The first ATP-dependent complex formed in pre-mRNA splicing is the prespliceosome, a 30 S complex. This reaction was investigated using partially purified fractions isolated from nuclear extracts of HeLa cells. Previous studies (Furneaux, H. M., Perkins, K. K., Freyer, G. A., Arenas, J., and Hurwitz, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4351-4355) have shown that DEAE-cellulose chromatography of nuclear extracts yielded two fractions (fractions I and II, eluted at 0.2 and 1 M NaCl, respectively) which carried out pre-mRNA splicing only when combined. Fraction II, alone and in the presence of ATP, supported the formation of the 30 S complex. In this report, we have separated fraction II into ribonucleoprotein and protein-rich fractions by isopycnic banding in CsCl. The combination of these two fractions completely replaced fraction II in prespliceosome formation; when supplemented with fraction I, they yielded spliced products. The CsCl fractions, like fraction II, efficiently converted pre-mRNA to the 30 S complex with high yields (30-70%). The 30 S complex was shown to contain pre-mRNA complexed to U2 small ribonucleoproteins and small amounts of U1 small ribonucleoproteins. The 30 S complex protected a 50-nucleotide region at the 3'-end of the intron from T1 RNase attack. This region included sequences spanning the branch site, the polypyrimidine stretch and the AG dinucleotide of the 3'-splice site.

When the 30 S complex was first generated with partially purified fractions, followed by the addition of a large amount of poly(U) or unlabeled pre-mRNA, the 30 S complex could be chased into a 55 S spliceosome complex by the addition of fraction I. These results support the conclusion, initially derived from kinetic data, that the 30 S complex is a precursor of the 55 S complex.

The splicing of pre-mRNA in vitro is preceded by the accumulation of a 55-60 S spliceosome complex (1-4). This complex, formed only in the presence of ATP, contains pre-mRNA splicing intermediates and a number of snRNPs (5, 6). The function of the spliceosome is to juxtapose exon ends so that they can be ligated together. This process must depend upon intermolecular reactions between proteins, ribonucleoprotein complexes, and the pre-mRNA. A smaller ATP-dependent complex of 30-35 S (prespliceosome), containing unaltered pre-mRNA, has also been observed. This complex, which is formed rapidly and accumulates prior to the formation of the spliceosome, is thought to be a precursor of the 55 S complex. However, altered pre-mRNAs that are either incapable of forming spliceosomes or support the formation of low levels of the spliceosomes can yield increased levels of the pre-spliceosome complex (7, 8, 24).

Formation of 55 S complex requires the presence of U1, U2, U5, and the U4-U6 snRNPs. While there is general agreement that U2, U5, and U4-U6 snRNPs are found in the spliceosome complex, there is controversy about the presence of U1 snRNP, although the integrity of U1 snRNP is essential for spliceosome formation and splicing (5, 6, 9). The 30 S complex has been shown to contain U2 snRNP (6, 9, 10), but like the 55 S complex, there is controversy about the presence of U1 snRNP in the smaller complex (5, 11).

A variety of components interact with pre-mRNA prior to the formation of the 30 S complex. The first complex that is formed results from the interaction of U1 snRNP with the 5' end of the intron-exon border (12, 13). This reaction occurs at 0 °C and is formed in the absence of ATP. A number of reactions at the 3' end of the intron-exon region have been reported. A poorly characterized protein, U2 snRNP auxiliary factor (U2AF), binds to pre-mRNA recognizing sequences around the 3'-splice site (14). This protein is required for the binding of U2 snRNP to the branch site of the pre-mRNA. Another protein, intron-binding protein also binds to the 3' end of the intron recognizing the same sequence, required for U2 snRNP binding (15, 16). The binding of intron-binding protein occurs at 0 °C and does not require ATP. The role of this interaction in prespliceosome formation is presently unclear.

Since the prespliceosome is the first ATP-dependent reaction in a series of discernible partial reactions that lead to splicing, we have focused on this reaction as a more systematic way of examining the more complex overall splicing reaction. We have previously shown that nuclear extracts of HeLa cells can be separated by DEAE-cellulose chromatography into two fractions that are both required for splicing (fractions I and II (17)). Fraction II, which is eluted from DEAE-cellulose at high salt concentrations, contains all of the snRNPs required for splicing. This fraction alone can convert pre-mRNAs to the prespliceosome complex (4). In the present communications...

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1 The abbreviations used are: snRNPs, small nuclear ribonucleoproteins; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; NEM, N-ethylmaleimide; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
tion, we utilized this fraction as the starting point for a study of the synthesis of the prespliceosome complex. We have examined CsCl isopycnic gradient centrifugation as a strategy for separating snRNPs from proteins. It was reported earlier that snRNPs retained their structural integrity on CsCl gradients (18). We have found that fraction II can be further resolved by this procedure into a RNP fraction and a fraction which is a mixture of "light" snRNPs and proteins. A combination of these fractions can replace fraction II in a variety of splicing reactions. The two fractions alone form the prespliceosome complex, and when supplemented with fraction I, the two CsCl fractions fully replace fraction II in the overall splicing reaction. The experiments presented here explore the fraction requirements for 30 S complex formation. We have detected protein fractions in addition to snRNPs that are essential for prespliceosome complex. Similar studies have been reported by Kramer (19) and by Krainer (20), and our results are in complete accord with their findings.

