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Composition and Functional Characterization of the Yeast Spliceosomal Penta-snRNP

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Summary

Pre-mRNA introns are spliced in a macromolecular machine, the spliceosome. For each round of splicing, the spliceosome assembles de novo in a series of ATP-dependent steps involving numerous changes in RNA-RNA and RNA-protein interactions. As currently understood, spliceosome assembly proceeds by addition of discrete U1, U2, and U4/U6•U5 snRNPs to a pre-mRNA substrate to form functional splicing complexes. We characterized a 45S yeast penta-snRNP which contains all five spliceosomal snRNAs and over 60 pre-mRNA splicing factors. The particle is functional in extracts and, when supplied with soluble factors, is capable of splicing pre-mRNA. We propose that the spliceosomal snRNPs associate prior to binding of a pre-mRNA substrate rather than with pre-mRNA via stepwise addition of discrete snRNPs.

Introduction

Eukaryotic pre-mRNA undergoes extensive modification and processing in the nucleus to convert it into mature mRNA for export to the cytoplasm, where it is subsequently translated into protein. An important processing step is the excision of intronic sequences by the spliceosome, a large complex containing five phylogenetically conserved small nuclear RNAs (snRNAs) assembled into small ribonucleoprotein (snRNP) particles and over 60 proteins (reviewed in Burge et al., 1999; Will and Lührmann, 2001). In the context of the spliceosome, introns are removed by two sequential transesterification reactions which splice the flanking exons together to generate the proper mRNA sequence.

Studies of spliceosome assembly in vitro, using extracts made from whole yeast cells or mammalian cell nuclei, have defined an order of interaction of the snRNPs with the pre-mRNA substrate. The first to interact with the pre-mRNA is the U1 snRNP (Ruby and Abelson, 1988; Séraphin and Rosbash, 1989; Du and Rosbash, 2001). U1 snRNP binds to the 5′ splice site in the absence of ATP, and it can thus commit a labeled pre-mRNA to splicing by forming the commitment complex. Subsequently, the U2 snRNP interacts with the branch-point sequence of the intron (Parker et al., 1987) to form the prespliceosome. Addition of the U4/U6•U5 tri-snRNP and the action of other non-snRNP-associated factors, such as the spliceosomal DExD/H box ATPases, complete spliceosome assembly (reviewed in Moore et al., 1993; Staley and Guthrie, 1998). While the scheme for spliceosome assembly has been supported by numerous studies in yeast and mammalian cell systems, the conditions used to determine the putative assembly intermediates were different from the conditions that support pre-mRNA splicing in vitro. Generally, discrete U1, U2, and U4/U6•U5 snRNP particles have been purified under salt concentrations which are incompatible with splicing in vitro. Furthermore, the spliceosome assembly intermediates were defined using native gel analyses following the addition of heparin, which is likely to disrupt all but the most stable complexes.

During the purification of the yeast U4/U6•U5 tri-snRNP (Stevens and Abelson, 1999), we noticed salt-dependent changes in the characteristics of the snRNP complexes. We purified the tri-snRNP at 250 mM KCl; as the monovalent salt concentration was lowered to 150 mM, we observed a U2•U4/U6•U5 tetra-snRNP which sediments at ~30S in glycerol gradients. A similar particle was observed in extracts prepared from human cells (Konarska and Sharp, 1988). When the salt concentration was reduced to 50 mM, a U1•U2•U4/U6•U5 penta-snRNP was observed which sedimented at ~45S. Pre-mRNA splicing in yeast extracts occurs at 50–120 mM for monovalent salt (Lin et al., 1985). These preliminary observations suggested that splicing activity might correlate with the presence of the penta-snRNP. We present evidence here that the penta-snRNP contains 85% of all known yeast splicing factors and is active in pre-mRNA splicing. When supplemented with soluble factors present in a micrococcal nuclease (MN) treated extract, purified penta-snRNP exhibits splicing activity. In mixing experiments, we show that U2 snRNP from one extract does not exchange with U2 snRNP associated with tri-snRNPs from another extract but that the U1 snRNPs can exchange to a modest extent. These results indicate that the previous idea of an order of "addition" of discrete U1, U2, and U4/U6•U5 snRNPs to the pre-mRNA substrate during spliceosome assembly likely resulted from disruptions of native tetra- and/or penta-snRNP particles when these were subjected to destabilizing conditions in previously reported assays. We propose that spliceosome assembly in yeast proceeds by binding of the pre-mRNA substrate to a large preassembled particle rather than by the stepwise addition of discrete snRNPs as previously thought.

Results

Purification of a 45S Ribonucleoprotein

We have purified an ~45S ribonucleoprotein particle from yeast *S. cerevisiae* whole-cell splicing extract under conditions conducive to pre-mRNA splicing in vitro. The integral U4/U6•U5 tri-snRNP protein Prp4 was epitope tagged using a carboxy-terminal His-Polyoma (CHP) tag (Stevens, 2000). Large-scale preparations of yeast splicing extract were immunopurified on a poly-

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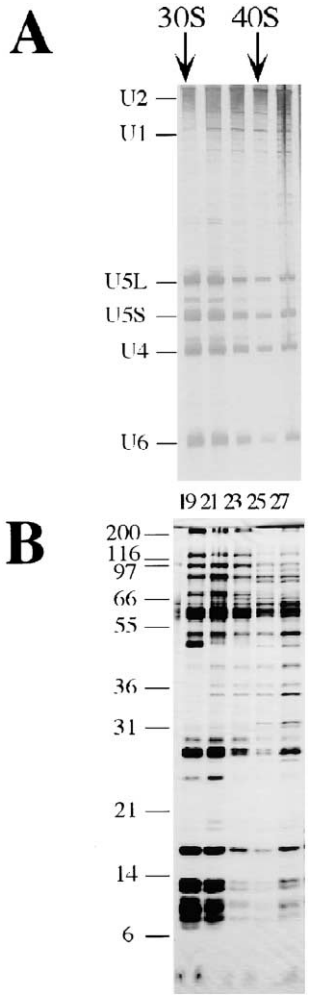

Figure 1. A Particle Containing All Five Spliceosomal snRNAs Was Isolated from Yeast Extracts

(A) Silver-stained snRNAs derived from a glycerol gradient separation are shown. The U1, U2, U4, U5S, U5L, and U6 snRNAs are noted. Fractions from the top of the gradient to the bottom were loaded from left to right, respectively. Penta-snRNP was found in fractions 21–27, and tetra-snRNP was found in fractions 18–20.

(B) Silver-stained proteins were from the same fractions as in (A). Gradient fraction numbers are noted between panels.

oma monoclonal antibody column at low salt concentrations (50 mM NaCl) and selectively eluted with the polyoma peptide as previously described (Stevens, 2000). The column eluate was layered onto a 10%–30% glycerol gradient to effect the separation of the snRNPs containing the tagged Prp4 protein. At monovalent salt concentrations of 50 mM, a U2•U4/U6•U5 tetra-snRNP and a U1•U2•U4/U6•U5 penta-snRNP are separable from the tri-snRNP by glycerol gradient sedimentation (Figure 1). The penta-snRNP sediments at ~35–50S and contains the U1, U2, U4, U5, and U6 snRNAs (Figure 1A). As detailed below, this particle contains almost all known U1, U2, and U4/U6•U5 snRNP proteins (Figure 1B) (Caspary et al., 1999; Gottschalk et al., 1998, 1999; Stevens and Abelson, 1999). As this particle contains all of the spliceosomal snRNPs, we tested the purified penta-snRNP for splicing activity.

