Affinity Chromatography of Splicing Complexes: U2, U5, and U4+U6 Small Nuclear Ribonucleoprotein Particles in the Spliceosome

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The splicing process, which removes intervening sequences from messenger RNA (mRNA) precursors is essential to gene expression in eukaryotic cells. This site-specific process requires precise sequence recognition at the boundaries of an intervening sequence, but the mechanism of this recognition is not understood. The splicing of mRNA precursors occurs in a multicomponent complex termed the spliceosome. Such an assembly of components is likely to play a key role in specifying those sequences to be spliced. In order to analyze spliceosome structure, a stringent approach was developed to obtain splicing complexes free of cellular contaminants. This approach is a form of affinity chromatography based on the high specificity of the biotin-streptavidin interaction. A minimum of three subunits: U2, U5, and U4+U6 small nuclear ribonucleoprotein particles were identified in the 35*S* spliceosome structure, which also contains the bipartite RNA intermediate of splicing. A 25*S* presplicing complex contained only the U2 particle. The multiple subunit structure of the spliceosome has implications for the regulation of a splicing event and for its possible catalysis by ribozyme or ribozymes.

SPLICING OF MESSENGER RNA (MRNA) PRECURSORS (PRE-mRNA) occurs by a two-step cleavage-ligation process (*1–3*). In the first step, cleavage at the 5′ splice site accompanies linkage of the 5′ end of the intervening sequence via a 2′,5′-phosphodiester bond to an adenosine residue at the branch site (*2–4*). This generates a bipartite intermediate containing the 5′ exon RNA and a lariat RNA containing the entire intervening sequence (IVS) and 3′ exon. In the second step, spliced RNA and the free intervening sequence RNA are generated by cleavage at the 3′ splice site and linkage of the exons by a normal 3′,5′-phosphodiester bond.

Before the first cleavage-ligation step, a multicomponent, 40 to 60*S* complex is assembled on mammalian (50 to 60*S*) (*5, 6*) and yeast (40*S*) (*7*) pre-mRNA substrates. Such a complex, termed spliceosome, was anticipated since the two RNA's in the intermediate would have to be confined in a single structure for efficient reaction. As would be expected, the two RNA's in the intermediate (the 5′ exon and the lariat IVS –3′ exon) are a distinctive feature of the spliceosome. Other requirements for formation of the spliceosome are (i) incubation in the presence of adenosine triphosphate (ATP) (*5–7*), (ii) activity of U1 small nuclear ribonucleoprotein particles (snRNP's) as demonstrated by inhibition with specific antiserum to U1 snRNP (*5, 6*), and (iii) functional splice site sequences in the pre-mRNA (*5, 6*). Mutations that attenuate or block spliceosome formation include point mutations at the 5′ and 3′ splice sites (*6, 7*), deletion of the polypyrimidine tract near the 3′ splice site (*6*), and, in yeast only, mutations in the branch site (*7*).

A characteristic lag is observed before the appearance of the products of splicing during incubation of reaction mixtures made from extracts of mammalian cells (*8–10*). Such sigmoidal reaction kinetics reflects the requirement for assembly of the spliceosome as an early step in the splicing process. Consistent with this observation, the 60*S* complex can be converted into the products of splicing with reaction kinetics lacking the characteristic lag (*5*).

The constituents and their arrangement in the spliceosome are of interest since the structure of the spliceosome is probably the final determinant in the specificity of splicing. The snRNP's are likely candidates for spliceosome components. First, various antisera specific for the U class of snRNP's have been found to inhibit splicing (*1, 11, 12*). These data, in combination with results of experiments where splicing was inactivated by oligonucleotide-directed cleavage of either U1 or U2 snRNA's (*13, 14*) demonstrate the importance of snRNP's in the process. Furthermore, immunoprecipitation with specific antisera has provided evidence for the presence of U1 and U2 snRNP's in the 50 to 60*S* spliceosome (*5*). Experiments in which protection against ribonuclease was combined with immunoprecipitation have demonstrated binding of three distinct snRNP's with specific regions of the pre-mRNA (*13, 15*). By this criterion, U2 snRNP protects the branch site region of pre-mRNA in an ATP-dependent fashion (*13*; also, for protection experiments, see *16*). U1 snRNP protects the 5′ splice site region in an ATP-independent fashion (*13*) and another snRNP, possibly U5 snRNP, protects the 3′ splice site region of pre-mRNA in an ATP-independent fashion (*15*). These data suggest that a minimum of three snRNP's interact stably with pre-mRNA, and it therefore follows that these are likely constituents of the spliceosome.

In this article, we present a method for affinity purification of the

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spliceosome and analysis of its snRNP constituents. The affinity purification scheme is based on the specificity of the interaction of biotin and streptavidin. This technique has been used successfully to isolate plasma membrane proteins (*17*) and hormone receptors (*18*).