MATERIALS AND METHODS

Preparation of pre-mRNA—The plasmid pKT1 and its construction were previously described (17). The plasmid was linearized with Scal and transcribed with SP6 RNA polymerase (21). All transcripts were capped using an 18 Snuclease with an activity of 115,000 cpm/ 

Splicing Reaction Mixtures—Reaction mixtures (50 µl) contained 20 mM HEPES-KOH buffer, pH 7.6, 2 mM MgCl2, 2 mM DTT, 2% polyethylene glycol 6000, 0.4 mM ATP, 20 mM creatine phosphate, 40 mM of labeled pre-mRNA, and the fractions as indicated. Mixtures were incubated at 30 °C for 2 h, then adjusted to 0.5 mM ammonium acetate, 0.5% SDS, 10 mM EDTA, and Escherichia coli tRNA (5 µg) was added. The mixture was diluted to a final volume of 0.4 ml, extracted with phenol-chloroform (400 µl), chloroform (400 µl), and precipitated with 1 ml of ethanol. Reaction products were analyzed on an 18% polyacrylamide gel containing 8.3 M urea and run in the presence of TBE. Gels (16 X 14 X 0.04 cm) were run at 19 mA, until the xylene cyanole reached the bottom of the gel. After drying and autoradiography, products were excised and quantitated by counting in the presence of scintillation fluid.

Complex Formation and Analysis—The reaction conditions were the same as those used for measuring splicing except that the incubation period was usually 1 h at 30 °C. Following incubation, 6 µl of a dye mixture (50% glycerol, 1% Nonidet P-40, and 0.1% each of bromphenol blue and xylene cyanole) was added, and the reaction was further resolved by this procedure into a RNP fraction and a fraction which is a mixture of "light" snRNPs and proteins.

Complexes were also analyzed by sucrose gradient centrifugation. Both 5% and 11% sucrose gradients containing 20 mM HEPES-KOH buffer, pH 7.6, 2 mM MgCl2, 1 mM DTT, and 0.1 M KCl were used. Sucrose gradients were centrifuged in an AH650 rotor ( Sorval) at 48,000 rpm at 4 °C (5 ml), or in the TH641 ( Sorval) at 35,000 rpm at 4 °C (11 ml), as indicated in the figure legends.

CsCl Isopycnic Gradient Centrifugation—The preparation of fraction I, II, and III was previously described (4, 17). A brief outline of this procedure is presented below (see "Discussion").

The material that banded at a density of 1.42 gm/ml (fraction IIR) supported the splicing of pre-mRNA when supplemented with nuclear extract pretreated with micrococcal nuclease (Fig. 1B). This activity observed with this nucleoprotein fraction was selectively isolated. After adjusting the salt concentration of the sucrose gradient fractions to 0.15 M KCl, a 50% suspension of anti-biotin-agarose beads (Bethesda Research Laboratories (40 µl), previously equilibrated with a wash buffer containing 20 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40, and 0.15 M KCl was added (per 1 ml) and the mixture incubated for 1 h at 4 °C. The beads were washed five times with the wash buffer (1 ml each time), and the complex was eluted by incubating the immobilized complex at 25 °C for 15 min with wash buffer containing 0.1% SDS, 10 mM Tris, 5 mM sodium acetate, and 0.5 mM EDTA. The RNA was extracted with an equal volume of phenol-chloroform (1:1), chloroform, and precipitated with ethanol (2.5 volumes). The reconstituted fractions were then analyzed by centrifugation at 55 °C in a solution containing 0.125 M sodium phosphate, pH 6.4, 0.25 M NaCl, 7% SDS, 10% PEG (6000), 1 mM EDTA, and 50% formamide. SP6 RNA polymerase-generated RNA probes (1.75 X 1011 cpm/pmol of labeled RNA) were hybridized to the membranes containing anti-biotin-agarose beads. Hybridization was usually carried out overnight at 55 °C. Membranes were washed several times in 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1% SDS, at 55 °C and several times with the same buffer containing 0.03 M NaCl. The membranes were subjected to autoradiography for 24-48 h with an intensifying screen at ~70 °C.

RESULTS

Separation of an snRNP-rich Fraction from CsCl Gradient Centrifugation—Previous studies from this laboratory indicated that fraction II, in the presence of ATP and labeled pre-mRNA, supported the formation of a 30 S complex (4). Thus, all of the components essential for the synthesis of this putative intermediate in splicing (prespliceosome) are present in this fraction. Fraction II was subjected to CsCl isopycnic banding in order to resolve RNA, ribonucleoprotein, and protein. As shown in Fig. 1, this procedure resulted in the separation of protein, snRNPs, and free RNA. Some resolution of the snRNPs was also observed, most likely due to differences in their ratio of protein to RNA. Thus, U2 and U6 snRNPs banded at a higher density than U1 and U5 snRNPs (Fig. 1A).