The Yeast Penta-snRNP Possesses Splicing Activity that Requires Additional Soluble Factors

The ability of the penta-snRNP to associate with pre-mRNA and catalyze pre-mRNA splicing was assayed using the standard conditions for yeast splicing extract (Lin et al., 1985). The penta-snRNP alone did not assemble a spliceosomal complex with pre-mRNA, nor was it able to effect pre-mRNA splicing (Figures 2A and 2B, lane 6). Addition of MN-treated yeast extract, itself unable to form complexes or to splice (Figures 2A and 2B, lane 7), to the penta-snRNP fraction produced a rapid accumulation of a spliceosomal complex equivalent to the A1 complex (Cheng and Abelson, 1987) (Figure 2A). Excision of this A1-like complex from the gel followed by RNA elution and separation of the splicing products revealed that the complex contained 86% unspliced pre-mRNA, 6% first-step splicing intermediates, and 8% splicing products (data not shown). A time course experiment showed the gradual increase of a prespliceosome complex (the B complex in Cheng and Abelson, 1987) which decreased upon accumulation of the slower mobility A1-like complex at later time points (Figure 2A, lanes 8–12). Serial dilution of the MN-treated extract added to the penta-snRNP strongly diminished pre-mRNA splicing (data not shown), indicating that splicing required approximately stoichiometric amounts of soluble factor(s) from the extract. Splicing activity also required the intact particle, since the extracted snRNAs from the penta-snRNP, when added to the MN-treated extract, did not assemble spliceosomes and were not sufficient to reconstitute splicing activity (Figures 2A and 2B, lane 14). Penta-snRNP-mediated assembly and splicing showed the normal dependence on pre-mRNA splice site and branchpoint sequences, as tested using 5′ splice site mutant (G1C), branchpoint mutant (U257A), and 3′ splice site mutant (AC/AC) pre-mRNA substrates (data not shown).

The penta-snRNP was purified by two different glycerol gradient procedures and was assayed for splicing activity. In Figure 3A, the 35–50S species sedimented near the middle of the gradient. Splicing activity (dependent on addition of micrococcal nuclease-treated extract) peaked in the ~45S region. Heavier species containing U1 snRNA were observed in the gradient (Figures 3A and 3C, fractions 22–26); however, they were not competent for splicing. In Figure 3B, the 60S and 80S ribosome particles were pelleted, and again splicing activity was found in the 35–50S region near the pellet. The latter sedimentation conditions were employed to characterize all of the proteins in the penta-snRNP (see below).

Endogenous Penta-snRNPs Are the Major Precursors to Spliceosomes in Yeast Extracts

Although penta-snRNP was functional when added to MN-treated extract, we could not conclusively demonstrate that the penta-snRNP functioned as an intact particle in those experiments. A test for the integrity of functional penta-snRNP was carried out in a mixing experiment. In these experiments, we took advantage of the existence of viable yeast mutants in which the U1 or U2 snRNAs contain a large deletion of nonessential sequence (U1δ and U2δ snRNAs). Extracts were pre-

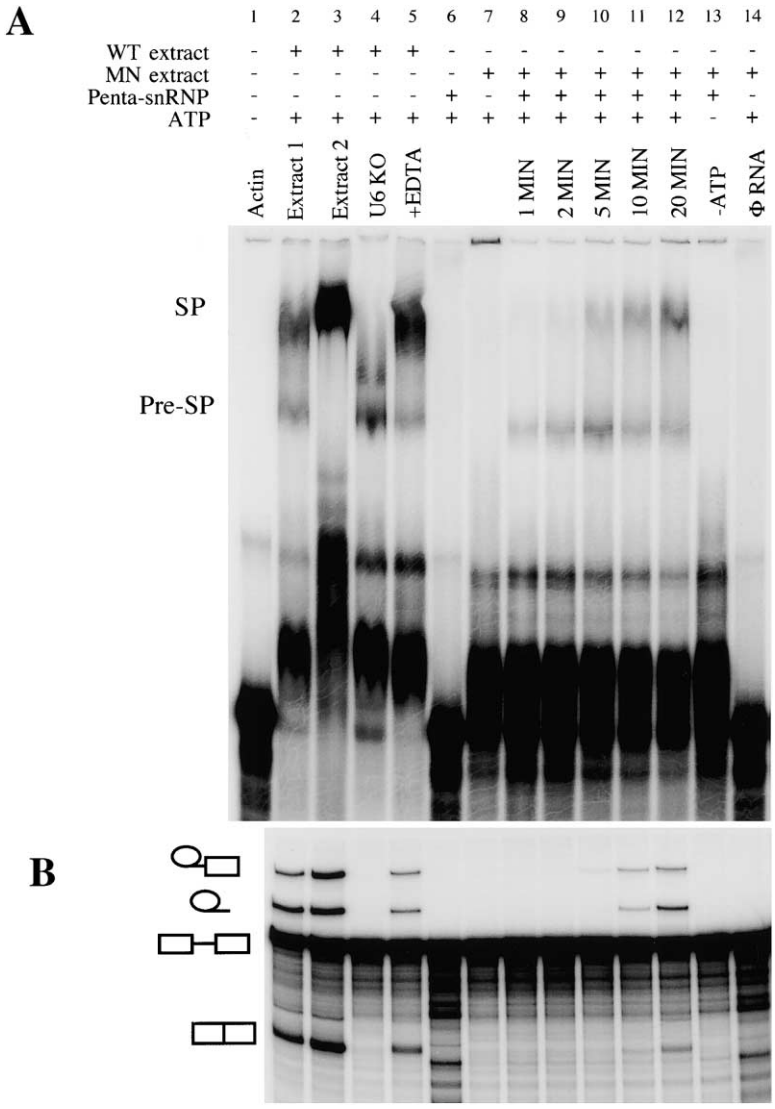

Figure 2. Pre-mRNA Splicing Is Catalyzed by the Penta-snRNP

A time course for spliceosome assembly and splicing of ^{32}P-labeled pre-mRNA was conducted in micrococcal nuclease-treated yeast extract (MN extract) complemented by gradient-purified penta-snRNP.

In (A), spliceosomal complexes were assayed by native gel electrophoresis. Spliceosome (SP) and prespliceosome (Pre-SP) species are noted. The "Actin" lane contains only the ^{32}P-labeled actin pre-mRNA. Extract 1 was used for all samples except for lane 3. For the U6 knockout (U6 KO) lane, endogenous U6 in wild-type extract was depleted by oligonucleotide-directed RNase H digestion, and U2-containing prespliceosomes accumulate. The EDTA lane contains 2 mM EDTA to block the activity of fully assembled A1 spliceosomes (Cheng and Abelson, 1987).

In (B), pre-mRNA splicing products are noted. Reactions depleted of ATP indicate that both spliceosome assembly and splicing require ATP (lane 13). Phenol-extracted and -precipitated snRNAs from penta-snRNP are not sufficient for reconstitution of spliceosome assembly and splicing in MN-treated extract (lane 14).

pared from a strain containing wild-type snRNAs as well as an epitope-tagged Prp4 protein (WT-tagged extract) and from strains containing no epitope tags and shortened U1δ or U2δ snRNA (U1δ or U2δ extract). All extracts were competent for in vitro pre-mRNA splicing (data not shown). In the accepted model of spliceosome assembly, U1 and U2 snRNPs act independently of the U4/U6•U5 tri-snRNP, suggesting that mixtures of shortened and WT U1 or U2 snRNA should associate functionally with the tagged tri-snRNP when bound to pre-mRNA. After incubating the separate or mixed extracts with biotinylated pre-mRNA under standard splicing conditions, tri-snRNP-containing particles were immunopurified by virtue of the epitope-tagged Prp4 protein and were gently eluted from the affinity column. From this pool, functionally engaged spliceosomes were then affinity purified using streptavidin beads. Associated snRNAs were recovered after proteinase K digestion, phenol extraction, and ethanol precipitation and were subjected to primer extension analysis. All splicing reactions after 10 min received a chase of additional ATP and an excess of nonbiotinylated actin pre-mRNA to eliminate detection of recycled snRNPs after a round of splicing.

