Genetic depletion indicates a late role for U5 snRNP during in vitro spliceosome assembly

Bertrand Séraphin*, Nadja Abovich and Michael Rosbash

Howard Hughes Medical Institute, Department of Biology, Brandeis University, Waltham, MA 02254, USA

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ABSTRACT

The pre-mRNA splicing pathway is highly conserved from yeast (*S. cerevisiae*) to mammals. Of the four snRNPs involved in splicing three (U1, U2 and U4/U6) have been shown to be essential for in vitro splicing. To examine the remaining snRNP, we utilized our previously described genetic procedures (Seraphin and Rosbash, 1989) to prepare yeast extracts depleted of U5 snRNP. The results show that U5 snRNP is necessary for both steps of pre-mRNA splicing and for proper spliceosome assembly, i.e., addition of the U4/U5/U6 triple snRNP. The prior steps of U1 and U2 snRNP addition occur normally in the absence of U5 snRNP.

INTRODUCTION

The process of pre-mRNA splicing is highly conserved from yeast to mammals (1,2). Many of the trans-acting factors required for splicing are also conserved, and these include the four snRNPs (U1, U2, U4/U6, and U5) (3) that associate with the pre-mRNA during the spliceosome assembly process, much of which precedes the first cleavage and ligation reaction (4–12). U1, U2 and U4/U6 have been shown to be essential for in vitro splicing and have been assigned a specific role in spliceosome assembly (13–25).

The role of U5 snRNP is less clearly defined. On the one hand, it has been suggested that an early step in spliceosome assembly might consist of 3' splice site recognition by U5 snRNP (26). On the other, two different studies (an in vivo study in yeast and an in vitro study in fractionated Hela extracts), have raised the possibility that U5 snRNP might not be essential for the first step of splicing, 5' splice site cleavage and lariat formation (27,28). The relatively indirect approaches used to date are due, at least in part, to the fact that active U5 snRNP is difficult to remove from splicing extracts. This is because it is refractory to the normal oligonucleotide hybridization procedures necessary for targeted RNase H digestion or for antisense depletion procedures (29).

To remove U5 snRNP, we turned to genetic procedures that have been previously used to deplete extracts of U1 snRNP and

of U2 snRNP(21). Both steps of pre-mRNA splicing were defective in extracts that lack U5 snRNP. U4/U6 snRNP addition was also defective, consistent with the notion that U5 snRNP and U4/U6 snRNP are added to the pre-mRNA as a multi-snRNP complex. U1 and U2 snRNP addition, however, occurred normally, indicating that U5 snRNP plays no detectable role in splice site recognition or the early steps of spliceosome assembly that precede U4/U5/U6 snRNP addition.

MATERIALS AND METHODS

Strains, snRNA Depletions, and Northern Blot Analysis

To construct a galactose-regulated yeast U5 snRNA gene, we first isolated a *Dra*I-SalI fragment containing the yeast U5 snRNA coding sequence from plasmid pUC-7HpNc (a kind gift of Bruce Patterson). This fragment was ligated together with a *Nco*I-SalI backbone fragment from plasmid pBS88 (21) and a *Nco*I-*Dra*I fragment encompassing the Gal-U1 promoter of plasmid pBS72 (21). (Plasmid pBS72 contains the Gal-U1 construct in a pTZ backbone.) The resulting plasmid, pBS202, is identical to plasmid pBS88 except that the U1 snRNA coding sequence has been replaced by the U5 snRNA coding sequence. Because both of the *Dra*I sites used in the construction are located 10 nucleotides upstream of the transcription start site of either U1 or U5 snRNA, the spacing between the transcription start site of the U5 snRNA gene and the U1 TATA box is conserved. We also generated a plasmid, pBS204, to disrupt the chromosomal copy of the yeast U5 snRNA gene. We inserted a blunt-ended *Hpa*I-SalI fragment encompassing the LEU2 gene into plasmid pUC-7HpNc. The LEU2 gene was inserted between the *Dra*I site located 36 nucleotides upstream of the U5 snRNA start site and the *Hinc*II site located at position 34 in the U5 snRNA coding sequence. DNA of plasmid pBS204 was cleaved with *Xba*I and *Sac*I and used, together with DNA from plasmid pBS202, to co-transform yeast strain BS-Y17 (MATa, leu2–3, leu2–112, ura3–52, trp1–289, arg4, ade2) as previously described (21). A LEU⁺ GLUCOSE[–] strain, BS-Y119, was selected for further studies. The structures of the disrupted chromosomal copy of the U5 snRNA gene and of the Gal-U5-containing plasmid in this strain were verified by Southern blotting.