Affinity purification method. The problems inherent in the identification of the components of the spliceosome are predominantly related to contamination by U1 and U2, the abundant snRNP's. That is, in the glycerol gradient system used to resolve the 60*S* spliceosome, fractions in the 60*S* region are contaminated by snRNP's that trail from the 10 to 15*S* region. To purify the spliceosome from free or loosely bound factors, a scheme based on affinity purification of pre-mRNA was developed (Fig. 1). In this scheme, pre-mRNA was synthesized in the presence of a low level of the biotin-UTP (uridine triphosphate) conjugate (*19*). The resulting biotin-labeled pre-mRNA was used as a substrate for splicing under standard conditions in a nuclear extract of HeLa cells. The entire reaction mixture was fractionated by sedimentation in a glycerol gradient, and complexes from distinct peaks were selected by binding to streptavidin-agarose beads. The high affinity of the biotin-streptavidin interaction (dissociation constant = $10^{-15}M$) (*20*) gave specific retention of these complexes. The beads were extensively washed, and specific, bound components were eluted and analyzed.

Transcription reactions contained ^{32}P-labeled UTP to monitor the percentage of incorporation of biotin-UTP and to allow detection of pre-mRNA–related species. Conditions were established where an average of five biotin-UTP residues were incorporated in a 465-nucleotide (nt) RNA chain. Biotin-UTP (Fig. 2A) is a poor substrate for SP6 polymerase. However, at a biotin-UTP level of 1.25 percent of the total nucleoside triphosphate pool, an 80 percent yield of product was obtained compared to a control reaction which lacked biotin-UTP (Fig. 2A). In addition, this amount of biotin-UMP (uridine monophosphate) incorporation did not result in the partitioning of biotin-labeled RNA into the interphase during the extraction with phenol.

The biotin-labeled pre-mRNA was compared as a substrate for splicing with the normal, unmodified substrate. Incubation of the biotin-labeled substrate in a HeLa cell nuclear extract showed that the RNA products and intermediates of the reaction appeared with the same time course and had the same gel mobility as those of the unmodified substrate (Fig. 2B).

The ability of the biotin-labeled pre-mRNA to form splicing complexes was also tested in comparison to the control substrate. The pattern of complexes obtained with the two substrates was essentially identical (Fig. 2C). To reduce the binding of nonspecific factors to splicing complexes, heparin, a negatively charged polymer, was added to splicing reactions before they were subjected to fractionation on glycerol gradients. The pattern of complexes obtained by this method is shown in Figs. 2C and 3A. Three complexes containing full-length pre-mRNA were resolved. These complexes sediment at 15*S*, 25*S*, and 35*S*. Formation of the latter two complexes required the addition of ATP. The 35*S* complex is the spliceosome as judged by the presence of both RNA's characteristic of the intermediate in splicing. The 25*S* complex is probably a prerequisite complex in the formation of the spliceosome (*21*). Material in the 15*S* region formed in variable amounts (i) in the absence of ATP (Fig. 3A), (ii) during incubation at 4°C, and (iii) with an RNA substrate lacking known splice sites (*22*). Thus some fraction of the 15*S* material probably represents nonspecific binding of cellular components to RNA. The efficiency of formation of the 25 and 35*S* complexes with biotin-labeled pre-mRNA was identical, within experimental error, with that of normal substrate RNA (*23*).

Analysis of the RNA components of the affinity purified spliceosome reveals the presence of U2, U4, U5, and U6 RNA's.

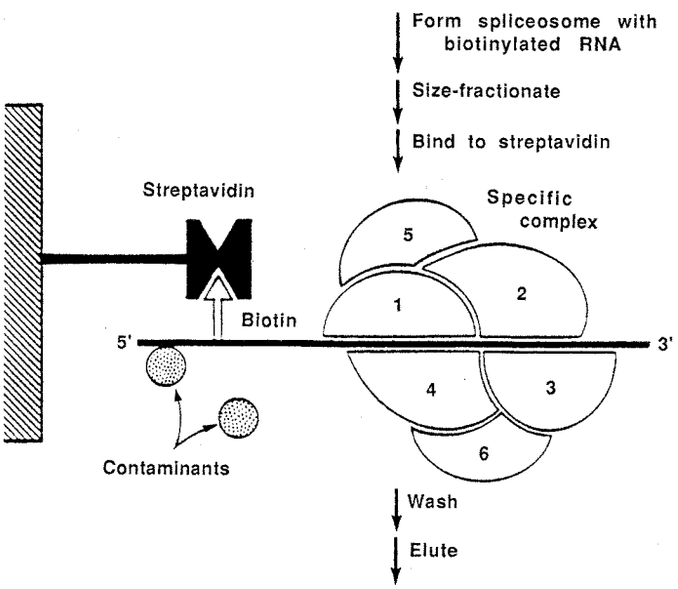

Fig. 1. Scheme for affinity purification of spliceosome components. A hypothetical splicing complex is shown (see text).

The RNA components of the 35*S* spliceosome have been identified as follows. A saturating level of biotin-labeled pre-mRNA was incubated in a HeLa cell nuclear extract for 15 minutes, and the complete reaction was treated with heparin and then sedimented in a glycerol gradient to fractionate the various complexes (Fig. 3A). Material from each distinct sedimentation class, 15*S*, 25*S*, and 35*S*, was then incubated directly with streptavidin-agarose to select those complexes containing biotin-labeled pre-mRNA. The streptavidin-agarose–bound material was extensively washed and individual components were eluted. The cellular RNA species present were labeled as follows. Eluted fractions were extracted with phenol, precipitated with ethanol, and incubated with [^{32}P]pCp and T4 RNA ligase. This method labels only those RNA species with a 3′ hydroxyl group. As a control, identical splicing reactions lacking pre-mRNA (Fig. 3B, lanes 2, 4, and 6) were fractionated according to size and treated in parallel with those containing biotin-labeled substrate (Fig. 3B, lanes 1, 3, and 5).

