Pre-mRNA binding to the yeast U2 small nuclear ribonucleoprotein (snRNP) during prespliceosome formation requires ATP hydrolysis, the highly conserved UACUAAC box of the branch point region of the pre-mRNA, and several factors. Here we analyzed the binding of a radiolabeled 2'-O-methyl oligonucleotide complementary to U2 small nuclear RNA to study interactions between the UACUAAC box, U2 snRNP, and Prp5p, a DEAD box protein necessary for prespliceosome formation. Binding of the 2'-O-methyl oligonucleotide to the U2 snRNP in yeast cell extract was assayed by gel electrophoresis. Binding was rapid, enhanced by ATP, and dependent on the integrity and conformation of the U2 snRNP. It was also stimulated by Prp5p that was found to associate physically with U2 snRNP. In vitro heat inactivation of the temperature-sensitive prp5-1 mutant extract decreased oligonucleotide binding to U2 and the ATP enhancement of binding by 3-fold. Furthermore, the temperature-sensitive prp5-1 mutation maps to the ATP-binding motif I within the helicase-like domain. Thus the catalytic activity of Prp5p likely promotes a conformational change in the U2 snRNP.

The splicesome is a large, dynamic ribonucleoprotein particle that catalyzes nuclear pre-mRNA splicing. It is composed of multiple factors including five snRNPs and numerous non-snRNP proteins, which assemble on the pre-mRNA (1, 2). It undergoes a number of conformational changes during its transit through a splicing cycle. At least seven such changes are disruptions of RNA base pairings (3–5), whereas other changes involve protein-protein and RNA-protein interactions. Some of these changes are probably catalyzed by DEXD/H-box proteins, eight of which are known to be required for the splicing pathway in the yeast Saccharomyces cerevisiae (6).

DEXH/D-box proteins are numerous in both prokaryotes and eukaryotes and are involved in diverse biological processes, but they all have a similar domain with six or seven signature motifs (7, 8). In the archetypal DEAD box family member, EIF4A4, these motifs encode RNA-dependent ATPase and RNA unwinding activities (9, 10). To date the splicesomal DEXH/D box proteins are fulfilling the prediction that they catalyze the RNA rearrangements in the splicesome. They are required at steps in which ATP hydrolysis and splicesomal structural changes both occur; most have been shown to have RNA-dependent ATPase activity; and some have even been found to have RNA unwinding activity (6). Nevertheless, the actual targets of the splicesomal DEAD box proteins are unknown. Some of the proteins may not even unwind RNA but instead act as an RNAse to remove protein bound to RNA (11, 12).

Two of the first DEAD-box proteins required for yeast pre-spliceosome formation on the pre-mRNA are Sub2p (13–15) and Prp5p (16). The prespliceosome is an early intermediate in splicesome formation. It immediately succeeds the binding of the U1 snRNP and at least two proteins (Bbp and Mud2p) to the pre-mRNA. During prespliceosome formation, Bbp and Mud2p are displaced from the pre-mRNA (17, 18); ATP is hydrolyzed (19); and U2 snRNP binds to the pre-mRNA (20, 21). During U2 binding or shortly thereafter, the U2 snRNA base pairs with the UACUAAC box of the pre-mRNA (22). One UACUAAC box nt, the branch point nt, remains unpaired (23), however, and will eventually initiate the first transesterification reaction of splicing catalysis (2, 5).

Although Sub2p is thought to remove Bbp and Mud2p from the pre-RNA (13–15, 24), the target and mechanism of action of Prp5p in prespliceosome formation are unknown. In vitro, Prp5p enhances deoxyoligo-directed RNase H cleavage of U2 snRNP (25), and the ATPase activity of the Prp5 is stimulated by U2 snRNA (26). These two results suggest that U2 snRNA is the target of the Prp5p. This idea is attractive because U2 alternates between two conformations involving stem-loop II in its 5' end region, but only one conformation (stem-loop IIA) forms the prespliceosome efficiently (27, 28). Genetic data as well indicate that Prp5p somehow interacts with U2 (16, 29, 30). However, it has not been shown whether or not Prp5p physically associates with the U2 snRNP.