* Present address: European Molecular Biology Laboratory, Postfach 10.2209, Meyerhofstrasse 1, D-6900 Heidelberg, FRG

Splicing Extracts, Analysis of Splicing and Complex Formation

Yeast extracts were prepared from the indicated strains of 16 h growth in medium containing 4% glucose (21). Splicing assays, and native gel electrophoresis were as described (21). Procedures for streptavidin affinity selection of splicing complexes formed on biotinylated substrates as well as subsequent snRNA analysis have also been previously described (25).

RESULTS

We constructed a yeast strain containing a disrupted chromosomal copy of the yeast U5 gene complemented by a plasmid containing a U5 snRNA transcription unit under the control of the Gal-10 upstream activator sequence (Gal-U5). As previously described for similar Gal-U1 and Gal-U2 constructs (21,30), switching carbon sources from galactose to glucose repressed transcription from the Gal-U5 gene. Yet cell growth continued for 16 h (and was normal for at least 10 h), during which time the U5 snRNA content was diluted substantially (27) (data not shown).

In extracts derived from these depleted cells (Δ U5 extracts), U5 snRNA is undetectable, indicating that there is less than 5% of the wild type level of U5 snRNP (Figure 1, compare lane 4 with lanes 1–3). The depletion protocol has little effect on the levels of the other 4 snRNAs, and the extent of U5 depletion (Figure 1, lane 4) is comparable to what has been previously reported for U1 depletion (Figure 1, lane 2) and U2 depletion (lane 3) (21,30).

Figure 1. snRNA Depletions and Northern Blot Analysis. Yeast extracts were prepared from strains BS-Y46 (wild type), BS-Y82 (Δ U1), BS-Y88 (Δ U2), and BS-Y119 (Δ U5). Aliquots of each extract were separated into pellet (P) and supernatant (S) fractions (25). RNA was prepared from equivalent amounts of whole extract (lanes 1–4), pellets (lanes 5–8), and supernatants (lanes 9–12) and electrophoresed on 4% acrylamide, 7M urea gels. The snRNAs were visualized after blotting and hybridization to radioactive probes (25). Lanes 1, 5 and 9; wild type extract. Lanes 2, 6 and 10; U1-depleted extract. Lanes 3, 7 and 11; U2-depleted extract. Lanes 4, 8 and 12; U5-depleted extract.

Centrifugation of a wild-type extract under moderate salt conditions leads to a partitioning of yeast snRNPs between pellet and supernatant fractions (25) (Figure 1, compare lanes 5 and 9). Depletion of U1 or U2 snRNP had only a modest effect on the distribution of the remaining snRNAs between the pellet and supernatant fractions (Figure 1, lanes 6, 7, 10 and 11). In a Δ U5 extract, however, a major change was readily visible; all of the U4 and U6 snRNAs were in the supernatant fraction (Figure 1, lanes 8 and 12). This observation suggests that the U4/U6 snRNP present in the pellet fraction was associated with U5 snRNP in a U4/U5/U6 triple snRNP particle and that the U4/U6 snRNP present in the supernatant fraction was free U4/U6 snRNP. Depletion of U5 snRNP thus resulted in loss of the U4/U5/U6 triple snRNP particle (Figure 1, lanes 5 and 8).