Selection of components bound to the biotin-labeled pre-mRNA from the 35*S* spliceosome fraction yielded RNA species that comigrated with U2, U4, U5, and U6 RNA's (Fig. 3B, lane 5). Selection of components with streptavidin-agarose from the equivalent fractions of the control gradient were not enriched in these snRNA's. Also purified from the 35*S* fraction containing the biotin-labeled spliceosome was an unidentified RNA species X that migrated in the vicinity of transfer RNA (75 to 85 nt). These results suggest that this set of snRNA's form specific complexes with the pre-mRNA substrate in the spliceosome.

Material from the 25*S* region of the above gradient was analyzed in the same way. Selection of RNA bound to the biotin-labeled pre-mRNA from the 25*S* complex yielded enrichment for only one cellular RNA species, U2 snRNA (Fig. 3B, lanes 3 and 4). Other cellular RNA species were present at equivalent levels in the biotin-labeled and control reactions. This represents nonspecific background in the experiment since the amount is independent of the presence of biotin-labeled pre-mRNA substrate. Purification of the 15*S* complex did not reveal any specific RNA components bound to biotin-labeled pre-mRNA (Fig. 3B, lanes 1 and 2).

The specificity of the biotin-streptavidin selection is shown by the use of pre-mRNA lacking biotin (Fig. 3B, lane 7). As we anticipated, no snRNA's above background were selected during chromatog-

raphy of these 35*S* fractions on streptavidin-agarose. This control shows that the streptavidin-agarose matrix by itself does not select snRNP components from fractions containing spliceosomes.

To demonstrate that genuine complexes were purified, rather than nonspecific complexes formed after gradient fractionation, the following experiment was done. Immediately before selection on streptavidin-agarose, biotin-labeled substrate was added to the 25 or 35*S* region of a control gradient that lacked substrate RNA. These mixtures were then purified as described above. No snRNA's were purified by binding to streptavidin-agarose (Fig. 3C, lanes 2 and 5) even though these fractions of the gradients contained abundant snRNP's before chromatography (*23*).

To further identify the RNA species purified from the 35*S* spliceosome, the [^{32}P]pCp-labeled RNA's were subjected to partial ribonuclease (RNase) T1 digestion. The distribution of G residues relative to the labeled 3′ terminus dictates the pattern obtained; thus, comparison of partial digestions of two RNA's should reveal their relatedness. The RNase T1 products of the affinity purified species (Fig. 4A, single line above lanes) were resolved in parallel with ^{32}P-labeled snRNA markers (Fig. 4A, cross-hatched bar above lanes). The patterns of partial digestion products of the RNA species eluted from the affinity purified 35*S* complex matched those of the corresponding marker snRNA's (Fig. 4A). Thus, both comigration in gels and comparison of patterns of RNase T1 partial digestion suggest that the 35*S* spliceosome contains U2, U4, U5, and U6 snRNA's. Interestingly, U1 snRNP, a factor shown to be essential for splicing, was not enriched in either the 25*S* or 35*S* complex (see below).

To ensure that the RNA's were correctly identified, [^{32}P]pCp-labeled snRNA's U2, U4, U5, U6, and X from the 35*S* spliceosome were also subjected to partial RNase T1 digestion in parallel with purified U1 snRNA. The pattern of U1 snRNA digestion products did not match any of the RNA's isolated from the 35*S* spliceosome, including species X (Fig. 4B). Hybridization of purified species X to M13 single-strand recombinants containing sequences complementary to the entire human U1 gene showed that this species is not related to U1 RNA (*23*). These data provide evidence that U1 snRNA is not present in the 35*S* spliceosome.

A Western blot analysis was made in order to confirm the presence of genuine snRNP proteins in the 35*S* spliceosome (Fig. 5). Complexes in fractions sedimenting at 25*S* and 35*S* were purified as described above, and the proteins were separated by gel electrophoresis. The resolved proteins were transferred by electrophoresis to a nitrocellulose sheet and probed with monoclonal antibodies specific for either the Sm- or U1-snRNP antigen. The Sm-specific antibody detects the Sm antigen, which is present on polypeptides B, B′, D, and E, common to snRNP's of the U class; whereas the U1-specific antibody detects only the 68-kD polypeptide (*24–26*).

The prominent Sm-specific polypeptides B and B′ were found in both the 35*S* (Fig. 5, lanes 9 and 10) and 25*S* complexes (*23*). The remaining Sm-specific polypeptides D and E were not detected.

