodies, consistent with their report of loss of U1 snRNPs during electrophoresis (data not shown). Anti-Sm antibodies were able to immunoprecipitate precursor RNA under these conditions, consistent with maintenance of U2 snRNP-spliceosome association in the presence of heparin. Under

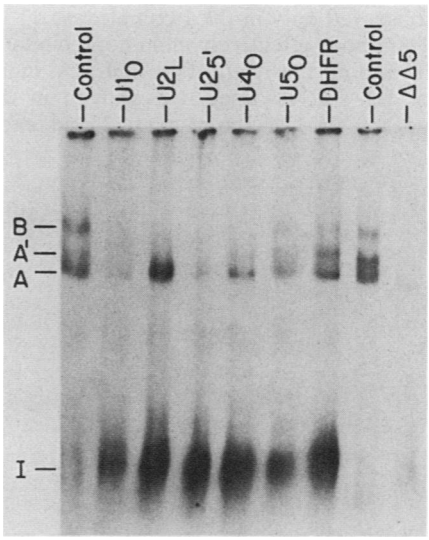

FIG. 3. Formation of complex B requires U1, U2, and U4 snRNPs. Assembly reactions with wild-type MINX precursor RNA (20-min reactions) with extracts in which individual U snRNAs had been cleaved by oligonucleotide-directed RNase H cleavage were analyzed on RNP gels. $U1_0$ (nucleotides 1 to 16 of U1 RNA), $U2_L$ (nucleotides 28 to 42 of U2 RNA), $U2_5'$ (nucleotides 1 to 16 of U2 RNA), and $U4_0$ (nucleotides 66 to 85 of U4 RNA) directed cleavage of over 95% of their target U RNAs and completely inhibited splicing activity. $U5_0$ (nucleotides 1 to 16 of U5 RNA) and DHFR (a sequencing primer not complementary to any U RNAs) directed no observable U RNA cleavage and did not inhibit splicing activity. Control lanes received no oligonucleotide. A comparison assembly with the ΔΔ5′ deletion substrate is also shown. RNP complexes are indicated by letters.

our gel conditions (EDTA), U1-specific antibodies were able to immunoprecipitate both precursor and reaction intermediate RNAs. Therefore, the conditions we used should maintain U1 snRNP association with assembled complexes during electrophoresis.

To further address the association of U1 snRNPs with gel-fractionated spliceosomes, RNP complexes were eluted from the gel matrix and subjected to immunoprecipitation. Because endogenous snRNP-containing complexes might comigrate with complexes formed on exogenously added RNA, immunoprecipitation was done in the presence of large amounts of unlabeled precursor RNA. The presence of competitor RNA ensured that any labeled RNA released during elution would not bind to simultaneously released endogenous complexes during immunoprecipitation. This was especially important for U1 snRNPs which associate with precursor at 0°C in the absence of ATP (7–9, 24). Free U1 snRNPs migrate faster than the A, A′, and B complexes in this gel system and should be major contaminants for only the eluted I complexes.

RNP complexes I, A, and B were excised from a native gel and eluted into Roeder buffer D (11) and subjected to immunoprecipitation with Sm and RNP antibodies (Fig. 4). The eluate used for immunoprecipitation was displayed on denaturing RNA gels before immunoprecipitation. The gel was stained to assess the relative amount of eluted RNA and added competitor RNA (lanes 1 to 3) and autoradiographed to assess the RNA composition of each eluted complex (lanes 4 to 6). Added competitor RNA was estimated to be present in at least 50-fold excess compared with eluted precursor or U1 RNA. Labeled precursor RNA was observed in all three complexes. Intermediate RNAs were observed in the B and A complexes (their presence in A reflects contamination of A with A′ and B). Ligated product RNA was observed only in the I complexes.

The RNP antibody effectively immunoprecipitated precursor RNA from both the A and B complexes, indicating the presence of U1 snRNP-associated antigen in these complexes (lanes 10 to 12). Lariat-exon 2 and exon 1 were immunoprecipitated from the B complex, indicating retention of U1 snRNPs within the B complex after 5′ splice junction cleavage. Free lariat was only weakly immunoprecipitated, and ligated exons were not immunoprecipitated, suggesting loss of U1 snRNPs after exon ligation. U1-specific immunoprecipitation of complexes in the I region was also detected, although the level of this precipitation was low. We do not know whether the detection of U1 snRNP-containing complexes from this area of the RNP gel reflected reassociation of gel-eluted complexes with gel-eluted U1 snRNPs or breakdown of larger U1-containing complexes. Anti-Sm antibodies immunoprecipitated B, A, and I complexes containing all RNAs except ligated exons (Fig. 4, lanes 13 to 15). They were noticeably better at immunoprecipitating released lariat than were RNP antibodies, suggesting that an snRNP other than U1 is still associated with complexes containing released lariat.