Like the Δ U1 and Δ U2 extracts, Δ U5 extracts made from cells depleted of U5 snRNA for 5, 10, or 16 h (U5–5; U5–10; U5–16) supported little or no in vitro splicing (Figure 2, lanes 1–6). Depletion for 5 h, estimated to reduce U5 snRNP levels 5-fold (data not shown), was insufficient to eliminate splicing (lane 4) whereas no splicing was detected in the extracts depleted for 10 and 16 h (lanes 5 and 6, respectively). The Δ U5–10 extract was able to complement the Δ U1 and the Δ U2 extracts (lanes 8 and 9) almost to the same extent that the Δ U1 and Δ U2 extracts were able to complement each other (lane 7). A less efficient complementation was also observed for the Δ U5–16 extract (data not shown).

Figure 2. In Vitro Splicing Assays. Splicing reactions were carried out with either 4 μ l of a single extract (lanes 1–6) or 2 μ l of each of the indicated extracts for complementation (lanes 7–9) with the Δ 2 substrate as described previously (21). The lariat intermediate and lariat intron (indicated by the top and bottom arrows, respectively) were assayed on a 15% acrylamide denaturing gel. Lanes 1, 2 and 3; extracts prepared from BS-Y46, BS-Y82 and BS-Y88, respectively, after 16h growth in glucose. Lanes 4, 5 and 6; extracts prepared from strain BS-Y119 after 5, 10 and 16h growth in glucose, respectively. Lane 7; complementation between extracts in lanes 2 and 3. Lane 8; complementation between extracts in lanes 2 and 5. Lane 9; complementation between extracts in lanes 3 and 5.

Spliceosome assembly was also significantly reduced in the $\Delta U5$ extracts ($\Delta U5-16 < \Delta U5-10 < \Delta U5-5$; Figure 3, lanes 2–4). Yet U2 snRNP addition to the substrate still occurred as the complexes that formed in these depleted extracts have been previously shown to contain U2 snRNP (21). Also, oligonucleotide-directed RNase H digestion of U2 snRNA in the $\Delta U5$ extracts resulted in the disappearance of these complexes and the appearance of commitment complexes (21,31), the U1 snRNP-containing complexes that form prior to the addition of U2 snRNP (CC; Figure 3, lane 6 and data not shown). Significantly, no CC accumulation was detectable in the $\Delta U5$ extracts, indicating that U2 snRNP addition was efficient enough to convert all of the commitment complexes to prespliceosome complexes.

The analysis could not, however, assess the addition of U4/U6 snRNP. (This is because in this system some of these presplicing complexes also contain U1 snRNP; as yeast U1 and U2 snRNAs are so much larger than U4, U5, and U6 snRNAs, complexes that contain U4/U6 snRNP are not well resolved from those that lack it (21)(data not shown). As a consequence, we assayed U6 snRNP addition by affinity selection of the pre-mRNA substrate (Figure 4). In a $\Delta U5$ extract, U6 snRNP addition was not detected (Figure 4A, lane 1), i.e., the levels were comparable to what was detected with a control (splicing-incompetent) substrate (lane 2) or with mutant extracts in which U2 snRNP addition activity (prp9, lane 4), or U4/U6 snRNP activity (prp6 and prp4, lanes 5 and 6, respectively), had been heat inactivated (25). U2 snRNP addition was assayed in parallel with the expected results, namely, that only the extract in which U2 snRNP activity had been inactivated (lane 4) and the control substrate (lane 2) were defective for U2 snRNP addition. Results with a $\Delta U2$ extract were identical to those obtained by heat inactivating the prp9 strain (25).

Figure 3. Native Gel Analysis of Splicing Complexes. Standard splicing reactions were carried out with the $\Delta 2$ substrate. The extracts in lanes 6 and 7 were pre-incubated with an oligonucleotide complementary to U2 snRNA (18). The splicing complexes (SP) and the commitment complexes (CC) were resolved on native gels (21). Lane 1; wild type extract. Lanes 2, 3 and 4; $\Delta U5$ extracts from 5, 10 and 16h of depletion. Lane 5; $\Delta U2$. Lane 6; $\Delta U5$ (10h). Lane 7; wild type extract.

As expected from the splicing complementation results (Figure 2), the addition of a second depleted (or mutant) extract was able to complement the U6 addition defect of the $\Delta U5$ extract (Figure 4B). This was true for a $\Delta U2$ extract (lane 3) as well as an inactivated prp9 extract (lane 4) and inactivated prp6 and prp4 extracts (lanes 5 and 6, respectively).