A schematic diagram of our test for functional U1 and U2 snRNPs in the context of penta-snRNP particles is shown in Figure 4A. A test for exchange of U2 snRNPs in mixed extracts is shown in Figure 4B. Where indicated, extracts were mixed and incubated prior to use in a large splicing reaction (see Experimental Procedures). Total RNA from each extract was subjected to primer extension analysis, and the U2 extension products are shown for WT U2 (lane 1) and for U2δ (lane 2). Controls containing an intronless, biotinylated RNA (lane 3), no pre-mRNA (lane 4), or nonbiotinylated actin pre-mRNA (lane 5) resulted in no purification of U2 snRNA. Splicing reactions conducted using WT-tagged extract only were subjected to the two affinity chromatography steps. As expected, the WT U2 snRNA was affinity selected whether or not the extract was depleted of ATP (Figure 4B, lanes 6 and 7). Although the association of U2 snRNP with pre-mRNA after ATP depletion may seem contradictory to the traditional view of yeast spliceosome assembly, spliceosomes containing U2•U4/U6•U5 snRNPs, or yeast

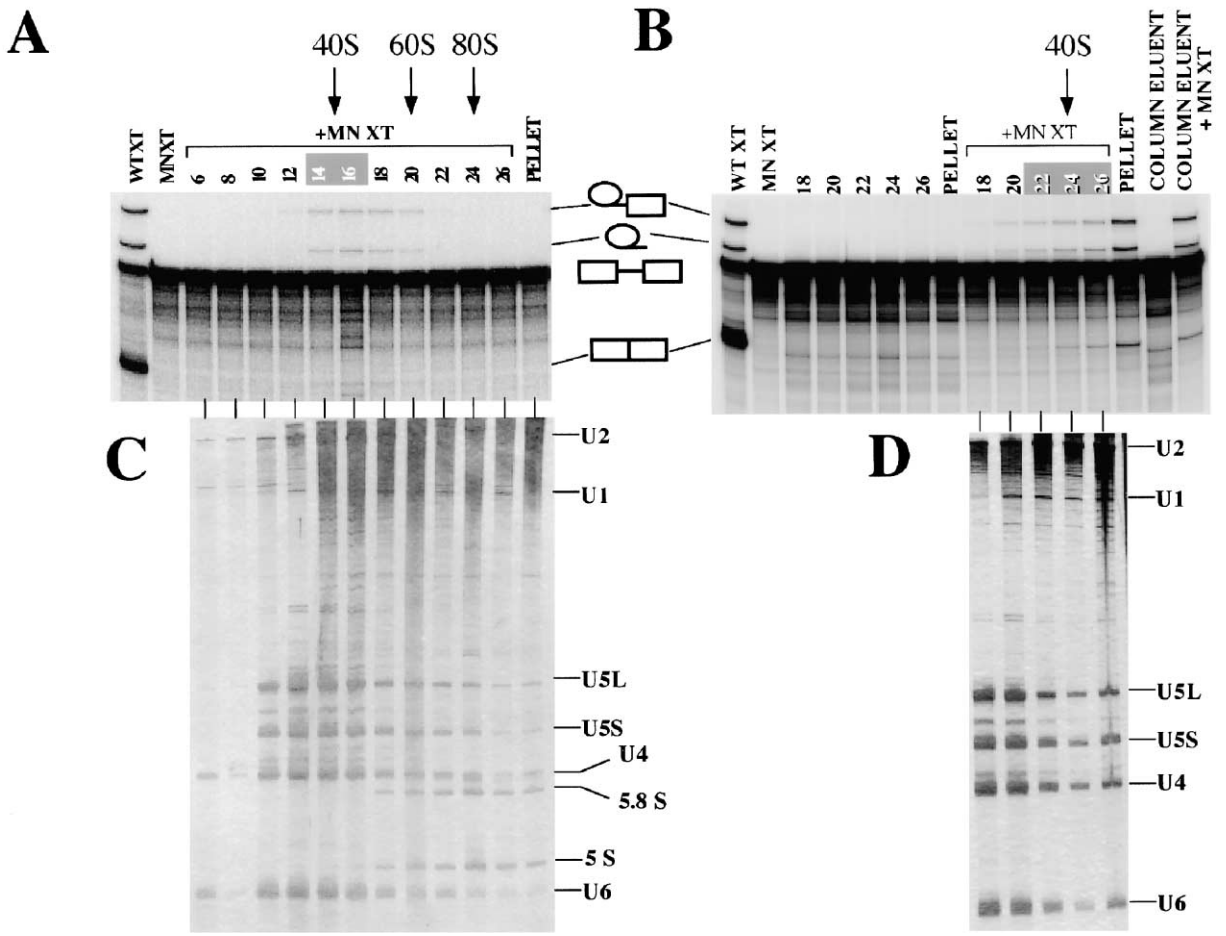

Figure 3. Penta-snRNP Splicing Activity Sediments at ~45S in Glycerol Gradients

(A) Glycerol gradient separation of the affinity-purified penta-snRNP was performed under centrifugation conditions which resolve larger complexes. The splicing activity of the gradient-purified penta-snRNP fractions using ^{32}P-labeled pre-mRNA in MN-treated yeast extract peaks at ~45S. XT, extract.

(B) Glycerol gradient separation of the affinity-purified penta-snRNP was performed under centrifugation conditions which pellet the 60S and 80S ribosomes. These centrifugation conditions were used to purify the penta-snRNP for mass spectrometry analysis. The fractions with splicing activity correspond to those in (A); however, the active fractions were additionally assayed for splicing without addition of MN-treated yeast extract, and splicing activity was shown to depend on the presence of MN-treated extract. The fractions used for mass spectrometry analysis are highlighted, and equivalent fractions are similarly highlighted in (A).

(C and D) The silver-stained RNAs extracted from each fraction. The sedimentation behavior of the 40S, 60S, and 80S ribosomes is indicated.

A complexes, have been visualized on native gels for extracts depleted of ATP (Cheng and Abelson, 1987; D.E.R., unpublished data). Parallel reactions using the U2δ extract only did not yield any U2δ snRNA by the affinity purification scheme as there was no epitope tag on the Prp4 protein in this extract (Figure 4B, lanes 8 and 9). To functionally test for the ability of the short and full-length U2 snRNPs to associate with biotinylated pre-mRNA, splicing reactions were subjected to the streptavidin affinity step only. Lanes 12–15 of Figure 4B demonstrate that both extracts contain U2 snRNPs that are functionally capable of interacting with pre-mRNA; lane 16 shows that there is no bias between short and full-length U2 in interacting with pre-mRNA after mixing the two extracts. Mixing WT-tagged and U2δ extracts and subjecting the subsequent splicing reaction to the two-step affinity purification resulted in recovery of only the WT U2 snRNA, indicating that short U2δ snRNP was unable to mix with the WT U2 snRNP associated with epitope-tagged tri-snRNP (Figure 4B, lanes 10 and 11). We conclude that competent U2 snRNP is stably associated with tri-snRNP in penta- or tetra-snRNP complexes prior to specific binding of pre-mRNA. Tri-snRNP-associated U2 snRNPs cannot exchange under the ionic conditions optimal for pre-mRNA splicing in vitro.

The U2 snRNP component of the penta-snRNP can, however, reassociate with U4/U6•U5 snRNPs in a stochastic manner when exposed to high salt. A mixture of WT-tagged and U2δ extracts was dialyzed to high salt and then returned to splicing buffer conditions by dialysis. The resulting material was subjected to Prp4 affinity chromatography, and the resulting coimmunopurified U2 snRNAs were equally mixed as shown in Figure 4B, lane 17. This experiment demonstrated that high salt can induce dissociation and scrambling of the penta-snRNP components. Dialyzing back to splicing

conditions caused both U2 snRNPs from the mixed extracts to reassociate with the tagged tri-snRNP.

Experiments analogous to those described above for the U2 snRNP assays were conducted for the U1 snRNP (Figure 4C). The controls were identical to those used in the U2 experiment and gave equivalent results. The mixing experiment (analogous to Figure 4B, lanes 10 and 11), however, demonstrated that after both chromatography steps, some short U1δ snRNA had exchanged with WT U1 and was recovered with Prp4-tagged spliceosomes, although U1δ was only 16% of the total purified U1 snRNA (Figure 4C, lanes 10 and 11). This demonstrated that approximately one-sixth of the U1δ snRNP participated in spliceosome assembly and that it associated with epitope-tagged U2•U4/U6•U5 tetra-snRNP (see above) either before or after binding of pre-mRNA. Strikingly, the majority of spliceosome assembly in both samples was initiated by penta-snRNPs—at least 68% of the total yield (allowing for a maximum of 16% each of exchangeable free U1δ and free WT U1 snRNPs). To ensure that the deletion of sequence in the U1 and/or U2 snRNAs did not introduce an experimental bias, the CHP epitope tag was moved to the Prp4 protein in the U1δ and U2δ strains. Extracts from these strains were used in conjunction with a "WT extract" containing WT snRNAs and an untagged Prp4 protein in experiments analogous to those described above. The U1δ- and U2δ-tagged extracts gave equivalent results as originally observed for the WT-tagged extract above, indicating that there was no bias introduced by our choice of sequence deletions in U1δ and U2 snRNAs (compare lane 11 of Figure 4C with lane 8 of Figure 4D and compare lane 11 of Figure 4B with lane 16 of Figure 4D). In the affinity-purified spliceosomes, the ratio of WT U1 versus U1δ snRNA from the tagged extract was 1:7 or 14% (Figure 4D, lane 8), similar to the level of U1 mixing observed for the alternately tagged extracts (Figure 4C, lane 11). Thus, the functional integrity of the penta-snRNP particles was confirmed.