The α complex formed with substrates deleted for 5′ splice junctions was also analyzed by immunoprecipitation of gel-isolated complexes. A low level of immunoprecipitation of the α complex was observed with U1-specific antibodies (Fig. 4, lane 20). This level was lower than that observed with isolated I complex (lane 21) and was, therefore, considered a background level attributable to whatever phenomenon created the observed I complex immunoprecipitation. Notice that anti-Sm antibodies were significantly better at immunoprecipitation of the α complex (lane 22) than the I complex (lane 23), indicating that true association of snRNPs with α complexes should result in a higher signal for α complex immunoprecipitation than for I complex immunoprecipitation.

Protection of 5′ splice junctions against oligonucleotide-directed cleavage in gel-isolated complexes. The ability of antibodies directed against snRNP antigens to immunoprecipitate gel-isolated complexes containing splicing intermediates suggested that snRNP antigens remain associated with radiolabeled RNA during electrophoresis, elution, and immunoprecipitation. To further analyze the association of factors with RNA in gel-isolated complexes, we used oligo-

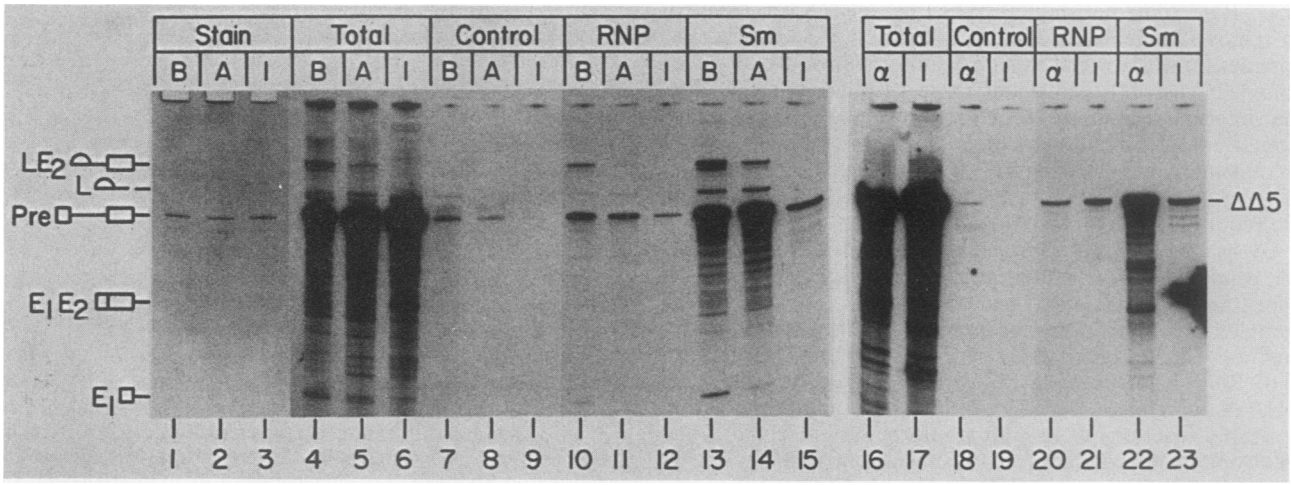

FIG. 4. RNP complexes A and B contain the RNP antigen. RNP complexes containing radiolabeled MINX or ΔΔ5′ precursor RNA were visualized by brief autoradiography of an RNP gel and excised. Complexes B, A, and I were eluted into extract buffer containing an excess (with respect to eluted U1) of unlabeled competitor substrate RNA. Total eluted RNA was analyzed by gel electrophoresis on denaturing gels that were silver stained to detect added unlabeled precursor RNA (lanes 1 to 3) and autoradiographed to visualize eluted radiolabeled RNA (lanes 4 to 6 and 16 to 17). Fractions of the eluted RNA equal to that shown in the total sample in lanes 4 to 6 were immunoprecipitated with control human sera (lanes 7 to 9 and 18 to 19), U1-specific RNP serum (lanes 10 to 12 and 20 to 21), or Y12 monoclonal Sm antibodies (lanes 13 to 15 and 22 to 23). Immunoprecipitated RNAs were analyzed on denaturing gels. Complex B was contaminated by a small amount of complex A and vice versa. The intermediates of the reaction are indicated schematically. LE_2, Lariat-exon 2; E, exon.