Stoichiometry of snRNP's. The approximate stoichiometry of the snRNA's in the 35*S* complex was determined by recovering the [^{32}P]pCp-labeled RNA species (Fig. 3B, lane 5, and 3C, lane 4) from gels and measuring the radioactivity (Table 1). In order to

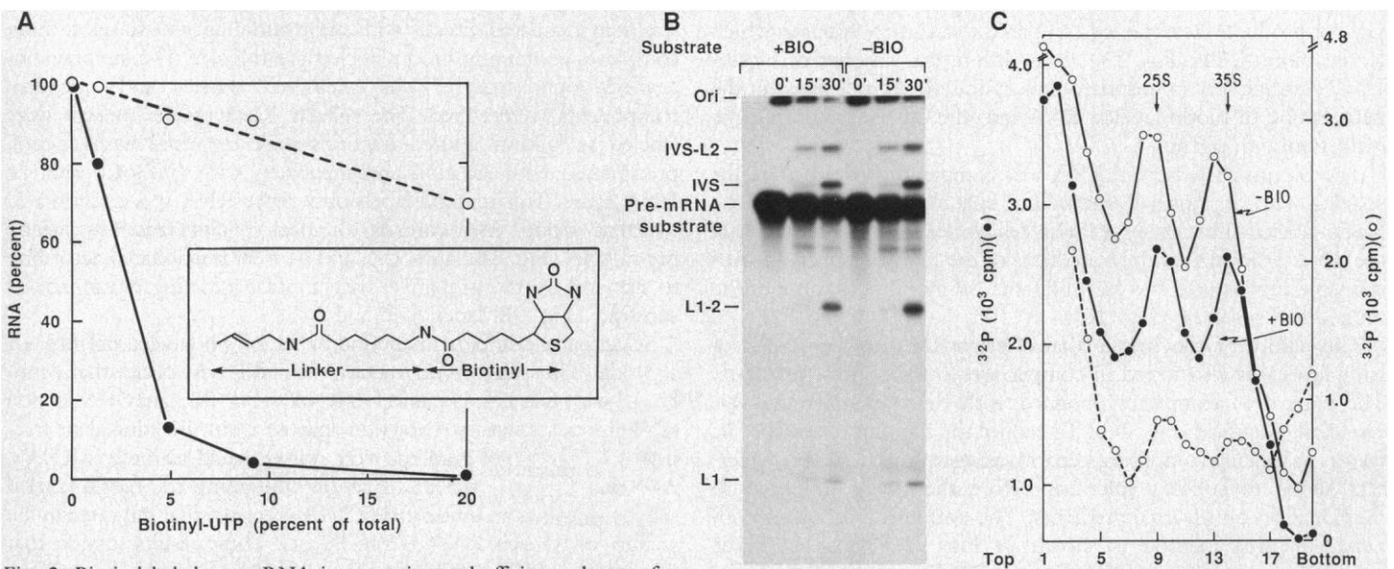

Fig. 2. Biotin-labeled pre-mRNA is a genuine and efficient substrate for splicing. (A) Efficiency of SP6 transcription in the presence of biotin-UTP (closed circles) and efficiency of RNA recovery after phenol extraction (open circles) are shown. The structure of the biotin-containing side chain linked to position 5 of UTP is shown (inset). The transcription curve shows the percentage of RNA product (percentage DE81 filter-bound counts per minute) as a function of the amount of biotin-UTP (percentage of the total nucleoside triphosphate pool). (B) [^{32}P]UTP-labeled substrate RNA's with (+BIO) or without (−BIO) biotinyl residues were tested in parallel for splicing activity in a HeLa nuclear extract. The RNA species were purified and resolved on a 10 percent polyacrylamide, 8*M* urea gel to assay for the intermediates (IVS-L2 and -L1) and products (IVS and L1-2) of splicing. (C) Similarly, 15-minute splicing reactions were analyzed directly by sedimentation in glycerol gradients to compare the relative efficiency of splicing complex formation on biotin-labeled (closed circles) or unlabeled (open circles) substrate RNA's. Splicing reactions were treated with heparin before gradient fractionation to reduce nonspecific binding of nuclear extract components to the RNA substrate (legend to Fig. 3). Heparin treatment changed the sedimentation coefficient of the spliceosome from 60*S* to 35*S* and could strip specific as well as nonspecific components from the complex. Heparin is known to dissociate protein-DNA interactions; however, the open complexes of RNA polymerase and DNA promoter sequences are resistant to treatment with heparin (*41*). A parallel splicing reaction but without heparin (dashed line) is shown for comparison; under these conditions the 60*S* complex was pelleted. Because of the similarity of the profiles containing substrate RNA's labeled with (+BIO) or without (−BIO) biotinyl residues, the ordinates of the two graphs are not identical. RNA precursor was synthesized from plasmid pRSP1 (*4*) digested with Bgl I. Transcription reactions were performed as described (*42*) except that the concentration of the following components was: ATP, CTP, GTP, and UTP, 0.5 m*M* each; [α^{32}P]UTP, 1.0 mCi/ml; DNA template, 0.02 mg/ml; and SP6 RNA polymerase, 2 U/μl; biotin-UTP, 0.025 m*M* (biotin-11-UTP; BRL). RNA transcripts were purified by gel electrophoresis (*1*). Analytical splicing reactions for (B) (0.025 ml), or preparative reactions for (C) (0.3 ml) were performed in a HeLa cell nuclear extract, as described (*5*).

calculate the molar ratios of the various snRNA's it is necessary to know the efficiency of [^{32}P]pCp labeling of each species since considerable variations in efficiency have been observed (*27*). This was estimated by [^{32}P]pCp labeling of purified nuclear RNA's and deriving a specific activity (counts per minute per micromole) for each snRNA species. The specific activities were then used to calculate a molar ratio for each snRNA species (Table 1).