The material that banded at a density of 1.42 gm/ml (fraction IIR) supported the splicing of pre-mRNA when supplemented with nuclear extract pretreated with micrococcal nuclease (Fig. 1B). The activity observed with this nucleoprotein complex (1.42 gm/cm) was relatively stable. When subjected to a second CsCl isopycnic gradient centrifugation, it banded at the same density and showed an identical ability to support splicing when supplemented with nuclear extracts pretreated with micrococcal nuclease (results not presented). Reconstitution of splicing activity with fraction IIR peaked at a density that contained little U1 RNA, suggesting that either the amount of U1 snRNP in this fraction was augmented by the
as described under "Materials and Methods," fractions were collected RNAs in the CsCl fractions. After banding in a Sorvall T-865 rotor from the bottom of the tube, and dialyzed against buffer A containing various U-rich RNAs are indicated. B, splicing activity of various fractions. The curve (W) was obtained by assaying individual units/ml of micrococcal nuclease). The curve (A-A) was obtained pretreated with micrococcal nuclease (30 min at 30 OC using 200 CsCl fractions supplemented with nuclear extract (0.1 mg of protein) separated on a 10% denaturing polyacrylamide gel. The position of fraction I (DEAE-cellulose step) as described under "Materials and Methods." micrococcal nuclease-treated crude extract or that the low levels present in this fraction were ampie to support splicing. A second peak of splicing activity (IIIL), banding at a density of 1.35 g/ml, was detected when fraction IIIR and fraction I pretreated with micrococcal nuclease were added to each CsCl gradient fraction (Fig. 1B). This second peak of splicing activity corresponded to a region enriched in U1 and U5 RNAs, as well as in proteins. This peak of activity did not coincide with the protein profile, suggesting that the activity contained in this fraction may not only be due to a protein factor.

When fraction I was pretreated with micrococcal nuclease, both fractions IIIR and IIIL were required for splicing (Fig. 2). Previous studies showed that fraction I treated with micrococcal nuclease supported splicing when combined with fraction II. This suggested that the snRNPs and additional protein factors essential for splicing in fraction II, were distributed between fractions IIIR and IIIL.

We believe that a protein component(s) that functions in splicing is present in the protein-enriched CsCl fraction 11 or 12 (Fig. 1). This component(s) supported splicing when added to nuclear extracts that were heated at 45 °C for 15 min (result not shown). Such heated extracts alone did not support splicing. In addition, preincubation of fraction III with Ca^{2+} alone, for 15 min at 30 °C, inactivated its ability to support any of the reactions involved in splicing. This inactivation most likely represented proteolysis rather than an activation of an endogenous RNase. After incubation with Ca^{2+}, the U-rich RNAs present in fraction III were intact (result not shown).

Fraction II Can Be Replaced by the CsCl Fractions for the Synthesis of the 30 S Prespliceosome Complex—Fraction II alone, incubated with ATP and pre-mRNA, yielded a 30 S complex (4). This complex, first identified by sucrose gradient centrifugation, can be assayed by its gel mobility (8). The complex was isolated following sucrose gradient centrifugation and was shown to migrate with the same mobility as the complex detected by gel electrophoresis (Fig. 3). Two fractions (IIIR and IIIL), isolated by CsCl banding, were required to form the prespliceosome complex (Fig. 4, A–C). As shown, the amount of the prespliceosome formed was proportional to the concentration of both IIIR and IIIL and required ATP. The rate of prespliceosome complex formed (Fig. 4D) with both fractions, showed a lag of 5 min followed by a linear reaction for about 30 min at which time, approximately 40% of the input pre-mRNA had been converted to the 30 S complex.

We have previously shown that after incubation of fraction II with fraction Ib (which was isolated by Biorex 70 chromatographic separation of fraction I to yield fraction Ia and Ib), the 55 S spicisome containing 5' exon and the intron-exon lariat accumulated (4). As shown in Fig. 4E, the CsCl gradient fractions, supplemented with fraction Ib, supported the accumulation of a 55 S complex. It was noted that with increasing concentration of fraction Ib, the 30 S complex migrated significantly slower than the complex formed in the presence of the CsCl fractions alone. This change in mobility of the 30

![Fig. 1. The distribution of snRNPs and splicing activity after CsCl isopycnic centrifugation. A, distribution of U-rich RNAs in the CsCl fractions. After banding in a Sorvall T-865 rotor as described under "Materials and Methods," fractions were collected from the bottom of the tube, and dialyzed against buffer A containing 0.1 M KCl. RNA was extracted from individual fractions (0.1 ml) and separated on a 10% denaturing polyacrylamide gel. The position of various U-rich RNAs are indicated. B, splicing activity of various fractions. The curve (O—O) was obtained by assayign individual CsCl fractions supplemented with nuclear extract (0.1 mg of protein) pretreated with micrococcal nuclease (30 min at 30 °C using 200 units/ml of micrococcal nuclease). The curve (A—A) was obtained by measuring splicing activity of each fraction supplemented with fraction I (DEAB-cellulose step) as described under "Materials and Methods."](http://www.jbc.org/content/2806/10/2806.full)