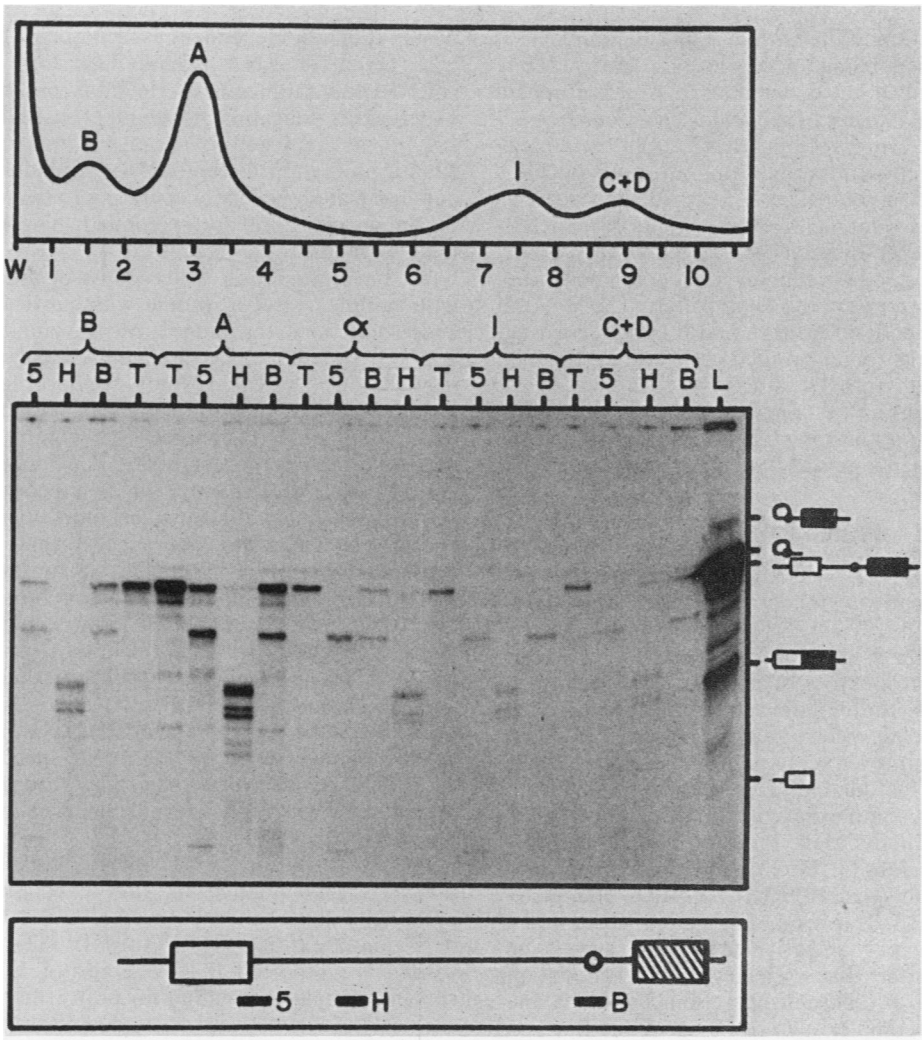

FIG. 5. 5′ splice junctions are protected against oligonucleotide-mediated cleavage in complexes A and B. A preparative splicing reaction was fractionated by RNP gel electrophoresis, and the gel was cut into 0.5-cm slices. Each slice was counted (top panel) to identify individual RNP complexes. An identical lane was exposed overnight to confirm complex identification. B, A, I, and C/D (the bottom portion of the I region in which product complexes first appear) were identified by counting gel slices. Some amount of the α complex was detected on the trailing edge of the complex B peak (3.5 to 4.0 cm) on the overnight autoradiogram and was used as a source of this complex. Complexes were eluted from individual gel slices and subjected to oligonucleotide-directed RNase H cleavage with oligonucleotides (12-mers) complementary to 5′ splice junctions (5), internal intron sequences (H), and branch-point sequences (B) (middle panel). Relative location of the sequences complementary to the utilized oligonucleotides is shown in the cartoon (open box, exon 1; hatched box, exon 2; circle, branch point). Actual nucleotide map position for each oligonucleotide is indicated in Materials and Methods. An equal amount of eluted material was used for each protection and was also displayed directly (T lanes). The RNA content of the reaction analyzed on the RNP gel is also indicated (L lane). After oligonucleotide-mediated cleavage, RNA products were resolved on a 7 M urea RNA gel. On the exposure presented, only bands resulting from cleavage of precursor RNA are visible. Protection is detected by appearance of a band of length equal to precursor RNA; no protection results in cleavage bands smaller than precursor RNA. Precursor, intermediate, and end-product RNAs for the total reaction (lane L) are indicated to the right.