Biochemical assays to detect unwinding of a generic RNA substrate by Prp5p have not been successful (26). This may be because Prp5p acts as an RNAse on an RNA-protein substrate, or it requires a specific substrate or protein cofactor as do some helicases (31, 32). The function of Prp5p in vivo does depend on other proteins as revealed by genetic assays. The Ts prp5-1 and prp5-3 mutations are synthetically lethal with any one of several mutations in the multimeric SF3 complex (16, 29, 33). The human SF3 complex binds via one of its components,
constructed in two steps as follows: step 1, the 3.9-kb Prf fragment of 
Pprr5p from pGD532 was purified by gel electrophoresis and circularized by ligation under dilute conditions; and step 2, the circularized fragment was cut with BsuHI and SacI (removing 1.68 kb of coding sequence), then ligated to pS450 cut with a 1-kb XhoI fragment of pS24 (pSpp∆1815::LEU2) was confirmed by ligation to a BclI-XhoI PRP5 fragment amplified by PCR with oligos sOR42 and sOR43 to pS12 cut with BclI and XhoI.

The GST tag was fused to the N terminus of the Prp5p as follows. The 2.5-kb EcoRI-SalI fragment from pGD-Pprr5p was ligated into the EcoRI and SacI sites of pRS423 to create pSR281. The SacI-EcoRI fragment from pGAL1 promoters and GST fragment was fused upstream and out-of-frame to PRP5 ORF by cloning into pSR281 cut with SmaI and EcoRI to create pSR282. The PRP5 ORF and GST were put in-frame by ligating hybridized oligos sOR177 and 178 into pSR282 cut with EcoRI and NdeI to create plasmid pSR284.

Yeast Strains—The following S. cerevisiae strains were obtained from others: RL172 (Matα prp5-1) from R. Last and J. Woolford (see Ref. 44); and H170–wt (Matα leu2-3,112 his4-619 [pY2 U3::Ura3 (Ycpln-LEU2-U2wt)] and H170–C2U (Matα leu2-3, 112 his4-619 y2y2 U3::Ura3 (Ycpln-LEU2-U2C261)). From M. Ares (45). Haploid strains with the indicated prp mutations (16) and wild type haploid Tsr1210 (46) were as described. Diploid DSR1515 was made by mating SYRwtg (Matα his3Δ200 hist7 leu2-3,112 yrw4-2) and SYRwt (Matα his3Δ200 leu2-3,112 pip3-4) and transformation to His prototrophy at 23°C. Seven restriction endonucleases were tested for gapping. DNA gapped with NcoI and SpeI yielded only Ts yeast cells when 30 independent transformants were assayed. Plasmids (pTS3R342-d1, pTS3R342-d5, and pTS3R42-e4) from 3 of the 30 yeast clones were isolated and amplified in Escherichia coli as described (48). All 3 plasmids conferred Ts growth when tested in Tsr1210Δ (by transformation in WCE). The reactions were next incubated with 5 mM MgCl2, DTT, and 20% (v/v) extract in 20 mM [CaCl2, DTT, 50 mM DTT, and 20% (v/v) glycerol. The binding reaction was mixed on ice and then initiated by C4, Five-
To analyze any effect of ATP on oligo binding or GST selection (see below), the reactions were first incubated with 5 mM glucose to deplete ATP or water at 23 °C for 5 min and then chilled on ice. The binding reaction was then initiated by the addition of radiolabeled oligo to 5 nM and either water or ATP (to 2 mM) and incubated at 23 °C.