The approximate stoichiometry of snRNA's in the 35*S* complex suggests that U2, U4, U5, and U6 snRNA's are each present at one copy per 35*S* complex (Table 1). Others have shown that U2 and U5 snRNP's are distinct complexes which sediment at 10*S* (*28*) and 8*S* or 30*S* (*25, 29*), respectively, while U4 and U6 snRNA's are associated in a single complex which sediments at 10*S* (*29, 30*). We then infer that the three snRNP's, U2, U5, and U4+U6, are present in approximately unit stoichiometry in the 35*S* spliceosome. The sum of the molecular weights of the three snRNP particles (0.6×10^6 to 1.1×10^6 daltons) (*31*) is within the range expected for a complex that has a sedimentation coefficient of 35*S*.

A role for the U4+U6 RNP particle. Our method for purification of splicing complexes is a novel adaptation of affinity chromatography based on the specificity of the biotin-streptavidin interaction. A biotin-labeled pre-mRNA was used as a substrate for the formation of splicing complexes, and the resulting complexes were selected by binding to streptavidin-agarose. This method for splicing complex purification permits direct identification of any factors stably bound to pre-mRNA. This is particularly important in the study of the role of snRNP's in splicing as it does not rely on antibody reagents specific for snRNP's. Using affinity chromatography, we have identified four distinct snRNA constituents of the 35*S* spliceosome: U2, U4, U5, and U6 snRNA's.

Previous work has suggested that U1, U2, and possibly U5 RNP particles might be involved in the pre-mRNA splicing process (*1, 11–15*). A role for the U4+U6 particle has heretofore not been suggested, although it has been proposed that this particle, specifically U4 snRNA, might be involved in polyadenylation (*32*). Our results suggest strongly that the U4+U6 particle functions in the splicing process. It should be noted that the substrate RNA used here contains no polyadenylation site.

An additional component, species X, was identified from the 35*S* spliceosome region (Fig. 3). This species does not comigrate with any of the HeLa cell snRNA species that have been identified to date. It may be a 3′ terminal fragment of one of the snRNA's, U2,