![Fig. 2. Two CsCl fractions replace fraction II in splicing. Fraction I was pretreated with micrococcal nuclease (200 units/ml for 30 min at 30 °C) to eliminate cross-contaminating snRNPs. Splicing reactions were carried out for 2 h (as described under "Materials and Methods") after which time the RNA was extracted and separated on an 18% denaturing polyacrylamide gel. All reactions were carried out in the presence of micrococcal nuclease-treated fraction I (60 μg of protein). In lane 1, fraction II (25 μg of protein) was added in addition to micrococcal nuclease-treated fraction I; lanes 2 and 4 were assayed with fraction III (8.5 μg of protein) or IIIR (1 μg of protein), respectively, while in lane 3, fractions III, IIR, and fraction I were all combined. The positions of the different RNA products formed in the splicing reactions are schematically presented next to that individual species.](http://www.jbc.org/content/2806/10/2806.full)
The 30S complex was also noted in reactions containing large amounts of CsCl fractions (data not shown).

Both fractions were tested for their sensitivity to N-ethylmaleimide (NEM) treatment, heat, and micrococcal nuclease in order to characterize the components present in each fraction (Table I). When each fraction was treated with NEM, and then supplemented with the untreated fraction, prespliceosome formation was either unaffected (IIR) or was reduced by only 30% (III). In contrast, when both CsCl fractions were treated with NEM, their combination resulted in a marked decrease in 30S complex formation (10-fold). This observation suggests that a minimum of three components are necessary to form the 30S complex and that an NEM-sensitive component is distributed between III and IIR. We have established by chromatographic separation that fraction III contains two components, one of which is NEM sensitive as described below. While both fractions showed some heat lability, fraction IIR was more stable than the lower density fraction IIL; this RNP-rich fraction was partially resistant to heat, and micrococcal nuclease, it was not clear whether this fraction contained an essential RNP or only essential proteins. Subsequent studies indicated that an essential protein component(s) was present in fraction IIL that was required for 30S complex formation. Since fraction III was inactivated by incubation in the presence of 1 mM CaCl₂ without the addition of micrococcal nuclease, it was not clear whether this fraction contained an essential RNP or only essential proteins. Subsequent studies indicated that an essential protein component(s) was present in fraction III that was required for 30S complex formation.

Determination of Sequences of pre-mRNA in the 30S Complex Protected against RNase Attack—The sequenced regions of the pre-mRNA in the 30S complex were determined using T1 RNase digestion. For this purpose, 30S complex was formed with the CsCl fractions III and IIR (Fig. 5). After synthesis of the 30S complex, reaction mixtures were digested with increasing amounts of T1 RNase and then subjected to gel-electrophoresis (Fig. 5A). A significant portion of the labeled pre-mRNA was resistant to T1 RNase digestion, and this material, complexed to RNA-binding components, migrated to the same position as observed with the parental 30S complex. The RNase-resistant material was eluted from the
ment involved incubation on ice for 20 min followed by the addition of DTT (10 mM). Prior to the heat treatment, fractions were centrifuged at 12,000 X g for 2 min. Following heat treatment they were again centrifuged before they were assayed. Treatment with micrococcal nuclease was for 30 min at 30 °C using a final concentration of 200 units of micrococcal nuclease/ml. The amount of complex obtained with fraction IIL alone (6%) has been subtracted from reactions in which this fraction was added to IIR. 100% activity represented 30 fmol of pre-mRNA incorporated into the 30 S complex.

Fractions added and treatment | Complex formed
--- | ---
IIR + IIL | 100
IIR | 0
IIL | 6
IIR + IIL (NEM) | 68
IIR (NEM) + IIL | 96
IIR (NEM) + IIL (NEM) | 11
IIR (NEM) + IIL (45 °C, 5 min) | 2
IIR (45 °C, 5 min) + IIL | 100
IIR (100 °C, 5 min) + IIL | 22
IIR + IIL (45 °C, 5 min) | 55
IIR + IIL (100 °C, 5 min) | 0
IIR (micrococcal nuclease) + IIL | 2

As previously reported by us (7) and others (5, 6), the 5'-end of the intron appears to play no role in the formation of the 30 S complex since pre-mRNAs containing alterations in the 5'-end support 30 S formation. In contrast, regions involving the polypyrimidine stretch and the AG dinucleotide at the 3'-splice site, all at the 3'-end of the intron (Fig. 5C). These results are analogous to the results obtained by Kramer (19) and Konarska and Sharp (8) (24).

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**TABLE I**

Properties of fractions required for prespliceosome complex formation

Reaction mixtures (as described under "Materials and Methods"), containing the fractions treated as indicated above, were incubated with pre-mRNA at 30 °C for 1 h. Where indicated, 10 μl of fraction IIR (0.57 mg protein/ml) and 2 μl of fraction IIL (12.5 mg protein/ml) were used. The 30 S complex was separated by electrophoresis on a native composite gel, and the band corresponding to the prespliceosome complex was excised and counted. NEM (5 mM) treatment involved incubation on ice for 20 min followed by the addition of DTT (10 mM). Prior to the heat treatment, fractions were centrifuged at 12,000 X g for 2 min. Following heat treatment they were again centrifuged before they were assayed. Treatment with micrococcal nuclease was for 30 min at 30 °C using a final concentration of 200 units of micrococcal nuclease/ml. The amount of complex obtained with fraction IIL alone (6%) has been subtracted from reactions in which this fraction was added to IIR. 100% activity represented 30 fmol of pre-mRNA incorporated into the 30 S complex.