nucleotide-directed RNase H cleavage to demonstrate the protection of consensus sequences in gel-isolated complexes. Complexes B, A, α, and I were eluted from gel slices and subjected to RNase H protection analysis (Fig. 5). Oligonucleotides complementary to the 5′ splice junction, the branch point, or internal intron sequences were used to probe the association of factors with these regions (Fig. 5). Each oligonucleotide was used in an amount giving complete cleavage of precursor RNA in the absence of extract. The oligonucleotide complementary to non-consensus sequences within the intron provided an internal control. This region should be free of specific factors in all complexes. Owing to the minimal length of the intron in MINX precursor RNA, there was some concern that the binding of factors at the 3′ end of the intron might give misleading protection at the 5′ end of the intron. The oligonucleotide complementary to internal intron sequences directed cleavage of RNA in all complexes from either whole extract (data not shown) or from gel slices. Therefore, these sequences are not protected by specific proteins, and 5′ and branch-point protection results could be considered independently.

The oligonucleotide protection assay shown in Fig. 5 was performed on gel-isolated complexes from RNP gel analysis of a 90-min splicing reaction with wild-type MINX precursor RNA. The reaction was frozen and thawed multiple times before RNP gel electrophoresis to produce some amount of

the α complex in addition to the normal A and B complexes. Areas of the gel corresponding to complexes B, A, α, I, and C/D (the lower region of the I complex in which product complexes first appear) were excised and used as a source of individual complexes. The precursor RNA in all the eluted complexes was cleaved with the internal intron oligonucleotide. 5′ splice junction sequences were protected against cleavage in complexes from the A and B regions of the RNP gel but not from the α or I regions of the gel. Branch-point sequences were protected in precursor RNA contained in B, A, and α complexes but not in the I complexes. The level of 5′ splice junction protection for the A and B complexes was equivalent to the branch-point protection. We interpret this experiment to indicate that U1 snRNPs are bound to 5′ splice junctions in the A and B complexes and are providing the observed protection and that U2 snRNPs are bound to the branch-point sequence in the B, A, and α complexes.

DISCUSSION

Elucidation of the process of RNA splicing requires an understanding of the pathway for formation and disassembly of the spliceosome (15, 23, 31). Several fractionation techniques have been utilized to examine spliceosome assemblies formed after introduction of exogenous RNAs to in vitro splicing extracts. Both gradient centrifugation (2, 6, 8, 12, 13, 17, 20a, 26) and gel electrophoresis (18, 19, 22, 27, 28) identified initial complexes containing precursor RNA, larger active complexes containing reaction intermediates, and smaller terminal complexes containing reaction products. On sucrose gradients these three types of complexes have sedimentation values of 20 to 35S, 50 to 60S, and 20 to 30S, respectively, with substrate RNAs of 400 to 500 nucleotides containing a single intron. On native gels, initial complexes migrate faster than active complexes, suggesting a larger size for the latter. Because each of the fractionation systems reported utilizes different separation conditions, the relationship between complexes in one system and those in another is not clear. Furthermore, it is not clear whether any of the systems is resolving intact assemblies or only portions thereof. For example, although gradient-fractionated active complexes contain U1 snRNPs as defined by immunoprecipitation (2, 8, 13), complexes fractionated by either of the gel systems of Konarska and Sharp (18, 19) do not. We found conditions under which complexes containing U1 snRNPs survive electrophoresis. Our system resolves preactive and active complexes that contain U1 snRNPs. Therefore, we suggest that U1 snRNPs are stable components of the active splicing complex and can be observed as such under optimum conditions.

Assembly of the preactive complex required ATP, sequences in both the 3′ and 5′ portions of the intron, and U1 and U2 snRNPs. Therefore, the first stable assembly that we observed required participation of both ends of the intron and the factors that recognize each end. This result is in agreement with recent experiments suggesting that 5′-binding factors influence recognition of the 3′ end of the intron and vice versa (9, 20a, 22, 30, 32, 33). Initial assembly of the spliceosome, therefore, may be best conceived not as independent binding of individual factors recognizing separated consensus sequences, but rather as concerted recognition and definition of an intron by multiple snRNPs and associated factors.