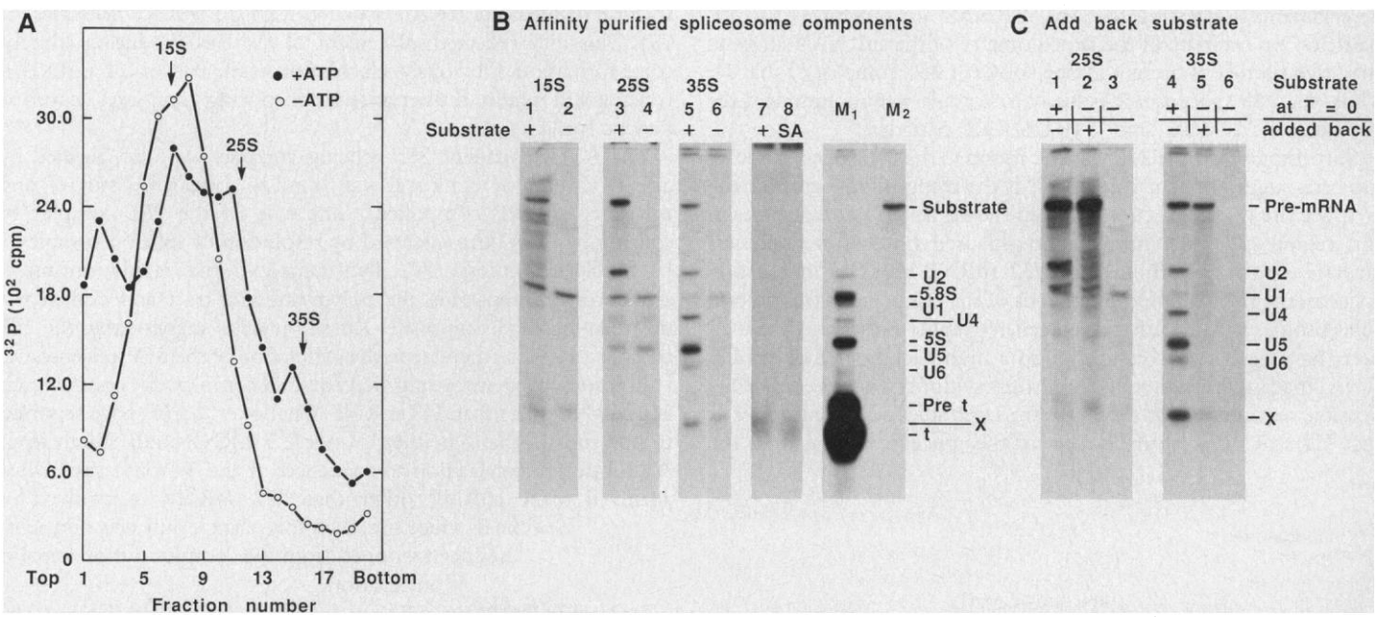

Fig. 3. Detection of snRNA components of the affinity purified, 35*S* spliceosome. Biotin- and [^{32}P]UTP-labeled pre-mRNA was incubated for 15 minutes in a HeLa cell nuclear extract to generate splicing complexes. These reactions were treated with heparin and size-fractionated by sedimentation in glycerol gradients. (A) ^{32}P profile of glycerol gradient used for affinity chromatography (+ATP). A splicing reaction incubated without ATP (−ATP) serves as a marker for the ATP-independent 15*S* peak. The gradient fractions containing the various biotin-containing complexes (15*S*, fractions 7 and 8; 25*S*, fractions 11 and 12; 35*S*, fractions 15 and 16) were then selected by binding to streptavidin-agarose. After the selected complexes were washed, individual components were eluted. To detect RNA species present in the eluted fractions, these were extracted with phenol, precipitated with ethanol and labeled at the 3′ end with [^{32}P]pCp and T4 RNA ligase. Control splicing reactions (B, lanes 2, 4, and 6) were those incubated without substrate. As another control, a non-biotinylated substrate RNA was used to form splicing complexes, and the 35*S* gradient fractions were selected on streptavidin-agarose (B, lane 7). For the matrix control, only gradient buffer was added to the streptavidin-agarose (B, lane 8, designated SA). For the M1, RNA purified from the total nuclear extract was labeled as above to serve as markers; for M2, biotin-labeled substrate RNA was used to generate complexes. (C) As a control, the 25*S* (C, lane 2) and 35*S* (C, lane 5) regions of a gradient lacking substrate RNA were combined with biotin-labeled substrate immediately before the addition to streptavidin-agarose. Preparative scale (0.3 ml) splicing reactions, constituted as described (*5*), contained saturating levels (0.01 mg/ml) of pre-mRNA. The reaction mixtures were incubated for 15 minutes at 30°C, and heparin (4 mg/ml) was added; incubation was continued for 8 minutes at 30°C. The entire reaction was sedimented in glycerol gradients containing 0.025*M* KCl as described (*5*), except that the centrifugation time was 5.5 hours. Size markers were sedimented in parallel gradients and without heparin treatment. Gradients were collected in 20 fractions, 0.550-ml each, and the gradient profile was obtained by scintillation counting of a 20-μl sample of each. Two fractions containing the peak fraction for the 15*S*, 25*S*, and 35*S* peaks were then added to 50 μl of packed streptavidin-agarose (BRL). Streptavidin-agarose had been previously washed in 1 ml of buffer A (10 percent glycerol, 20 m*M* Hepes, *p*H 7.5, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA) and 1*M* KCl, followed by two washings with 1 ml of buffer A plus 0.025*M* KCl containing carrier bovine serum albumin (BSA) at 0.1 mg/ml, glycogen at 0.1 mg/ml, and 1 m*M* UTP. Binding was carried out for 1.5 hour at 4°C in the presence of carrier. The beads were washed five times with 1 ml of buffer A containing 0.1*M* KCl; the bound material was eluted at 90°C, over a 5-minute period in 1 percent SDS, 0.001*M* EDTA, and 50 μg each of BSA and glycogen. The eluate was extracted with phenol and precipitated with ethanol, and labeled with [^{32}P]pCp and T4 RNA ligase (*27*). RNA species were resolved on a 10 percent polyacrylamide, 8*M* urea gel.

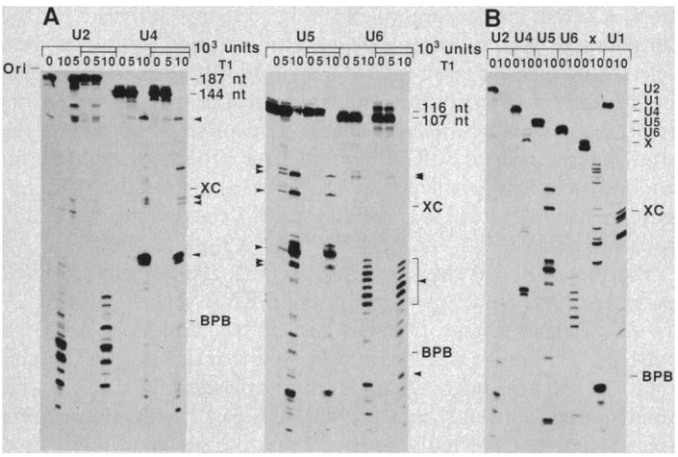

Fig. 4. Partial T1 digestion of affinity-purified RNA species identifies known snRNA's. (A) [^{32}P]pCp-labeled RNA species isolated from 35*S* gradient fractions by selection on streptavidin-agarose (single line above lanes) were gel-purified and treated with RNase T1 under conditions to give partial digestion (*43*). Markers for the known snRNA's (cross-hatched bar above lanes) U2, U4, and U6 were purified from the 15*S* region, and U5 snRNA was purified from the 30*S* region of a gradient-fractionated HeLa cell nuclear extract. Marker snRNA's were [^{32}P]pCp-labeled, gel-purified and treated with RNase T1 in parallel with the affinity purified RNA species. Products of RNase T1 digestion were resolved on a 20 percent polyacrylamide 8.3*M* urea gel. Positions of common T1 fragments are indicated by arrowheads. XC, xylene cyanole; BPB, bromophenol blue. (B) U1 snRNA purified from the 15*S* region of a gradient-fractionated nuclear extract was treated as in (A), in parallel with affinity purified snRNA species U2, U4, U5, U6, and unidentified RNA species X.