U4 and U6 snRNAs Are Base Paired in the Purified Penta-snRNP

An indication that the penta-snRNP is not a spliceosome comes from a determination of U4/U6 base pairing in the isolated penta-snRNP complex. During spliceosome assembly, U4 and U6 base pairing is completely disrupted to allow mutually exclusive U2/U6 and intramolecular U6 helices to form. After splicing, U2 and U6 snRNPs must release the mRNA and lariat-intron products, and U4 and U6 snRNPs must rejoin before the next round of splicing.

We therefore assayed the base pairing status of the U4 and U6 snRNAs in purified penta-snRNP (a glycerol gradient fraction equivalent to fraction 16 in Figure 3A was used for this analysis). Phenol-extracted penta-snRNP RNA was precipitated with ethanol, resuspended in low-salt gel loading buffer, and loaded on a native polyacrylamide gel. Bands containing U6 snRNA were assayed by Northern hybridization using primers made by random hexamer priming on a PCR fragment of U6 DNA. To determine the extent of any U4/U6 reannealing that may occur during manipulation of the snRNAs, a split portion of the same penta-snRNP fraction was heated to 95°C prior to phenol extraction, etc. As a marker for U6 snRNA, an identical portion of the penta-snRNP was similarly processed but heated immediately before loading onto the gel. These samples are shown in Figure 5A. The penta-snRNP sample heated prior to extraction (lane 1) shows free U6 snRNA and no detectable U4/U6 duplex, indicating that no appreciable reannealing of U4 and U6 RNAs occurred during sample processing. As expected, the sample heated immediately prior to loading on the gel shows entirely free U6 snRNA (lane 2). The unheated penta-snRNP sample shows only base-paired U4/U6 snRNAs and no detectable free U6 snRNA (lane 3). To test for ATP-driven unwinding of U4/U6 duplexes in the penta-snRNP, a sample of penta-snRNP was incubated at 4°C for 30 min in the presence of ATP, and only free U6 snRNA was found (lane 4). The penta-snRNP contains only base-paired U4 and U6 snRNAs and therefore cannot be a fully assembled spliceosome or a postsplicing lariat-intron-bound complex.

Quantitation of snRNAs in Isolated snRNP Complexes

Nucleic acids extracted from the peak fractions of affinity-purified tri-snRNP, tetra-snRNP, and penta-snRNP were subjected to an RNase protection assay to quantitate the relative levels of the snRNAs present in each purified particle. The protecting oligonucleotides for this procedure are shown in Figure 5B. The tri-snRNP snRNAs are present at a 1:1:1 mole ratio. In the tetra-snRNP, the U2, U4, U5, and U6 snRNAs are present at a 1:1:1:1 mole ratio. In the penta-snRNP, the U1 component is substoichiometric; the ratio of U1, U2, U4, U5, and U6 snRNAs is 0.7:1:1:1:1, indicating that the "penta-snRNP" fraction analyzed is most likely a mixture of tetra-snRNP and penta-snRNP particles.

The U1•U2•U4/U6•U5 Penta-snRNP Contains All Previously Identified snRNP Proteins

The affinity-purified 45S particle that contains all five spliceosomal snRNAs and is active in splicing when supplemented with soluble factors was found to contain 85% of all proteins that are known to be associated with yeast pre-mRNA splicing. Preparative amounts of the penta-snRNP were purified by pooling fractions in the 35–50S region (Figures 1 and 3D, fractions 23–27) from several glycerol gradients, extracting the proteins with phenol and precipitating them from the organic phase with acetone. The sedimentation conditions were selected to pellet 60S and 80S particles and thereby avoid contamination with ribosomes (Figures 3B and 3D). The resuspended penta-snRNP proteins were separated by SDS-PAGE and stained with Coomassie blue G-250 (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/9/1/31/DC1). The entire lane of penta-snRNP proteins was fractionated by cutting it into ~4 mm wide gel slices, and the proteins contained in these gel bands were identified by tandem mass spectrometry (Davis and Lee, 1997).

Relevant data for the penta-snRNP proteins identified by mass spectrometry are presented in Table 1. All ten known yeast U1 snRNP proteins (Fortes et al., 1999; Gottschalk et al., 1998), ten known and one novel yeast

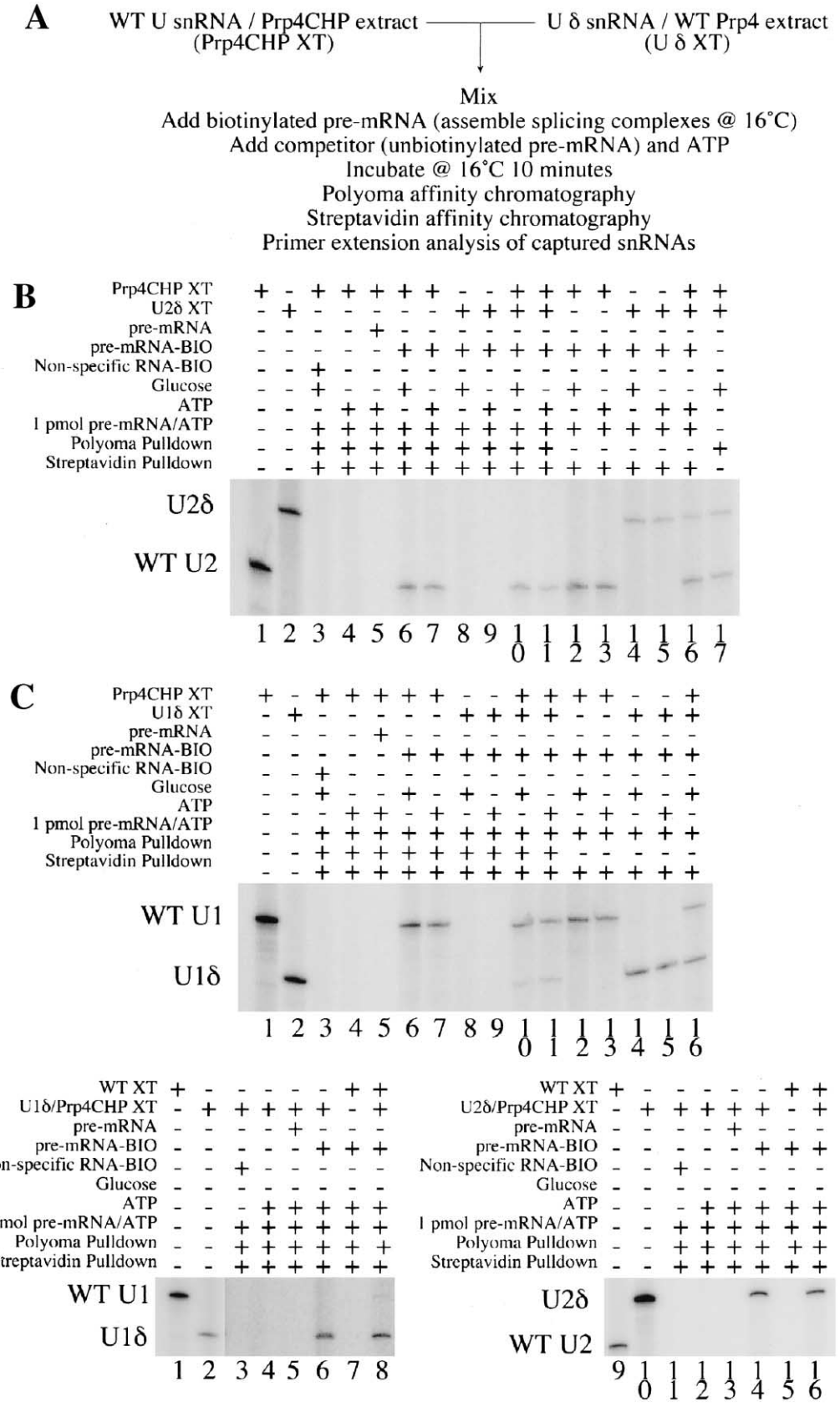

Figure 4. Penta-snRNPs Are the Predominant Native, snRNP-Containing Species that Initiate Yeast Spliceosome Assembly In Vitro

(A) Schematic diagram representing the steps performed in these experiments. Note that in (D), the epitope tag is contained in the extract with shortened snRNA, which is the reverse of (A–C). XT, extract.