A number of experiments investigating snRNP interactions with the spliceosome during splicing have suggested independent association of U1 or U2 snRNPs with precursor RNA (2, 7, 8, 12–14, 18, 19, 29). Our data would suggest that observation of independent association of U1 or U2 snRNPs with precursor RNA results from loss of one of these snRNPs under utilized experimental conditions. Many of the experiments indicating independent association of individual snRNPs have utilized RNase protection techniques. Perhaps RNase protection disrupts RNA-dependent snRNP-snRNP communication normally operating to define an intron, such that aberrant, normally prohibited, single snRNP interactions with substrate occur.

We have previously observed association of U1 snRNPs with complexes reconstituted with substrates deleted for 5′ splice junctions, as judged by the ability of U1-specific antibodies to immunoprecipitate these complexes from splicing reactions (33). In this study, we observed that complexes lacking 5′ splice junction sequences could be observed on gels but had different mobility than complexes reconstituted with wild-type precursor RNA. Furthermore, we were unable to immunoprecipitate gel-isolated complexes containing substrates deleted for 5′ splice junctions with U1-specific antibodies. Therefore, we suggest that U1 snRNPs are present on assemblies containing substrates without 5′ splice junction sequences but that this association is unstable to gel electrophoresis. Because the only known consensus sequences remaining in our 5′ deletion substrates were those at the 3′ end of the intron, we suspect that the association of U1 snRNPs with substrates deleted for 5′ splice junctions is occurring at the 3′ end of the intron. Substrates deleted for 3′ splice junction sequences form no specific complexes as assayed by gel electrophoresis or sucrose gradients, supporting an initial role for 3′ intron sequences in assembly (2, 12, 13, 18).

Initial association of U1 snRNPs with the 3′ end of the intron is substantiated by the reduction in spliceosome immunoprecipitability observed after U1 RNA cleavage or depletion of extracts for U1 snRNPs (33). In this communication, we reported that cleavage of U1 RNA inhibited specific complex formation for both wild-type and 5′ splice site deletion substrates. Coupled with the difference in electrophoretic mobility between wild-type and 5′ deletion A

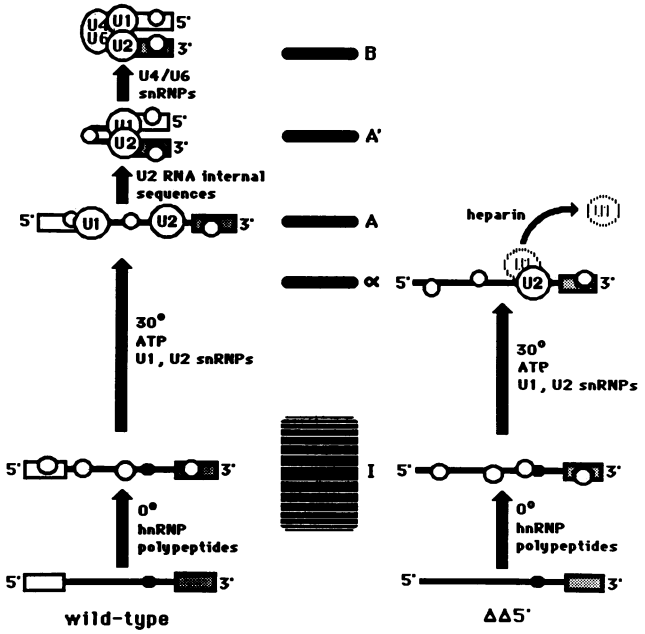

FIG. 6. Early splicing complexes revealed by gel electrophoresis. Gel complexes are identified with probable snRNP composition.

complexes, the sensitivity of complex formation to U1 cleavage suggests that the initial contact of snRNPs with precursor RNA is via a simultaneous binding of U1 and U2 snRNPs near the 3′ splice junction followed by association of U1 snRNPs with the 5′ splice junction to produce pre-splicing complex A on RNP gels.

Figure 6 summarizes the gel electrophoretic pattern of early splicing complexes observed by our system and the probable snRNP involvement in each. The first snRNP-containing complex is the A complex containing U1 and U2 snRNPs. The A′ complex is depicted as a conformational change of the A complex, dependent on nucleotides 28 to 42 of U2 RNA. Addition of U4 and U6 snRNPs (5, 16) is indicated as the difference between complexes A′ and B. We have no information about the participation of U5 snRNPs in the formation of the complexes we see on gels. The most obvious difference between the scheme shown in Fig. 6 and those promoted by other laboratories (15, 23, 31) is the lack of any complex containing U1 but not U2 snRNPs or vice versa. We favor the interpretation that no such complexes are normal assembly intermediates but are instead produced by experimental manipulation. We do acknowledge that our gel conditions may prohibit visualization of single snRNP complexes in the same way that other gel systems prevent retention of U1 snRNPs.

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