Quenched samples were fractionated on a 3.2% (v/v) polyacrylamide gel (50:1 acrylamide/bisacrylamide) in TPF8 buffer (48 mM Tris phosphate (pH 8 at 0 °C), 1.5 mM magnesium acetate) at 4 °C for 16–20 h at 5.5–6.7 V/cm. Radiolabeled bands were visualized by autoradiography and quantitated in dried gels with a Molecular Dynamics Storm 840 PhosphorImager and ImageQuant software version 5.0. Data were analyzed in Minibat version 13 by the Anderson-Darling test for normal distribution, the Bartlett test for variance homogeneity, one-way ANOVA for comparing means to 100%, and Tukey’s multiple ANOVA for all other comparisons of means (50, 51). Only a 95% confidence level could be calculated for the one-way ANOVA. The data in Figs. 2B and 8B were normalized to the 0-min values and analyzed by Tukey’s multiple ANOVA comparisons.

Northern Blot Analyses—RNAs in native and denaturing gels were electrophoretically transferred to PerkinElmer Life Sciences GeneScreen as described previously (52). Northern blots were incubated as described previously (52) with U-snRNA-specific probes made from DNA fragments amplified by PCR with oligos oSR115–oSR124 and radiolabeled by random deoxyoligo-primed extension (53).

Deoxyoligo-mediated RNase H Cleavage of the U2 snRNA—Reactions for deoxyoligo-mediated RNase H cleavage contained 2.2 mM DTT, 66 mM KPO4 (pH 7.4), 2 mM magnesium acetate, 3 20°C (in both) (50). The wild type 2′-OMe oligo mU2-wt is shown hybridized to U2 with the UACUAAC box designated by lowercase, underlined letters. The 5′ end of oligo mU2-dC has 4 modified deoxycytidines (dC) and inosine (I) instead of guanosine (see “Experimental Procedures”).

RESULTS

2′-OMe Oligonucleotide Binding to Components in Whole Cell Splicing Extracts—To study the interactions between Prp5p and U2 snRNPs, we modified previously published Lamos and co-workers (36, 57) to prepare the structure of the U2 snRNP in HeLa cell extracts. They tested several 2′-OMe oligos complementary to different regions of U2 snRNA and found that only oligos complementary to the 5′ end region of the snRNA hybridized to the U2 snRNP in splicing extracts. Based on their results and the sequence of a deoxyoligo, SRU2, previously used for targeted RNase H degradation of the yeast U2 snRNA (38, 58), we designed a 2′-OMe oligo, mU2-wt, complementary to the BpIR of U2 snRNA (Fig. 1). When mU2-wt hybridizes to U2, the “branch point nt” is predicted to bulge out from the helix. When this oligo was added to an in vitro splicing reaction at a concentration of 250 nM, it inhibited splicing of the exogenously added actin pre-mRNA substrate, whereas an oligo complementary to the 3′ end of the actin pre-mRNA substrate had no effect (data not shown). Additionally, we tested another oligo, mU2-dC (Fig. 1), similar to mU2-wt, but mU2-dC cannot form a bulged branch point nucleotide, and it has 4 modified deoxycytidines at its 5′ end that are not complementary to U2 snRNA. Nonetheless, the mU2-dC oligo also inhibited splicing at the same concentration as mU2-wt. These results suggest that mU2-wt and mU2-dC compete with the pre-mRNA for essential splicing factors.

We used native gel electrophoresis to assay binding of these oligos to factors in a whole cell extract (WCE) active for splicing. The conditions of the binding assay were nearly the same as those of an in vitro splicing assay (59). However, in the binding reaction, a radiolabeled 2′-OMe oligo was added instead of radiolabeled pre-mRNA splicing substrate. Aliquots of the reaction were taken during incubation at 23 °C and then fractionated by electrophoresis in a native gel to separate bound from unbound oligo.