U4, U5, or U6, or it may represent an unidentified snRNA, such as the HeLa equivalent of sea urchin U7 snRNA, which is similar in size (60 nt) (*33*). The relative quantity of species X selected in the 35*S* spliceosome is similar to that of the other RNA's (Table 1) and therefore its presence is significant.

The 35*S* spliceosome contains polypeptides with Sm-specific determinants suggesting that the snRNA's are present as snRNP particles. An estimate of the stoichiometry of the snRNA's suggests that each particle is present at one copy per 35*S* complex (Table 1). Thus, the 35*S* spliceosome is likely to contain a minimum of three subunits: the U2, U5, and U4+U6 RNP particles.

Surprisingly, U1 snRNP was not found in the 35*S* spliceosome. It has been suggested that U1 snRNP is the recognition factor for the 5′ splice site (*28*); in fact, it has been shown to bind to sequences in this region (*34*). We have previously used immunoprecipitation analysis to detect both U1 and U2 snRNP particles in the 60*S* spliceosome (*5*). Attempts at analysis of the nonheparin-treated 60*S* spliceosome, with the affinity chromatography technique described here, have been unsuccessful due to a high background of cellular RNA that sediments with the streptavidin-agarose beads. One possible explanation of the apparent contradiction noted above is that U1 snRNP is normally part of the spliceosome, but that the sedimentation conditions (that is, treatment with heparin) used in this study caused its removal. In this case, U1 snRNP would have to be much less stably bound than the other snRNP particles. An alternative possibility is that U1 snRNP is a factor that only acts transiently in splicing and is not a part of the fully assembled spliceosome. If this is the case, the previous findings that U1 snRNP binds pre-mRNA in an ATP-independent fashion would reflect the transient role of the particle. It is worth recalling in this regard that during incubation of pre-mRNA under splicing conditions, the fraction of substrate RNA associated with U1 snRNP decreases (*5, 13*). This may reflect displacement of the particle during spliceosome formation. Obviously, elucidation of the role of U1 snRNP in splicing will require further analysis of splicing complexes as well as genetic studies.

Table 1. Approximate stoichiometry of snRNA species purified from the 35*S* spliceosome.

snRNA	Fraction of total		Raw ratio‡
	^{32}P (cpm)*	RNA (10^5 μmol)†	
U2	0.1410	0.2766	1.00
U1			
U4	0.1489	0.2615	0.95
U5	0.3378	0.2991	1.08
U6	0.1305	0.3787	1.37
X	0.2056	ND	ND

*Numbers were determined by scintillation counting of the gel slices in Fig. 3, lanes 5 and Fig. 4C, and were adjusted by subtracting the background in the control lanes (Fig. 3B, lane 6, and Fig. 3C, lane 6). †These values are in counts per minute (cpm) but are corrected for variations in labeling efficiency. First, a specific activity (counts per minutes per microgram) for each snRNA was determined by densitometry scanning of the autoradiograph and of the negative of the ethidium bromide–stained gel containing snRNA's labeled with [^{32}P]pCp. This specific activity was then converted to counts per minute per micromole with the use of the known molecular mass of each snRNA (*49*). The snRNA's were isolated and labeled as in Fig. 3. To obtain the values in this column, the cpm values were normalized by the specific activity (cpm/μmol 10^{-5}) for U2 (0.5097), U4 (0.5693), U5 (1.1293), or U6 (0.3446) RNA's. These specific activities indicate that the labeling of U5 snRNA was two to three times more efficient relative to U2, U4, and U6 snRNA's. ‡Approximate molar ratio based on the assumption that one copy of U2 snRNA is present per 35*S* spliceosome. These values are qualified by the unkown variation in ethidium bromide staining of each snRNA species. ND; not determined.

The ATP-dependent 25*S* splicing complex was also purified by affinity chromatography and was found to contain U2 snRNP and no other snRNP component. The role of the 25*S* complex in splicing has also been analyzed by resolution of splicing complexes by gel electrophoresis (*21*). This complex forms rapidly on intron sequences encompassing the polypyrimidine tract and branch site upstream of the 3′ splice site. Kinetic analysis suggests that the 25*S* complex is an intermediate in the generation of the 35*S* spliceosome.

It is somewhat surprising that the 25*S* complex does not contain any snRNP other than U2 snRNP. Chabot *et al.* (*15*) have reported that an snRNP that is neither U1 nor U2 snRNP binds rapidly in an ATP-independent fashion to sequences at the 3′ splice site. Thus, again, if some snRNP other than U2 snRNP is involved in recognition of the 3′ splice site, then this particle only acts transiently in splicing or has been stripped from the complex during heparin treatment, or gradient purification.