(B) Test for the preassociation of U2 snRNP with affinity-tagged U4/U6•U5 tri-snRNP in spliceosome assembly. A yeast extract (Prp4CHP XT) containing WT snRNAs and an epitope tag on Prp4 protein was mixed or not with an extract (U2δ XT) containing no epitope tag and fully active, short U2δ RNA instead of WT U2. Subsequently, U2 snRNAs associated or not with the epitope-tagged tri-snRNP and biotinylated pre-mRNA were affinity purified. The primer extension products for WT U2 and internally deleted U2δ snRNA are noted on the left.

U2 snRNP proteins (Caspary and Séraphin, 1998; Caspary et al., 1999; Das et al., 1999; Gottschalk et al., 2001; Igel et al., 1998), and the thirteen known yeast U4/U6•U5 snRNP proteins (Gottschalk et al., 1999; Stevens and Abelson, 1999) were identified as components of the purified penta-snRNP. In addition to the snRNP-specific proteins, the common set of canonical Sm core snRNP proteins B, D1, D2, D3, E, F, and G was identified as being associated with this particle, as were six of the seven Sm-like (Lsm) proteins which are associated with U6 snRNA (Salgado-Garrido et al., 1999). Lsm5 was not detected.

Additional Known Splicing Factors Are Contained in the U1•U2•U4/U6•U5 Penta-snRNP

We identified the yeast Prp28 protein in the penta-snRNP (Table 1; see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/9/1/31/DC1) as well as in purified yeast U5 mono-snRNP (Stevens et al., 2001). Although the human Prp28 protein is stably associated with human U4/U6•U5 snRNPs (Teigelkamp et al., 1997), the yeast ortholog was not found in purified U4/U6•U5 snRNP (Gottschalk et al., 1999; Stevens and Abelson, 1999).

Thirteen splicing proteins, not previously known to be snRNP-associated, are contained in the penta-snRNP; these include Syf1, Syf2, and Syf3 proteins (Ben-Yehuda et al., 2000) and the yeast NineTeen Complex components (NTC), the eponymous Prp19, Cef1, Snt309, and Ntc20 (Chen et al., 1998; Tarn et al., 1994; Tsai et al., 1999). Orthologs of penta-snRNP proteins Cef1, Cwf2, Prp46, Syf1, Syf3, Ecm2, and Prp19 were found to be part of a 40S complex of spliceosomal snRNPs from *S. pombe* (McDonald et al., 1999). The Sad1 protein affects assembly of U4/U6 snRNP (Lygerou et al., 1999); Isy1 has two-hybrid interactions with Syf1 (Dix et al., 1999); Prp45 has two-hybrid interactions with Prp46 (Uetz et al., 2000); and Ecm2 has genetic interactions with U2 snRNA (Xu et al., 1998).

Other Proteins with Potential Links to Splicing Identified in the Penta-snRNP

A number of heat shock proteins were identified in the penta-snRNP isolate: Ssa1, Ssa2, Ssa3, Ssa4, Ssb1, and Ssb2. Previous studies have demonstrated that heat shock proteins have an affinity for splicing complexes in yeast (Bracken and Bond, 1999) and in mammals (Bond, 1988; Bennett et al., 1992).

The Xrn1, Pat1, and Lsm1-Lsm7 proteins constitute an mRNA degradation complex (Bouveret et al., 2000; Tharun et al., 2000), and they were present in the penta-snRNP preparation. Eight of the nine proteins of this complex were found: Lsm1, 2, 3, 4, 6, 7, Pat1, and Xrn1; Lsm5 was not found but is believed to be present endogenously. Deletion of the inessential *XRN1* gene conferred a pre-mRNA splicing defect (Johnson, 1997). Ninety percent of the Xrn1 complex is cytoplasmic (Johnson, 1997), but the 10% which is nuclear may be

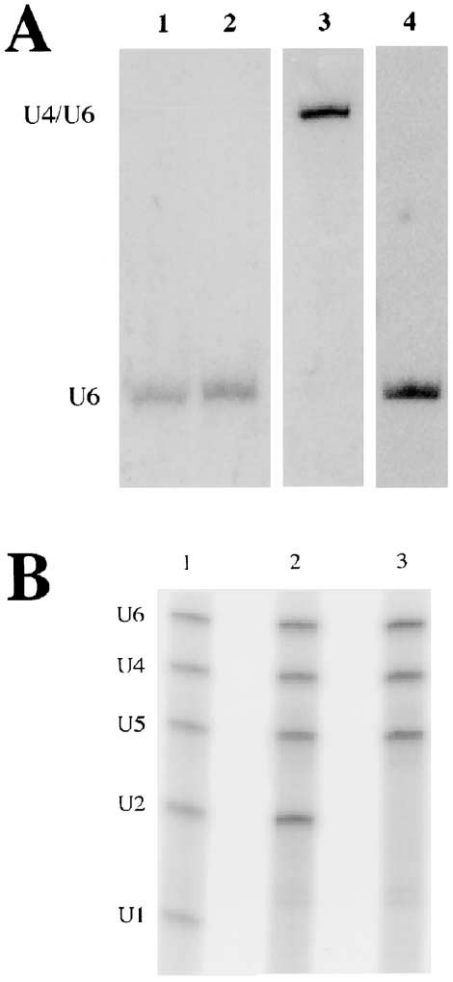

Figure 5. Penta-snRNPs Contain Stoichiometric Amounts of U2, U4, U5, and U6 snRNAs and Slightly Substoichiometric Amounts of U1 snRNA, and the U4 and U6 snRNAs Are Exclusively Base Paired

(A) Base pairing status of U4 and U6 snRNAs in the penta-snRNP. U6 snRNA was assayed by Northern hybridization for native 7% polyacrylamide (29:1) gel analysis of: penta-snRNP heated before phenol extraction and ethanol precipitation (lane 1), extracted and precipitated penta-snRNP snRNAs heated at 95°C just prior to loading on the gel (lane 2), extracted and precipitated penta-snRNP snRNAs loaded on gel with no heat treatment (lane 3), and penta-snRNP incubated with ATP prior to extraction and precipitation of the snRNAs which were not heat treated (lane 4). Identifications of the U6 and U4/U6 snRNAs are indicated.

(B) Quantitation of snRNAs in the purified snRNPs. RNA extracted from penta-snRNP (lane 1), tetra-snRNP (lane 2), or tri-snRNP (lane 3) was assayed for the presence of U1, U2, U4, U5, and U6 snRNAs using an RNase protection assay.

(C) Test for the preassociation of U1 snRNP with affinity-tagged U2•U4/U6•U5 tetra-snRNP in spliceosome assembly. Reactions were performed as in (B) using the epitope-tagged Prp4 extract (Prp4CHP XT), which was mixed or not with an extract (U1δ XT) containing no epitope tag, and fully active, short U1δ RNA instead of WT U1.

(D) Relocating the epitope tag to different strains does not affect the outcome of the experiment. Extract containing WT snRNAs and Prp4 protein (WT XT) was used with an extract containing epitope-tagged Prp4 and U1δ RNA instead of WT U1 (U1δ/Prp4CHP XT) (left side of panel) or tagged Prp4 and U2δ snRNA instead of WT U2 (U2δ/Prp4CHP XT) (right side of panel).