Five radiolabeled bands were obtained with either wild type oligo mU2-wt or mU2-dC (Fig. 2A and data not shown). Band 1 was the slowest migrating and most intense of all five bands. It weakly appeared during the course of the reaction in the presence or absence of ATP. Band 2 was the second most intense of the five bands when formed on mU2-wt and, as described below, contains the U2 snRNP. The amount of band 2 rapidly increased during incubation at 23 °C and was usually more abundant in reactions with added ATP compared with those depleted of ATP (Fig. 2B). Different extracts and extract prep-
arations varied as to the extent of ATP stimulation; the extracts most active for pre-mRNA splicing also gave the most ATP stimulation of band 2 formation. Usually 5–10% of the input oligo was in band 2 by 30 min in reactions with ATP. The amounts of radiolabeled mU2-wt oligo bound to U2 snRNP in band 2 were proportional to the concentration of radiolabeled oligo in the reaction over the range of concentrations tested (1–20 nM) and were similar for mU2-wt and mU2-dC (data not shown). Band 3 formed in samples kept on ice and was most intense at early times. It decreased after only a few minutes of incubation at 23 °C for oligo mU2-wt and more slowly for mU2-dC (Fig. 2A and data not shown). The amount of band 3 varied considerably among different extracts. Band 4 was the second most abundant band formed with mU2-dC oligo (data not shown) but was barely detectable with mU2-wt. It increased in abundance with time, more so in reactions depleted of ATP than those with added ATP. Extraction and analysis of band 4 from the gel indicated that oligo mU2-dC was still intact and radiolabeled (data not shown). Finally, band 5, which was the most intense of the five bands, comigrated with unbound oligo.

![Diagram](image-url)
An Intact U2 snRNP Was Required for Formation of Band 2. A, 2'-O-Me mU2-wt oligo binding assay of extract with cleaved U1 or U2 snRNA. The U1 or U2 snRNA in WCE was first cleaved by deoxyoligo-mediated RNase H. Either 3.2 μM SRU1 deoxyoligo complementary to the 5' end of U1 snRNA (U1, lane 4), 250 nM SRU2 deoxyoligo complementary to the BpIR of U2 snRNA (U2, lane 2), or water (−, lanes 1 and 3) was added to WCE. The reactions were incubated in splicing buffer with 0.2 mM ATP at 23 °C for 30 min. ATP to 2 mM and radiolabeled mU2-wt oligo were then added and the reactions incubated for another 30 min at 23 °C. The samples were resolved by native gel electrophoresis and visualized by autoradiography. Arrowheads B2 and B3 indicate bands 2 and 3; however, band 3 is not visible in this exposure. B, comparison of oligo binding and migration of spliceosomal snRNPs in SRU2-treated and untreated extracts. WCE was incubated with splicing buffer and either water (−, lane 1) or 100 nM deoxyoligo SRU2 (U2, lane 2) at 23 °C for 30 min. Radiolabeled mU2-wt oligo and additional ATP were then added and the reactions incubated for another 30 min at 23 °C. The samples were resolved by native gel electrophoresis and visualized by autoradiography as shown in the left panel. Arrowheads B2 and B3 indicate bands 2 and 3, although band 3 is not visible in this exposure. The RNAs in the gel were then assayed by Northern analysis with radiolabeled probes for U1 and U6 snRNAs and visualized by autoradiography as shown in the middle and right panels. The RNA bands are indicated by their snRNA contents. The radiolabeled mU2-wt oligo in the gel was not retained during the Northern analysis so it cannot be seen in the two right panels. C, Northern analysis of U1 and U2 snRNAs extracted from SRU2-treated and untreated extracts. WCE was incubated with splicing buffer, 0.2 mM ATP, and either water (−, lane 2) or 100 nM deoxyoligo SRU2 (U2, lane 3) at 23 °C for 30 min. The RNAs in the reactions were then extracted, fractionated by denaturing PAGE, and detected by Northern analysis by simultaneous hybridization with probes for U1 and U2 snRNAs. Radiolabeled MspI restriction endonuclease fragments of pBR322 DNA were used as size markers (M) in lane 1.