Role of snRNP's in splicing: Implications for regulation? The finding that multiple snRNP's are present in the spliceosome is intriguing for a number of reasons.

1) The requirement for assembly of multiple snRNP's into a single structure on a precursor RNA suggests an analogy with the assembly of the subunits of a ribosome (*35*). In the process of initiation of translation, a ribonucleoprotein particle, the 40*S* ribosomal subunit, combines with another RNA protein complex, initiator transfer RNA, mRNA and factors, to form a stable multicomponent complex at the site of the initiation codon (*36*). This process requires the binding of a high-energy cofactor, guanosine triphosphate (GTP), and hydrolysis of ATP, probably in a scanning process following recognition of the 5′ cap structure. The subsequent binding of another ribonucleoprotein particle, the 60*S* subunit, requires the action of a number of transient factors as well as hydrolysis of GTP. This process is superficially similar to the splicing process where multiple snRNP's are assembled on specific sites in the precursor RNA in an ATP-dependent fashion. The ultimate splicing complex is very stable and its structure specifies the sites of cleavage and ligation.

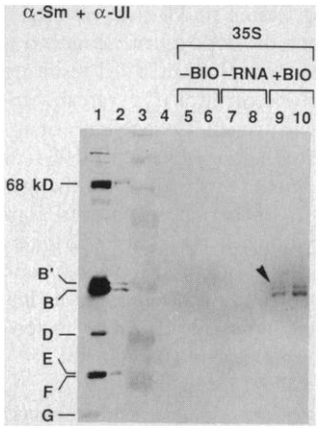

Fig. 5. Western blot analysis of affinity purified spliceosome proteins. To detect protein components, eluted fractions prepared as for Fig. 3 were precipitated with trichloracetic acid and resolved on a 12.5 percent discontinuous polyacrylamide–SDS gel (*44*). Proteins (200 to 6.2 kD) were transferred electrophoretically to nitrocellulose (*45*) and probed first with antibody to Sm (Y-12) (*46*) and antibody to U1 (2-73) (*47*), and then with a second antibody (see below) conjugated to alkaline phosphatase. Western blot was developed as described below. Total nuclear extract, 4 μl and 0.4 μl (lanes 1 and 2, respectively) provides markers for the U1-specific (68 kD) and Sm-specific (B′, B, D, and E) polypeptides. Prestained protein markers (lane 3) indicate approximate positions of 200, 97, 68, 43, 25.7, 18.4, 12.3, 6.2, and 3 kD. The nitrocellulose filter was blocked for 1 hour in 40 ml of blotto (5 percent Carnation nonfat dry milk, 0.05*M* tris, *p*H 8.0) (*48*), followed by incubation (1 hour) with 1 ml of primary antibody (approximately 4 mg) plus 20 ml of blotto. After the filter was washed three times with 40 ml of the blotto solution (10 minutes per washing), the second antibody (antibody to mouse IgG, conjugated to alkaline phosphatase; Promega Biotec) was incubated with the filter for 1 hour. The filter was washed in 40 ml of blotto for 10 minutes, and then washed with four 40-ml portions of 0.15*M* phosphate-buffered saline. Detection was by incubation of the filter with color substrates 5-bromo-4-chloro-3-indolyl phosphate (0.33 mg/ml) and nitro blue tetrazolium (0.17 mg/ml) in 0.1*M* tris, *p*H 9.5, 0.1*M* NaCl, 0.005*M* $MgCl_2$. Lane 4 is a blank lane; lanes 5 and 6, no biotin; lanes 7 and 8, no RNA substrate; lanes 9 and 10, biotinylated substrates. F and G are snRNP proteins not recognized by either antibody (α-Sm or α-U1).

2) The necessity for multiple snRNP's to assemble in a spliceosome structure is a possible control point for a splicing event. That is, altered amounts or ratios of snRNP's in the cell may affect regulation of the splicing process in situations where one or more snRNP is limiting. Another possibility is that a given snRNP in the complex may be replaced by a different, or modified, snRNP to give an altered rate of reaction. Such a situation may be important in the biological context of tissue specific splicing events (*37*). In this regard, the finding that different U1 snRNA's are produced during *Xenopus* development is quite interesting (*38*). This scenario requires flexibility in spliceosome structure to permit the incorporation of different combinations or types of snRNP's. Whether all spliceosomes contain the same set of snRNP's can be directly tested with the affinity chromatography protocol developed here.

3) The cleavage and ligation reactions during splicing may be catalyzed by the RNA constituents of the snRNP's in the spliceosome (*39*). That is, the role of snRNP's in the spliceosome may be to precisely align phosphate esters to be broken and joined. Here it is of interest that a mitochondrial pre-mRNA containing a group II intron, which generates products and intermediate RNA's that closely resemble those of a nuclear pre-mRNA splicing event, undergoes self-splicing (*40*). This finding suggests that the highly structured group II intervening sequence not only aligns phosphate bonds but is responsible for the catalytic activity that results in splicing. In the nuclear pre-mRNA case, that splicing activity may be intrinsic to the snRNA constituents of the spliceosome is an intriguing possibility.

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