Fractions added and treatment | Complex formed
--- | ---
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IIR (NEM) + IIL (45 °C, 5 min) | 2
IIR (45 °C, 5 min) + IIL | 100
IIR (100 °C, 5 min) + IIL | 22
IIR + IIL (45 °C, 5 min) | 55
IIR + IIL (100 °C, 5 min) | 0
IIR (micrococcal nuclease) + IIL | 2

As previously reported by us (7) and others (5, 6), the 5'-end of the intron appears to play no role in the formation of the 30 S complex since pre-mRNAs containing alterations in the 5'-end support 30 S formation. In contrast, regions involving the polypyrimidine stretch and the AG dinucleotide at the 3'-splice site, all at the 3'-end of the intron appear to be essential for the accumulation of this complex. Alterations of these regions (7) result in no complex formation with the two CsCl fractions (see Fig. 8) (24).

**Nucleoside Triphosphate Requirement for the Synthesis of 30 S Complex**—The formation of the prespliceosome complex was dependent upon the addition of ATP (Fig. 6A). The rate of complex formation was maximal between 0.4–0.8 mM. The presence of an ATP regenerating system substantially reduced the concentration of ATP required in the reaction to 0.05 mM. In addition, the yield of complex formed in the presence of ATP was dependent upon the addition of ATP (Fig. 6A). 

**Fig. 5. Analysis of the region of RNA in the 30 S complex resistant to RNase T1.** A. reaction mixtures (50 μl) containing fractions IIR (4 μg of protein) and IIL (19 μg of protein) were incubated with 0.1 pmol of [α-32P]GTP-labeled pKT1 pre-mRNA (5800 cpm/fmol of RNA) and other reagents as described under "Materials and Methods." B. analysis of RNase T1-resistant RNA. The RNase T1-resistant 30 S complex was excised from the native gel (lane 5 of panel A) and the RNA eluted with a solution containing TBE, 0.1% SDS, 0.5 mM CaCl2, and 0.5 mg/ml proteinase K. The mixture was incubated for 15 min at 37 °C and then extracted with phenol-chloroform, chloroform, and ethanol precipitated. This RNA was loaded onto an 18% polyacrylamide, 50% urea gel and electrophoresed in TBE. A 50-nucleotide band, the dominant band detected on the gel, was eluted with a solution containing 0.5 M ammonium
The experiment was repeated using biotinylated pre-mRNA as the substrate. This technique has been previously used to obviate the isolation of endogenous complexes in the analysis of the U-rich RNAs present in splicing complexes (5, 6). We have used immobilized anti-biotin in order to select complexes containing the biotinylated RNA substrate. This technique resulted in lower backgrounds than observed with streptavidin and permitted the elution of the complex using mild conditions (presence of biotin in the elution buffer). In the experiment described in Fig. 7, prespliceosome complex was first isolated by sucrose gradient centrifugation in order to obtain large amounts of this product. Complexes from the 30 S peak as well as the adjacent fraction (containing the ATP-dependent complex) were incubated with immobilized anti-biotin. After washing the immobilized anti-biotin several times, the RNA was eluted using an excess of free biotin at 4°C. The RNA was isolated, run on a denaturing gel, transferred to a nylon membrane, and probed for the presence of U1 and U2 RNA (Fig. 7). As shown, U2 RNA was detected in the prespliceosome complex as well as small amounts of U1 RNA.

**RNA Binding Reactions with Pre-mRNA**—In an attempt to develop a rapid assay for the synthesis of intermediates in the splicing reaction, we have explored a number of approaches. One such treatment, the resistance to T1 RNase, was used to isolate the 50-nucleotide oligomer complex to RNA binding activities (Fig. 5). The procedure was altered so that after incubation of the CsCl fractions with wild-type pre-mRNA, T1 RNase was added, and after a short incubation, the reaction mixture was then passed through a nitrocellulose filter. The bound material, representing protein associated

![Fig. 6](https://example.com/f6.png)

**Fig. 6. Influence of ATP and salt on prespliceosome complex formation.** Reaction mixtures as described under "Materials and Methods," with fractions IIR (3 µg of protein) and IIL (19 µg of protein), were incubated for 1 h at 30°C. A, titration with ATP alone (●—●) and in the presence of 20 mM creatine phosphate and 50 µg/ml creatine phosphokinase (C—C). B, the influence of NaCl added prior to incubation at 30°C (●—●), reaction mixtures (as described in A) after 1 h of incubation were adjusted to the NaCl concentration indicated, and after 20 min on ice the samples were diluted to 0.1 M NaCl and then loaded onto the gel (C—C). The value 100% was equivalent to 16.2 fmol of 30 S complex.