To determine which snRNPs, if any, comigrated with the radiolabeled bands, the RNAs in the native gel with radiolabeled mU2-wt in Fig. 2A were transferred to a membrane. During the transfer process, the radiolabeled oligo was lost as detected by autoradiography of the membrane. The membrane was then incubated sequentially with radiolabeled spliceosomal snRNA-specific probes (Fig. 2C and data not shown). Band 2 comigrated with the U2 snRNP which migrated as a single band. Although the U1 snRNP and U4/U6.U5 tri-snRNP migrated close to band 2, additional experiments described below indicated that they did not comigrate with band 2. Bands 3 and 4 did not comigrate with any snRNP. The low abundance of band 1 prevented definitive identification of any comigrating snRNP. Finally, there was no discernible difference in the migration of any snRNP due to the oligo (data not shown).

The Northern analysis also revealed that the U1 snRNP migrated as three distinct species. The slowest, form I, comigrated with the U5 snRNP consistent with previous gel electrophoretic (59), snRNPs (60), and purification assays (61, 62) indicating a complex containing the U1 and U5 snRNPs. Although this band in Fig. 2 was amorphous, other WCEs gave more defined bands (see, for example, Fig. 8). Form II contained only the U1 snRNP and migrated as a sharp band. The fastest, form III, partly ran with the U4/U6 snRNP. Nonetheless, there was little similarity between the hybridization patterns of form III U1 and U4/U6 indicating that the U1 and U4/U6 snRNPs did not migrate together as a complex.

An Intact U2 snRNP Was Required for Formation of Band 2—To determine whether formation of bands 2, 3, or 4 depended on the U2 snRNP, we tested whether or not they would form when the BpIR of U2 snRNA was first removed by deoxyoligo-targeted RNase H cleavage. The deoxyoligo SRU2 complementary to the BpIR directs cleavage of U2 snRNA by RNase H activity present in the extract. Such cleavage destroys the 5' end region of the U2 snRNA and inhibits prespliceosome formation (38, 58). Similarly, SRU1, a deoxyoligo complementary to the 5' end of U1 snRNA, specifically directs cleavage of U1 and prevents U1 snRNP from binding to the pre-mRNA and subsequent spliceosome formation (38, 59). We found that when U2 snRNA was cleaved, band 2 did not form with either the 2'-O-Me oligo mU2-dC (data not shown) or mU2-wt (Fig. 3A). In contrast, cleavage of U1 snRNA (Fig. 3A) or the addition of a control deoxyoligo that does not cleave an snRNA (data not shown) had no effect on band 2 formation. Bands 3 and 4 decreased when any one of the three deoxyoligos was added (Fig. 3A and data not shown). Thus band 2 depended specifically on the integrity of the U2 snRNP and normally contained the mU2 oligos bound to U2. The other two bands contained factors that could bind to oligos without any apparent sequence specificity. The low abundance of band 1 precluded its assessment in these assays.

To delineate further the relationship of band 2 with the spliceosomal snRNPs, we directly compared the migration of band 2 with the migration of the snRNPs in the SRU2-treated and untreated extracts. Oligo binding with radiolabeled mU2-wt was first assayed in untreated or SRU2-treated extracts by native gel electrophoresis (left panel in Fig. 3B). The spliceosomal snRNAs in the same gel were then detected by Northern analysis (middle and right panels in Fig. 3B). In the untreated extract, band 2 (as assayed by oligo binding) again comigrated exactly with the U2 snRNP (as assayed by Northern hybridization) but not with the U4/U6.U5 tri-snRNP. No
U2 snRNA in the treated extract was detected by Northern blot analyses. We found a single band corresponding to the U2 snRNA in the treated extract (Fig. 3). The amount of U2 snRNA in the treated extract was 50% that in the untreated extract, a difference similar to that in the native gel. The overall complementarity of the oligo mU2-wt was important for its binding to the U2 snRNP. A, binding assays of 2'-OMe oligos mU2-wt and mU2-bp in extract with cleaved U1 or U2 snRNA. U1 or U2 snRNA in WCE was first cleaved by deoxyoligo-mediated RNase H. WCE with either water (−, lanes 1 and 2), 3.2 μM SRU1 deoxyoligo complementary to the 5′ end of U1 snRNA (U1, lane 3 and 4), or 1 μM SRU2 deoxyoligo complementary to the BpIR of U2 snRNA (U2, lane 5 and 6) was incubated in splicing buffer with 0.2 mM ATP at 23 °C for 30 min. ATP to 2 mM and either radiolabeled mU2-wt or mU2-bp were then added and the reactions incubated for another 15 min at 23 °C. The samples were resolved by native gel electrophoresis and visualized by autoradiography. Arrowheads B1, B2, and B3 indicate bands 1–3. Arrowheads x and y indicate two new bands formed on mU2-bp. To visualize bands formed on both oligos at once, 2.5-fold more mU2-bp than mU2-wt was used in this assay. B, competition binding assays with mU2-wt and mU2-bp oligos. Radiolabeled mU2-wt oligo was mixed without (lane 1) or with either a 5-, 10-, 20-, or 50-fold molar excess of unlabeled mU2-bp (lanes 2–5) or mU2-bp (lanes 6–9), incubated in reactions with added ATP for 30 min at 23 °C, and analyzed as in A.