Table 1. Proteins Characterized by Mass Spectrometry

Protein	SGD ORF Name[a]	Calc. MW (kDa)	Number of Peptides Seq.
Known splicing proteins			
U1 snRNP proteins			
Prp39	YML046W	74.7	3
Snu71	YGR013W	71.3	3
Prp40	YKL012W	69.1	7
Prp42	YDR235W	65.1	2
Nam8	YHR086W	57	2
Snu56	YDR240C	56	4
Snp1	YIL061C	34.5	5
Mud1	YBR119W	34.4	3
Yhc1	YLR298C	32	2
Luc7	YDL087C	30	2
U2 snRNP proteins			
Rse1	YML049C	153.7	6
Hsh155	YMR288W	110	9
Prp9	YDL030W	63	7
Cus1	YMR240C	50.3	3
Prp21	YJL203W	33	13
Cus2	YNL286W	32.3	4
Prp11	YDL043C	29.9	11
Lea1	YPL213W	27.2	10
Hsh49	YOR319W	24.5	9
Snu17	YIR005W	17.1	7
Msl1	YIR009W	12.8	7
U4/U6•U5 snRNP proteins			
Prp8	YHR165C	279.5	22
Brr2	YER172C	246.1	30
Snu114	YKL173W	114	18
Prp6	YBR055C	104.2	29
Snu66	YOR308C	66.4	15
Prp31	YGR091W	56.3	12
Prp3	YDR473C	56	29
Prp4[b]	YPR178W	56	12
Spp381	YBR152W	34	3
Prp38	YGR075C	27.9	7
Snu23	YDL098C	22.6	13
Dib1	YPR082C	16.7	5
Snu13	YEL026W	13.5	3
Penta-snRNP specific proteins			
Syf1	YDR416W	100.2	8
Syf3	YLR117C	82.4	12
Cef1	YMR213W	67.7	7
Prp19	YLL036C	56.6	13
Sad1	YFR005C	52.2	5
Prp46	YPL151C	50.6	7
Prp45	YAL032C	42.4	18
Ecm2	YBR065C	40.2	1
Cwf2	YDL209C	38.3	11
Isy1	YJR050W	28.0	8
Syf2	YGR129W	24.8	2
Snt309	YPR101W	20.7	6
Ntc20	YBR188C	15.8	3
Sm/Lsm proteins			
SmB1	YER029C	22.4	14
SmD1	YGR074W	16.2	8
SmD2	YLR275W	12.8	5
SmD3	YLR147C	11.2	5
SmE1	YOR159C	10.4	1
SmF	YPR182W	10	2
SmG	YFL017W-A	8.5	2
Lsm2	YBL026W	11.2	2

continued

Table 1. Continued

Protein	SGD ORF Name[a]	Calc. MW (kDa)	Number of Peptides Seq.
Lsm3	YLR438C-A	10	2
Lsm4	YER112W	21.2	5
Lsm6	YDR378C	13.8	2
Lsm7	YNL147W	12	2
Lsm8	YJR022W	14.5	4
Proteins with a potential link to splicing			
Swi4	YER111C	123.7	7
Mbp1	YDL056W	93.8	2
Swi6	YLR182W	90.4	18
Xrn1	YGL173C	175.5	3
Pat1	YCR077C	83.4	2
Lsm1	YJL124C	20.2	5
Ded1	YOR204W	65.6	6
Ssa family[c]	YAL005C	69.6	6
Ssb family[d]	YDL229W	66.6	5
Asc1	YMR116C	34.7	11
Dhh1	YDL160C	57.4	3
Other proteins			
Pur5	YHR216W	56.5	8
YLR432W	YLR432W	56.4	11
YML056C	YML056C	56.2	6
YAR073W	YAR073W	44.3	5
Tef1	YPR080W	50	10
Nop1	YDL014W	34.3	4
Sis1	YNL007C	37.5	4
Tdh1	YJL052W	35.7	8
Gpm1	YKL152C	27.5	7
YLR016C	YLR016C	23.6	5
YCR063W	YCR063W	18.3	10
YDR428C	YDR428C	29.9	4
Rps0[e]	YGR214W	28	5
Rsp1	YLR441C	28.7	5
Rsp2	YGL123W	27.4	4
Rsp3	YNL178W	26.4	8
Rsp4	YHR203C	29.3	5
Rps5	YJR123W	25	3
Rsp7	YOR096W	21.5	6
Rsp9	YBR189W	22.1	3
Rsp12	YOR369C	15.3	7
Rsp13	YDR064W	17	4
Rsp14	YCR031C	14.5	9
Rps16	YDL083C	15.8	4
Rps18	YDR450W	17	5
Rsp19	YNL302C	15.8	4
Rsp20	YHL015W	13.8	3

[a] Stanford Saccharomyces Genome database http://genome-www.stanford.edu/Saccharomyces
[b] Calculated molecular weight of the Prp4 protein includes the epitope tag
[c] Ssa family proteins are highly similar and likely include the Ssa1, Ssa2, Ssa3, Ssa4 gene products
[d] Ssb family proteins are highly similar and likely include the Ssb1 and Ssb2 gene products
[e] Duplicated ribosomal protein genes are indistinguishable and may both be represented

associated with the spliceosome and may function as the proofreading enforcer (Guthrie, 1991).

Also present was a heterotrimeric RNA polymerase II activation complex containing Swi4, Swi6, and Mbp1. As no additional transcription factors were present in the penta-snRNP, it is unlikely that these three proteins were present because of contamination with RNA polymerase II complexes. Swi4 has two-hybrid interactions with Swi1, a Swi/Snf component, and Swi1 has two-hybrid interactions with five splicing factors (see database at http://portal.curagen.com): pre-mRNA binding proteins Bbp1 and Mud2, U2 snRNP proteins Msl1 and Cus1, and U1 snRNP protein Snp1. These factors are involved in early recognition of pre-mRNA splice sites, which may be coupled to transcription.

Discussion

The Penta-snRNP with Additional Soluble Factors Is Active in Splicing

We have shown that the yeast penta-snRNP is stable to affinity purification, to glycerol gradient sedimentation, and to challenges imposed by adding a competing extract in mixing experiments. Furthermore, the purified

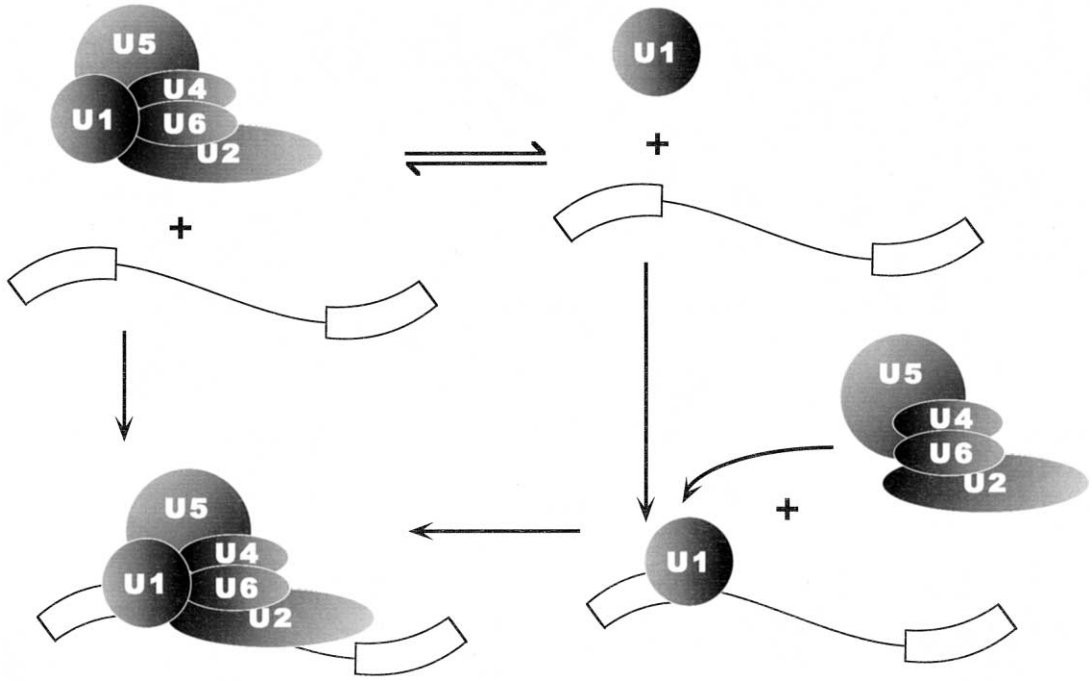

Figure 6. Model of the Modular Nature of Tetra-snRNP and Penta-snRNP Functions

Interaction of the penta-snRNP with a pre-mRNA substrate is schematically shown on the left side. The right side shows an initial interaction of U1 snRNP followed by recruitment of the tetra-snRNP. Both assembly pathways lead to the same functional spliceosome.

particle is able to splice pre-mRNA when supplied with MN-treated yeast splicing extract. Neither the purified penta-snRNP nor the MN-treated extract alone is able to form splicing complexes or to splice pre-mRNA. It remains to be determined what soluble factors from yeast extract are required to complement the splicing activity of purified penta-snRNP, but the list of candidate proteins is not large (see below). The isolation and purification of the penta-snRNP brings us closer to the eventual goal of studying pre-mRNA splicing in a fully defined system.