![Fig. 7](https://example.com/f7.png)

**Fig. 7. U2 RNA is associated with the prespliceosome complex.** Prespliceosome reaction were scaled-up 10-fold over that described under "Materials and Methods," and incubated with CsCl fractions IIR and IIL for 90 min. The reaction mixture was then applied to a 10–30% sucrose gradient containing 20 mM HEPES-KOH buffer, pH 7.6, 2 mM MgCl₂, 1 mM DTT, and 0.1 M KCl and centrifuged for 255 min at 48,000 rpm in a Sorvall AH650 rotor. Fractions were collected from the bottom and counted. Panel A, C—C, identical reaction in which biotin-labeled pre-mRNA was used; ●—●, reaction in which non-biotinylated pre-mRNA was used; Δ—Δ, reaction containing biotinylated pre-mRNA in which ATP was omitted. Fractions 5 and 6 were pooled (representing the 30 S region) as well as fractions 7 and 8 from each gradient (representing the nonspecific ATP-independent complex). Each pooled fraction was incubated with immobilized anti-biotin as described under "Materials and Methods." After several washings, the RNA was eluted, electrophoresed on an 8% polyacrylamide gel, transferred to a nylon membrane, and hybridized to a mixed probe for U1 and U2. Panel B, lanes 1–3, represent the fractions (7 and 8 of panel A) from the nonspecific region of the gradient without ATP, with ATP, and the reaction with non-biotinylated pre-mRNA, respectively. Lanes 4–6 of panel A) from the nonspecific region of the gradient without ATP, with ATP, and the reaction with non-biotinylated pre-mRNA, respectively. Each band corresponding to the U2 RNA was excised and the 32P content quantitated. Lanes 1–6 contained 225, 349, 190, 176, 910, and 118 cpm, respectively.
Formation of Prespliceosome Complex

fragments generated by RNase T1. A, effects of T1 RNase on pre-mRNA complexes formed with CsCl fractions. Reactions identical to those described in Fig. 5 were carried out with the exception that after RNase T1 digestion, the mixtures were filtered through nitrocellulose filters. After elution from the filters, the eluates were subjected to polyacrylamide-urea gel electrophoresis as described in Fig. 5. B, the influence of different nucleoside triphosphates on the binding reaction. Reaction mixtures were as described in panel A with 0.4 mM of each nucleoside triphosphates added. Where indicated, reactions which contained 20 mM creatine phosphate (CP) also contained creatine phosphokinase (30 &ml). After incubation of the mixtures with 5 units of T1 RNase, the 50 nucleotide fragment (24). All four of the RNAs examined in Fig. 8C showed a marked ATP-dependent decrease in the binding of fragments to the nitrocellulose filter.

The decreased binding of the RNA fragments to nitrocellulose was not dependent on RNase T1. Incubation of pre-mRNAs with ATP and the CsCl fractions reduced the amount of RNA bound to nitrocellulose 3-4-fold compared with similar reactions lacking ATP (data not presented). The relationship between splicing and the ATP-dependent alteration of the RNA binding reaction is now under investigation.

**Further Resolution of Fractions Required for 30 S Complex Formation**—As described in Table I, the activity with NEM-treated CsCl fractions suggested that more than two fractions are required for 30 S complex formation. Further fractionation of both 1IR and 1IL resulted in the isolation of the components required (Fig. 9). For this purpose, fraction IIR was treated with NEM and then used to monitor the synthesis of 30 S complex in reactions supplemented with purified fractions derived from IIL. Fraction IIL was subjected to the purification procedures outlined in the legend to Fig. 9 which resulted in the isolation of fraction IIL1 and IIL2. It was evident that fraction IIL1 contained one NEM-sensitive component while IIL2 contained a different NEM-sensitive component which was also present in fraction IIL1. Further fractionation of both fractions IIR and fractionation of IIL1 and IIL2 has revealed that at least three other components plus fraction IIR are required for 30 S complex formation (data not presented).

The 30 S Complex Can Be Chased into the 55 S Complex—The proposed pathway of spliceosome assembly suggests that the 30 S complex is first formed and then converted to the 55 S complex. However, the direct precursor-product relationship between these complexes has not been demonstrated. In order to determine whether the 30 S complex is a functional intermediate of the 55 S complex (i.e. spliceosome), the following experiments were carried out. The 30 S complex was generated by the incubation of pre-mRNA pKT1 (wild-type) with ATP and fractions IIL and to IIR. The further synthesis of RNA labeled with [α-32P]GTP (specific activity, 5800 cpm/fmol) was used and the reactions were as described in panel A. Each reaction mixture was incubated with 5 units of T1 RNase. the 5'-splice site mutant in which the 3'-end of the intron AG was changed to AC. In all cases, 100 fmol with T1 RNase-fragments, was eluted from the filter and then subjected to polyacrylamide-urea gel electrophoresis (Fig. 8). In the presence of ATP, a 50 nucleotide fragment was the dominant labeled product isolated (Fig. 8A, lanes 9 and 11). This 50-nucleotide oligonucleotide was hydrolyzed by RNase T1 and the products were determined. As was the case with the material eluted from the 30 S complex (Fig. 5B), oligonucleotides of 18, 14, and 8 nucleotides in length were detected (data not shown). This indicated that the region surrounding the branch site and the 3'-splice site were complexed and inaccessible to RNase T1. In the absence of ATP, however, there was a marked increase in fragments that bound to the filter (Fig. 8A). This suggests that ATP increased the non-specific binding activity. The nucleotide effect was specific since none of the other common ribonucleoside triphosphates tested replaced ATP (Fig. 8B). The ATP-mediated decrease in binding of T1 RNase fragments was not dependent on the formation of the 30 S complex. In Fig. 8C, pre-mRNAs formed from plasmid pGT3 (where the 5'-end of the intron, GU-GAGU, was mutated to CUGACU), which does support the formation of the 30 S complex (7), yielded the 50-nucleotide fragment after RNase T1 digestion. The other two mutated pre-mRNAs, pPY1 (a polypyrimidine tract mutant) and pIEC (where the 3'-end of the intron was changed from AG to AC), do not support 30 S complex formation and did not yield the 50-nucleotide fragment (24).
of the 30 S complex was almost completely blocked by the addition of poly(U). Fraction Ib was then added and the synthesis of 55 S complex was followed (Fig. 10A). As shown, 50% of the 30 S complex was converted to the 55 S complex after 15 min (Fig. 10A, lane 3). When the reaction was repeated with pre-mRNA pGT3 (where the 5'-end of the intron was mutated), the 30 S complex formed was not converted to the 55 S complex after the addition of fraction Ib. This observation is in keeping with the requirements for splicingosome formation. When the polypyrimidine mutant pre-mRNA (pPY1) was used in place of the wild-type pre-mRNA (pKT1), as expected, no 30 S or 55 S complex was detected.