To analyze the U2 snRNA in the SRU2-treated extracts, we extracted the RNAs from treated and untreated extracts, separated them by denaturing gel electrophoresis, and detected the U1 and U2 snRNAs by Northern blot analyses. We found a single band corresponding to the U2 snRNA in the treated extract (middle panel). No significant differences in the amount or migration of the other snRNPs were observed in the treated versus untreated extracts (right panel of Fig. 3B and data not shown).

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ple (from 0.01–4 μg of heparin per μl of extract), the faster band 2 migrated in the gel, the more compact and intense (up to 3-fold) it became, and the less it was enhanced by ATP. Band 3 decreased with increasing amounts of heparin until it was completely abolished. Band 4 as assayed on either oligo mU2-dC or mU2-wt showed no difference. Similar effects on bands 2 and 3 were observed when RNA (0.05–5 μg per μl of WCE) was added to the samples. However, because the amounts of heparin and tRNA which sharpened band 2 also inhibited splicing (Ref. 21 and data not shown), we did not use them in the assay because they could obscure physiologically relevant effects. Nonetheless, these data indicate that bands 2 and 4, but not band 3, were stable in the presence of a non-sequence-specific competitor. Judging by the effects of these polianons on the migration and amount of band 2, however, we think that there is at least one component of band 2 that is polianon-sensitive.

In conclusion, the binding of the wild type 2′-OMe oligos to the U2 snRNP was rapid, stimulated by ATP, and dependent on the integrity of the U2 snRNP. These are properties similar to prespliceosome formation on a pre-mRNA substrate. The binding was also sequence-specific; an oligo complementary to the 5′ end of U1 snRNA does not form band 2, and the mU2 oligos bound to the U2 snRNP were stable in the presence of polianons. However, unlike pre-mRNA, oligo mU2-wt binding was not affected by mutation U4A or A6C of the UACUAAC box but instead depended on the overall complementarity between the oligo and the U2 snRNA. Oligos mU2-dC and mU2-wt formed three additional bands when incubated in WCE. Neither band 1, 3, nor 4 depended on the integrity of the U1 or U2 snRNP nor comigrated with any specific snRNP. Oligo mU2-bp with only the UACUAAC box complementary to U2 snRNA formed bands 1 and 3 and two unique bands, x and y, none of which depended on U1 and U2. Additional experiments will be required to address the identities of bands 1, 3, 4, x, and y to determine whether these bands contain splicing factors.

The prp5-1 Mutation Mapped to the ATP-binding Domain of Prp5p—The DEAD box protein, Prp5p, has been shown to have ATPase activity (26) and to be required for prespliceosome formation (16). It was likely to be involved in the ATP stimulation of oligo binding to U2 snRNP. Previously, we showed that the Ts prp5-1 mutation inhibits prespliceosome formation in vitro (16); however, the identity of the prp5-1 mutation was not known. We therefore cloned the prp5-1 mutant allele by gap repair and sequenced it (see “Experimental Procedures”). The mutation is a single base change (G878A of the ORF) which would lead to the substitution glycine 293 with aspartate (G293D) in the protein. This substitution is 12 residues upstream of the glycine-lysine-threonine (GKT) triplet in the highly conserved, nucleotide-binding motif I within the putative helicase domain. This motif is important for ATP binding and hydrolysis in other DEXD/H box proteins (10).