During assembly of an active spliceosome, extensive base pairing between U4 and U6 RNAs is disrupted and replaced by U6 base pairing with U2 snRNA and an intramolecular U6 stem-loop. We show that U6 RNA is quantitatively base-paired with U4 RNA in the penta-snRNP and that this base pairing can be disrupted by incubating the penta-snRNP with ATP. This dissociation is presumably catalyzed by the RNA-dependent ATPase Brr2 protein (Raghunathan and Guthrie, 1998a). The finding of U4/U6 base pairing in the penta-snRNP is not consistent with the possibility that the penta-snRNP might be a fully assembled spliceosome or a postsplicing lariat-intron-bound complex, because U6 RNA is not base paired with U4 in such complexes. One could argue that the penta-snRNP might be a partially assembled spliceosome arrested prior to U4/U6 unwinding; however, it seems unlikely that we could have isolated a homogeneous collection of spliceosomes blocked at this early stage of spliceosome assembly. Furthermore, the penta-snRNP completely lacks proteins known to bind pre-mRNAs such as the cap binding complex (CBC) or hnRNPs, which bind to pre-mRNA with multimolar stoichiometry. Indeed, we similarly purified a pre-mRNA-containing complex from HeLa extract and found it to contain most known spliceosomal proteins, all known hnRNPs, and a CBC component (our unpublished data). The failure to detect any such pre-mRNA binding proteins in the purified penta-snRNP supports that it is not yet bound to pre-mRNA or its splicing products. Our combined findings strongly suggest that the penta-snRNP is a functional precursor to active yeast spliceosomes.

A Model for Penta-snRNP Function in Spliceosome Assembly

In the current model of yeast spliceosome assembly, U1 snRNP binds to pre-mRNA in conjunction with an as yet not fully defined set of extrinsic factors to form the commitment complex. The U2 snRNP then joins this complex in the first ATP-dependent step to form the prespliceosome (the yeast B complex). The mixing experiments we present in this paper (Figure 4) suggest instead that in extracts, the majority of active U1 snRNP binds to pre-mRNA in the context of a preformed, endogenous U1•U2•U4/U6•U5 penta-snRNP. Furthermore, the U2 snRNP component does not exchange freely prior to pre-mRNA binding; rather, it functions entirely within endogenous penta- and tetra-snRNP complexes. We review below a growing set of observations that support this altered view of spliceosome assembly.

There is a wealth of evidence for the existence of large, salt-sensitive snRNP complexes in both yeast and mammalian extracts. Zieve and Penman (1981) demonstrated the existence of the human U1 snRNA in a salt-

sensitive particle that sedimented at up to 150S in velocity gradients. Konarska and Sharp (1988) also noted multi-snRNP complexes in human extracts under modified salt conditions. Indeed, the salt sensitivity of the penta-snRNP is itself compelling evidence that the penta-snRNP is not a spliceosome bound to pre-mRNA, as previous work from this laboratory showed that the fully assembled spliceosome was stable to sedimentation in 400 mM KCl (Clark et al., 1988). A survey of the yeast pre-mRNA splicing literature shows that, at monovalent salt concentrations compatible with splicing, all five spliceosomal snRNAs can be coimmunoprecipitated using a monoclonal or polyclonal antibody to a selected spliceosomal protein. Among these proteins are Brr2 (Raghunathan and Guthrie, 1998a), Prp4 (Banroques and Abelson, 1989), Prp6 and Prp9 (Abovich et al., 1990), Prp18 (Horowitz and Abelson, 1993), and Prp21 (Arenas and Abelson, 1993). Additionally, an early indication of the 30S U2•U4/U6•U5 tetra-snRNP was suggested by data in a paper describing the Prp11 protein (Chang et al., 1988). Indeed, under salt conditions which favor the prevalence of the tetra-snRNP, Gottschalk et al. (1999) demonstrated that epitope-tagged Snu23, Spp381, and Snu66 proteins each immunopurified a U2•U4/U6•U5 tetra-snRNP. These various immunoprecipitation experiments were performed at increasing salt concentrations to determine which of the snRNPs were most stably associated with a particular protein, but the results are also consistent with the existence of the penta-snRNP under the low-salt conditions of splicing. Further evidence came from deletion of the *SNU17* gene, which caused a striking effect on spliceosome assembly in yeast (Gottschalk et al. 2001)—the deletion caused an immediate and exclusive accumulation of a particle consistent with a pre-mRNA/penta-snRNP complex. Therefore, the presence of Snu17 protein likely facilitates a rearrangement within these nascent particles such that they are destabilized and dissociate under native gel assay conditions. Thus, the typical observation of fragmentary complexes—the commitment and yeast B complexes—on native gels likely results from normal, programmed rearrangements within nascent penta-snRNP-containing spliceosomes, as mediated by Snu17 and other factors, which incidentally serve to destabilize the developing spliceosomes and make them susceptible to dissociation under various assay conditions.

Recent experiments have provided additional reasons to question a model of spliceosome assembly in which U1 and U2 snRNPs act discretely in the early steps of commitment and prespliceosome assembly. In human cells and in nematodes, Maroney et al. (2000) showed that the U4/U6•U5 component Prp8 can be crosslinked to the 5′ splice site in the absence of base pairing between the pre-mRNA substrate and U1 or U2 snRNP. Such blocking of base pairing would not necessarily disrupt the integrity of pre-mRNA/penta-snRNP complexes nor block all penta-snRNP functions. In a comparable study, a specific crosslink formed between U4 snRNA and the 5′ splice site in the absence of all branchpoint and 3′ splice site sequences (Johnson and Abelson, 2001). This result is difficult to explain in the context of the traditional assembly model. However, the results of both crosslinking studies are compatible with early interactions between the 5′ splice site and penta-snRNP.

Another recent result challenges the traditional view of spliceosome assembly—the mammalian commitment complex (or E complex) was found to contain both U1 and U2 snRNPs (Das et al., 2000) rather than only the U1 snRNP as had previously been thought. This indicated that purification of splicing complexes under conditions more similar to those required for splicing allows one to isolate larger, more functionally relevant complexes.

These results lead us to propose a modular model for spliceosome assembly as presented in Figure 6. For penta-snRNP-committed pre-mRNA splicing, we propose that the pre-mRNA interacts with the penta-snRNP as currently understood, with the 5′ splice site base pairing with the U1 snRNA component, with the branchpoint interacting subsequently with the U2 snRNA, followed by exchange of the U1-5′ splice site interaction for a U6 interaction (Staley and Guthrie, 1998, 1999), but all in the context of a 45S penta-snRNP particle. When a discrete U1 snRNP functionally commits a pre-mRNA to splicing, the tetra-snRNP is recruited as assembly proceeds. Clearly, there is a kinetic benefit to utilizing a preformed penta-snRNP, as illustrated by the mixing experiments (Figure 4), and perhaps the modular nature of these interactions can explain the stoichiometric excess of U1 snRNP in yeast extracts, as identifying and marking 5′ splice sites accurately is critical for the proper processing of pre-mRNAs. We believe, however, that the preferred use of the penta-snRNP versus free U1 snRNP in the mixing experiments reflects the importance of the penta-snRNP in yeast spliceosome assembly and splicing.

Yeast Pre-mRNA Splicing Proteins Not Found in the Penta-snRNP

The known splicing proteins which were not present in the penta-snRNP include pre-mRNA binding factors Bbp1 and Mud2, which are both important for early pre-mRNA recognition (Abovich et al., 1994; Berglund et al., 1997). The pre-mRNA cap binding complex was also absent (Lewis et al., 1996; Zhang and Rosbash, 1999), as were splicing factors required for the second step of splicing, such as Slu7 (Frank et al., 1992), Prp17 (Seshadri et al., 1996), and Prp18 (Horowitz and Abelson, 1993). The U6 snRNP protein Prp24, which is thought to anneal U4 and U6 snRNAs (Raghunathan and Guthrie, 1998b), was absent—perhaps not surprisingly given its absence from U4/U6•U5 tri-snRNP (Gottschalk et al., 1999; Stevens and Abelson, 1999). Noticeably absent were most RNA-dependent ATPases required for splicing: Prp2, Prp5, Prp16, Prp22, Prp43, and Sub2 (reviewed in Staley and Guthrie, 1998), which are thought to interact with the splicing machinery in *trans*.