Figure 4. Analysis of snRNAs in Splicing Complexes. Splicing reactions were carried out with the indicated extracts (above each lane) and a biotinylated substrate. After streptavidin agarose selection, U2 and U6 snRNAs associated with the substrate were analyzed by primer extension (25). Depletions ($\Delta 2$ and $\Delta 5$) were for 16 h. A. All reactions contained biotinylated $\Delta 2$ substrate except for lane 2 which contained a biotinylated substrate of a similar size derived from the first exon of the *Drosophila per* gene. Results from lanes 2–6 have been previously reported (25). B. Lanes 1 and 2; extracts made from prp6 and prp4 mutants, respectively. Lanes 3, 4, 5 and 6; complementation between $\Delta U5$ and $\Delta U2$, prp9, prp6 or prp4, respectively. The cDNA products of U2 and U6 snRNA primer extensions are indicated.

DISCUSSION

All of these data support the view that U5 snRNP is necessary for functional spliceosome assembly. In its absence, U4/U6 snRNP is not added to the pre-spliceosome that already contains U2 snRNP. Because this work has been done with a single pre-mRNA substrate, we cannot exclude the possibility that other substrates can undergo some splicing without U5 snRNP. However, identical conclusions have been independently reached by Brown and Beggs (personal communication), who examined yeast extracts inactivated or depleted of PRP8, a U5 snRNP protein. Identical conclusions have also been reached by Lamm et al., who used antisense procedures to deplete mammalian extracts of U5 snRNP (32).

Previous *in vivo* work in the yeast system indicated that depletion or inactivation of U5 snRNP lead to a severe inhibition of splicing (27,33). More recently, Newman and Norman uncovered mutations in U5 snRNA that suppressed a 5' splice site mutation (34). These observations suggest that U5 snRNP is important for the first step of splicing, consistent with the results reported here.

In the study that examined the consequences of U5 snRNP depletion (27), there was some increase in lariat intermediate which lead the authors to speculate that U5 snRNP might not be essential for the first step of splicing. A previous *in vitro* study in the mammalian system also suggested that U5 snRNP may not be essential for the first step of splicing (28). We suspect that these two observations were due to the pleiotropic *in vivo* effects of these depletion protocols on the one hand (27), and to the incomplete elimination of U5 snRNP from the *in vitro* experiments on the other (28). In any case, none of these studies identified the step at which spliceosome assembly was inhibited.

Our results indicate that the absence of U5 snRNP prevents U4 \ U6 snRNP addition, i.e., U4 \ U5 \ U6 snRNP are added together as a triple snRNP. As U1 and U2 snRNP addition occur normally, it would appear that the U5 snRNP plays no role in the early steps of splice site recognition and spliceosome assembly. This conclusion argues against the interpretation of some earlier work in mammalian systems (26). Taken together with studies that examined the effects of depleting or inactivating the other three splicing snRNPs, our results indicate that several central features of the spliceosome assembly pathway are conserved from yeast to man. These include the formation of a U2 snRNP-pre-mRNA complex and the subsequent formation of a U4/U5/U6 snRNP-U2 snRNP-pre-mRNA complex (A→B) (8,9,35). For yeast, there is additional evidence for the formation of a U1 snRNP-pre-mRNA commitment complex (CC) that precedes U2 snRNP addition (18,20,21,31), indicating a more complex pathway (CC→A→B).

In both systems, there are many undefined features of these complexes as well as evidence for additional assembly steps that are likely to reflect the addition or removal of multiple proteins, conformational changes, and/or the progressive weakening (or tightening) of snRNP and non-snRNP factors. For example, the relationship of U1 snRNP to both the A and B complexes is not well established (e.g., (11,12)), and the structure of the U4/U6 snRNP probably undergoes a substantial change during spliceosome assembly (6,8,9,11,36). As the core snRNP contents of the A and B complexes are unlikely to be altered by these ambiguities and spliceosome changes, we suggest that the resulting complexes be referred to as subcomplexes (e.g., A1, A2, B1, etc.) and that, where appropriate, the mammalian nomenclature be used for *S. cerevisiae*.

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