In Vitro Heat Inactivation of prp5-1 Mutant Extract Decreased mU2-wt Oligo Binding to the U2 snRNP—To determine whether Prp5p is required for 2′-OMe oligo binding to the U2 snRNP, we made WCE from Ts prp5-1 mutant strains grown at the permissive temperature and inactivated the extracts in vitro by mild heat treatments. Both the heat-treated and untreated extracts were then incubated in glucose at 23 °C for 5 min to deplete endogenous ATP. Finally, the extracts were assayed for splicing activity (data not shown) and for oligo binding to the U2 snRNP in the presence and absence of added ATP (Fig. 5). In the active prp5-1 extract, the binding of mU2-wt oligo to U2 snRNP in the presence of added ATP was about 2-fold that observed in reactions depleted of ATP (Fig. 5, A and B). Heat inactivation of this extract had two effects as follows: it reduced mU2-wt binding to about one-third that in the active extract; and it eliminated stimulation by added ATP. Heat inactivation of prp5-1 mutant extracts prepared from different strains, one with a different genetic background (strains RL172) and the other of similar background (SRY5-1c) gave similar results (data not shown). In contrast, heating of a wild type extract (Fig. 5, A and B) had no effect on oligo binding except to deplete effectively the extract of ATP. Due to variations in endogenous ATP levels among extract preparations, the conditions sufficient to deplete the heat-treated and unheated prp5-1 WCE of ATP were not sufficient to deplete the unheated wild type WCE shown in Fig. 5A. Longer incubation times in glucose at 23 °C did, however, show that this unheated wild type WCE could be depleted of ATP and that added ATP would then stimulate oligo binding (data not shown). As another control for the heat treatments, we found that heat inactivation of the mutant prp2-1 protein, which blocks a later step in the splicing pathway (66), also did not affect oligo binding (data not shown). These results suggested that the reduced oligo binding in the heated prp5-1 mutant extracts was due to inactivation of mutant prp5-1p. However, the reduction in oligo binding could have occurred indirectly if loss of Prp5 activity rendered U2 snRNP more sensitive to nuclease activity in the extract, thereby reducing the levels of U2 snRNA.

To investigate whether or not the reduced binding was due to U2 snRNA degradation, we directly compared the amounts of oligo bound with the amounts of U2 snRNA in inactivated and active prp5-1 extracts with added ATP. Oligo binding was first measured with radiolabeled mU2-wt oligo (left panel in Fig. 5C). The snRNAs in the same gel were then assayed by Northern hybridization (middle and right panels in Fig. 5C). Band 2 levels in inactivated prp5-1 extract averaged 35% in 3 independent determinations (± 9.5%, S.D.) and were significantly less (p < 0.01 by Student’s t test) than levels in active extract. In contrast, U2 snRNA levels in the inactivated extract (85 ± 10%) were not significantly different from that (100%) in active extract. The other snRNAs also did not detectably change (right panel in Fig. 5C and data not shown). Similar analyses of treated and untreated prp2-1 and wild type extracts showed no significant reductions in the amounts of U2 snRNA (data not shown). Thus, the reduction in oligo binding in the heat-inactivated prp5-1 extract was due to inactivation of mutant prp5-1p and not due to a reduction in the amount of U2 snRNA. We conclude that Prp5p was required for mU2-wt binding to the U2 snRNP and the ATP enhancement of binding.