Supramolecular Complexes: A Paradigm in Biology

Our newly revised view of the endogenous, pre-mRNA splicing machinery as a large, preassembled complex, or "penta-snRNP," results from our observations using epitope tagging of selected proteins and affinity chromatography. Yeast genetics and biochemistry have defined a large number of factors required for pre-mRNA

splicing. Eighty-five percent of the known splicing factors are present in the penta-snRNP (Table 1). These factors, together with the other 15% of known splicing factors, are likely to define a nearly complete complement of the factors required for pre-mRNA splicing. In total, these comprise 76 proteins and five RNA molecules—a total of about 1% of the functionally assigned genes in the yeast genome. As Sydney Brenner astutely observed, the seemingly impossible task of defining the action of the cell in terms of the interactions between its components is made simpler by macromolecular assemblages, because the overall coordination of the cell is likely to be defined by a smaller number of interactions between intrinsically complex modules. We propose that the penta-snRNP is such a module in yeast.

Experimental Procedures

Yeast Strains, Plasmids, and Reagents

Carboxy-terminally tagged Prp4 and Dib1 proteins were generated by a knockin procedure (Lafontaine and Tollervey, 1996) using plasmid pCHP424 (Stevens, 2000). Selection for integrants was performed using SD medium lacking tryptophan. Correct integration was determined by polymerase chain reaction (PCR) using a primer complementary to the Prp4 or Dib1 coding regions and another complementary to the knockin fragment followed by sequencing of the PCR product. These constructs were generated using haploid S288C strain BY4734. Large-scale yeast splicing extracts were prepared by the liquid nitrogen blending method using cells grown in a 200 liter fermenter, as previously described (Stevens and Abelson, 1999). Extracts were adjusted to 50 mM NaCl, 20 mM HEPES (pH 7.9), 8% glycerol, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin (Buffer D50) by dialysis. Extracts were passed through a polyoma antibody column (Stevens, 2000) at 5 ml/hr, washed with 75 column volumes of buffer D50, and eluted in D50 containing 100 μg/ml polyoma peptide (Stevens, 2000). PCR reactions were performed using a mixture of Taq DNA polymerase and Pfu DNA polymerase (Stratagene, La Jolla, CA). All chemicals were purchased from Fisher or Sigma.

Large-Scale Splicing Reactions and Spliceosome Affinity Purification

Strains used for the mixing experiments were: ySS344 (WT), SS19 (Δ192-506) from Paul Siliciano (used as U1δ), and U2Δ2 from Andrey Balakin (used as U2δ). Extract preparation was performed using the liquid nitrogen-cooled grinding method as previously described (Ansari and Schwer, 1995). The resulting extract was extensively dialyzed (3 × 2 hr in 4 l of D50 buffer). For ATP depletion, extracts were adjusted to 2 mM glucose and incubated at 23°C for 10 min. Five hundred microliter splicing reactions were assembled as previously described (Lin et al., 1985). For reactions in which mixing was required, extracts were combined and incubated at 4°C for 5 min prior to the addition of the other reaction components. These splicing reactions were incubated at 16°C for 10 min and placed on ice. Reactions were then adjusted by adding ATP to a final concentration of 2 mM and 1 pmol of unbiotinylated actin and were further incubated at 16°C for 10 min. For antipolyoma immunoaffinity chromatography, 20 μl of settled bed volume of anti-polyoma agarose beads were added to the splicing reactions, and these were incubated with mixing for 2 hr at 4°C. Beads were washed 5× with 1 ml of D150 buffer (same as D50 but with 150 mM NaCl) containing 0.05% NP-40. Elution was achieved by incubating the beads with 0.5 ml of D150 containing 0.1 mg/ml of polyoma peptide (Stevens, 2000). Eluted material was incubated with paramagnetic streptavidin beads (Promega, Madison, WI) for 15 min with agitation. Beads were recovered using a magnetic stand and washed 5× with 1 ml of D150 containing 0.05% NP-40. RNA was recovered from the beads by incubation in 400 μl of PBS containing 0.1% SDS and 20 μg/ml proteinase K followed by phenol extraction and ethanol precipitation. Oligonucleotides used for the primer extension analyses were: U1-δ-RT, 5′-CCTACCAAGACCTTCCAAAATTTTTC-3′; U1-WT-RT, 5′-GAATTTGGTGTCAAACTTCTCCAGGC-3′; U2-δ-RT, 5′-ACGGGA AGACGAGAGAAACATCAAGC-3′; and U2-WT-RT, 5′-CAAAAAG TAGTGAGACCTGACATTAGC-3′.

Glycerol Gradients, Electrophoresis, and Mass Spectrometry

Immunopurified material was layered onto a linear 10%–30% glycerol gradient made up in buffer D50 (see above) containing 0.1% NP-40. Various centrifugation schemes were employed depending on the particle separation required. Generally, gradients were centrifuged in an SW41 rotor at 29,000 rpm for 16 hr at 4°C. Fractions were collected manually from the top in 400 μl aliquots. Native polyacrylamide gels were prepared at 7% (29:1) in 0.5× TBE. Electrophoresis conditions were 200 V for 4 hr at 4°C. Total protein and nucleic acids were prepared as previously described (Stevens and Abelson, 1999). Proteins and nucleic acids were visualized by staining with silver (Blum et al., 1987) or Coomassie blue G-250. The entire lane of penta-snRNP proteins was fractionated by cutting it into ~4 mm wide gel slices. Coomassie-stained proteins in the gel splices were destained, reduced, alkylated, and digested using a modified Hellmann procedure (Hellmann et al., 1995). Peptides extracted from the gel were analyzed by LC/MS/MS using a custom-built capillary LC system interfaced to a thermoquest LCQ quadrupole ion trap mass spectrometer (Davis and Lee, 1998). Fragmented ion mass spectra were screened (Moore et al., 2000) and searched against the NCBI nonredundant database using the Sequest program (Eng et al., 1994). Database matches found by Sequest were then manually validated by comparing the actual and predicted spectra. Spectra of peptides from known contaminants such as human keratin and trypsin as well as single matches to non-yeast proteins were ignored.

Pre-mRNA Splicing Assays

Pre-mRNA splicing reactions were performed under standard conditions as previously described (Lin et al., 1985). Micrococcal nuclease treatment and quenching were performed as described (Yean and Lin, 1991). When penta-snRNP fractions were assayed for splicing, they constituted 40% of the reaction mixture, and MN-treated extract constituted an additional 40%. Actin pre-mRNA was uniformly labeled with [α-^{32}P] UTP, and 2 fmol of ^{32}P-labeled pre-mRNA was used in all splicing reactions (0.4 nM final concentration). Generally, the product mixture was split, and one half was run on a native PAGE gel (4% polyacrylamide, 80:1; 1× TAE) for 6.5 hr at 200 V at 4°C, and the other half was phenol extracted, precipitated, and run on a 7% denaturing polyacrylamide gel (29:1, 1× TBE) to visualize the radiolabeled splicing products using a Molecular Dynamics PhosphorImager screen.

Quantitation of snRNA

Peak fractions of tri-, tetra- and penta-snRNP were phenol extracted, and the total nucleic acid in each was precipitated with ethanol. The following oligonucleotides were used for RNase protection assays according to the manufacturer's instructions (Ambion): U1, 5′-AAAATAAATCAAAAATTATAAGATCCACCCG-3′; U2, 5′-CAG TTGTTACACTGAAAAGAACAGATACTACACTTGATCT-3′; U4, 5′-AAT TAAATTTCAACCAGCAAAAACACAATCTCGGACGAATCCTCACTG ATATGCGTATTT-3′; U5, 5′-AAATATGGCAAGCCCACAGTAACGGA CAGCTTTACCTGTTTCTATGGAGA-3′; and U6, 5′-CGAAATAAATCT CTTTGTAAAACGGTTCATCCTTATGCAGGGGAACTGCTGATCATC TCTGTATTGTTTC-3′.

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