Other experiments have been carried out which indicate that the 30 S complex can be directly converted to the 55 S complex. In these experiments, after 30 S complex formation, labeled pKT1 pre-mRNA was diluted 100-fold with unlabeled pKT1 pre-mRNA or reaction mixtures were adjusted to 0.13 M KCl and the 55 S complex was eluted from the gel with a solution containing TBE, 0.1% SDS, 0.5 mM CaCl2 and 0.5 mg/ml proteinase K. After 15 min at 37°C, the eluates were extracted with phenol-chloroform, chloroform, and ethanol-precipitated. Reaction products were then analyzed as described under splicing conditions described under "Materials and Methods" (lane 2). Reaction mixtures containing crude nuclear extracts (lane 1) were used to generate splicing products as markers.

exon lariat (Fig. 10B). In this experiment, the chase was carried out in the presence of 0.13 M KCl and the 55 S complex was eluted from the gel, digested with proteinase K, and then subjected to urea-acylamide gel electrophoresis (Fig. 10B, lane 2). A control, in which the pre-mRNA was incubated with crude nuclear extract (Fig. 10B, lane 1), served as markers for the expected products generated in the overall splicing system. Similar results were obtained with reaction mixtures in which poly(U) was used to inhibit 30 S complex formation. These results support the conclusion that the 30 S complex is a precursor of the 55 S complex.
The fractions isolated from nuclear extracts of HeLa cells and their activities in the splicing reactions are summarized in Scheme 1. Nuclear extracts fractionated on DEAE-cellulose yielded two fractions required for splicing (17). Materials eluted at high salt (fraction II) contained all snRNPs essential for the in vitro splicing but lacked other protein components necessary for splicing. This fraction alone was sufficient for the formation of the ATP-dependent 30 S prespliceosome complex. Based on the observations of Sri-Wadada et al. (18), we used isotopic CsCl gradient centrifugation to resolve the snRNPs in fraction II from protein-rich components. While U2 and U4-U6 snRNPs banded at a density distinct from the protein (1.44 g/ml), U1 and U5 snRNPs overlapped with the protein rich-peak (1.37 g/ml). This snRNP profile is similar to that observed by Leelay-Taha et al. (25) who demonstrated that the various snRNPs band at different buoyant densities in the presence of high concentrations of MgCl$_2$ (15 mM). The presence of MgCl$_2$ had no influence on the snRNP profile in our experiments.

Material that passed through DEAE-cellulose at 0.2 M NaCl (fraction I) was insensitive to treatment with micrococcal nuclease (17), suggesting that all of the snRNPs required for splicing were in fraction II. The reverse experiment could not be performed since fraction II was sensitive to incubation with CsCl in the absence of micrococcal nuclease (data not shown). When the CsCl gradient fractions were assayed for their ability to complement fraction I in the splicing reaction, it was found that fraction IIL was the only other component required. The requirement for fraction IIR was only observed when fraction I was treated with micrococcal nuclease. This suggested that the difference between fractions I and II was not due to the distribution of snRNPs but most likely reflected the presence of different protein factors in these fractions.

U2 snRNP is an essential component of fraction IIR, since blotting experiments detected U2 RNA in the 30 S complex. In the presence of fraction IIL purified U2 RNA supported the 30 S complex reaction (at 30% efficiency) when added in addition to micrococcal nuclease-treated fraction IIR. However, U2 RNA alone did not replace fraction IIR in the splicing reaction (data not shown). Differences were also observed with fraction IIL in splicing and in 30 S complex formation. Splicing activity was distributed over a broad peak (Fig. 1), near the top of the gradient, while the 30 S complex activity peaked in the top fraction. The activity at the top of the CsCl gradient may include the U2 AF protein factor previously described by Ruskin et al. (14), which was reported to be required for U2 snRNP binding to pre-mRNA. However, as described in Fig. 9 and in unpublished experiments carried out in our laboratory, at least three additional components isolated from the top of the CsCl gradient are involved in 30 S complex formation.

![Scheme 1](https://example.com/scheme1.png)

**Scheme 1. Fractions isolated from HeLa extracts and their splicing activities.**

- HeLa nuclear extract
- DEAE-cellulose
  - 0.2 M NaCl (I)
  - Biorex
    - 0.2 M (Ia)
    - 1 M (Ib)
  - 1 M NaCl (II)
    - CsCl
      - IIL (snRNPs 1 + 5)
      - IIR (snRNPs 2 + 4-6)

**Fraction added**
- a) II
- b) IIL + IIR
- c) IIL + IIR + Ib
- d) II + Ib
- e) I + Ib
- f) II + Ia + Ib

**Product formed**
- 30 S complex
- 55 S complex (cleaved products)
- Spliced

In order to determine which snRNPs are part of the 30 S prespliceosome, we analyzed the complex isolated from sucrose gradients or from native gels. The RNA present in this complex was extracted, run on denaturing gels, blotted to nylon membranes, and then hybridized to probes for the various U-RNAs. Using this direct analysis, U1 and U2 RNAs were detected. However, these RNAs were also detected in reactions carried out in the absence of pre-mRNA. The use of biotinylated pre-mRNA and its selective adsorption to immobilized streptavidin (5, 6) resulted in a high background of U1 and U2 RNA. We have altered this technique by using immobilized antibiotin as the affinity reagent to select biotin-labeled pre-mRNA complexes. Besides improving the background, the complex was readily displaced with free antibiotin.

Using this procedure, U2 RNA was detected as a stable component of the 30 S complex. U1 RNA was also detected as a component of the 30 S complex but present in much lower amounts than U2 RNA (10-fold). U1 snRNP may be weakly associated and thus easily dissociated from the 30 S complex or alternatively may function catalytically in the generation of the 30 S complex. U1 RNA was not detected by blotting native gels (8, 9), but we have found this technique to be less sensitive than the procedure we have used (data not shown). Our efforts to completely free fraction III of U1 snRNP failed. Attempts to cleave the 5'-end of U1 RNA with RNase H and complementary oligonucleotides were also unsuccessful. A population of U1 snRNPs resistant to this treatment was consistently observed. Bindereif et al. (5), concluded that U1 snRNP is involved in prespliceosome complex formation since U1 snRNP binds immediately to pre-mRNA and was detected in the 55 S spliceosome complex. The direct isolation of U1 snRNP in the prespliceosome complex was inferred but not directly demonstrated. The initial complex formed between U1 snRNP and pre-mRNA is more pronounced at 0 °C than at 30 °C (which is optimal for splicing) and does not require ATP.

The binding of U1 snRNP to pre-mRNA has also been examined in the yeast system by Ruby and Abelson (26). They observed that the binding of U1 snRNP to immobilized pre-mRNA required both the 5'-splice site as well as the UAC-UAC intron sequences. The latter sequence is uniquely essential for splicing in yeast. They also showed that RNase H degradation of U1 snRNP blocked subsequent binding of U2 snRNP and other snRNPs. These results indicate that U1 snRNP is essential for complex formation.

Our results do not define the role played by U1 snRNP in forming the 30 S splicing complex. We and others (7, 19) have found that pre-mRNAs containing altered 5'-intron sequences support 30 S complex formation as efficiently as do wild-type pre-mRNAs. Such altered structures do not form the 55 S spliceosome complex. Thus, the role of U1 snRNP...
in the accumulation of the 30 S complex in the HeLa splicing system is unclear at present.

We have shown that labeled 30 S complex can be converted to the 55 S complex upon addition of fraction Ib. The synthesis of the 55 S complex depended upon the presence of functional 5'- and 3'-intron sequences previously shown to be essential for prespliceosome formation (7).

The experiments described here suggest that the conversion of the 30 S complex to the 55 S complex most likely involves the interaction of snRNPs U4-U6 and U5 with the nucleoprotein-pre mRNA complex rather binding only to free pre-mRNA. The rapid synthesis of 55 S complex after the addition of poly(U) or the addition of a larger excess of unlabeled pre-mRNA supports this consideration.

The results presented here suggest that the prespliceosome complex contains at least, U2-snRNP, proteins, and pre-mRNA. The roles played by the multiple protein fractions and fraction IIR in this ATP-dependent reaction remain to be elucidated. The synthesis of the 30 S complex could be exploited to define the structure and biological activity of the U2 snRNP in a reaction critical for splicing but less complex than the overall reaction. Similar observations have been reported by Kramer (19) and by Krainer (20). They have also developed procedures for the separation of various fractions involved in the accumulation of intermediates in the splicing system. They, as well as we, have not as yet determined whether the isolated protein components which are free of the snRNPs are nuclear proteins or represent proteins weakly associated with snRNPs and other ribonucleoprotein complexes and thus easily dissociated. The further purification and characterization of these protein fractions should help resolve this problem.

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