Prp5p Physically Associated with the U2 snRNP in the Presence or Absence of the Oligo or ATP—Results from this and previous studies (16, 25, 26, 29) suggest that Prp5p physically associates with the U2 snRNP. To test this, we constructed a gene encoding a GST-Prp5p fusion and replaced the wild type, essential PRP5 gene with this construct in yeast cells by plasmid shuffling (47). The GST-Prp5p fusion was expressed and functional in yeast cells (Fig. 6A). We noted, however, that the cells were also viable but grew slowly when the PRP5 ORF was fused out-of-frame to GST. Although little or no full-length GST-PRP5 fusion protein was detected in this strain (Fig. 6A), apparently enough Prp5p was produced for viability. Certain rare translational events noted for other yeast ORFS (67, 68) could account for this production of Prp5p.

In vitro affinity selections of the GST-Prp5p fusion protein were conducted to determine whether Prp5p associated with the U2 snRNP. Splicing reactions with or without added ATP and with extracts containing wild type Prp5p or GST-Prp5p but without exogenously added pre-mRNA or oligo were incubated

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2 T. Quan and S. Ruby, unpublished results.
at 0 or 23 °C. The reactions were then incubated on ice with glutathione-Sepharose to select for GST-Prp5p. The coselected RNAs were extracted and analyzed by primer extension assays using spliceosomal snRNA-specific probes. We found that U2 snRNA, but not the other spliceosomal snRNAs, coselected with GST-Prp5p (Fig. 6B). This enrichment was independent of ATP as there was no difference between ATP-depleted and ATP-containing extracts. We conclude that Prp5p physically associated with the U2 snRNP in the absence or presence of ATP or pre-mRNA.

To determine whether Prp5p associated with the mU2-wt oligo, we repeated the GST-Prp5p selection experiments but monitored the radiolabeled oligo added to reactions. The oligo was enriched in the selected material from the in-frame extract as compared with either the out-of-frame extract (Fig. 7A) or to an extract with wild type Prp5p (data not shown). The association was also usually enhanced about 2-fold by added ATP, similar to the ATP stimulation in the oligo binding assays. To test whether the association of the oligo with Prp5p depended on U2, we assayed reactions in which the U2 snRNP was first cleaved by deoxyoligo-directed RNase H. Ablation of U2 abolished association of mU2-wt oligo with Prp5p (Fig. 7B). Thus, most of association of Prp5p with the oligo required a functional U2 snRNP and was probably indirectly mediated via the U2 snRNP. These data also suggest that Prp5p is not directly binding to the UACUAAC box of the pre-mRNA.
The Conformation of U2 snRNP Was Important for mU2-wt Oligo Binding—Prp5p has been implicated in a conformational switch of two phylogenetically conserved U2 RNA structures because prp5-1 is synthetically lethal with the cold-sensitive (Cs) C62U mutation in U2 snRNA (16, 29). In vivo, C62U arrests mitotic cell growth at temperatures below 25 °C and shifts the equilibrium between two structures causing the bulk of U2 snRNA to assume the conserved complementary stem (CCS) at the expense of stem-loop IIA (Fig. 1) at both permissive and non-permissive temperatures (27, 28). In vitro, C62U mutant extracts have reduced rates of splicing and prespliceosome formation with increasing reductions occurring with increasingly low temperatures (from 25 to 12 °C) (28).

To investigate the role of U2 RNA structure on oligo binding, we assayed the effect of the C62U mutation on 2'-OMe oligo binding. Active splicing extracts were prepared from the previously characterized, isogenic wild type and Cs mutant strains (27, 28) grown at the permissive temperature and tested for splicing and mU2-wt binding at 23 and 15 °C in vitro (Fig. 8). We found that the C62U mutant extract had significantly reduced splicing (data not shown) and oligo binding to U2 snRNP (Fig. 8A) at 15 °C compared with 23 °C. In contrast, the wild type extract was nearly equally active for oligo binding and splicing at both temperatures (Fig. 8A). There were also two
differences in the mutant band 2 compared with wild type. The mutant band 2 migrated noticeably slower than the wild type at either 23 or 15°C. The amount of mutant band 2 was also somewhat lower than wild type at 23°C, consistent with the slightly lower rate of splicing in the mutant extract versus the wild type extract at 23°C as noted previously (28). However, the differences in band 2 migration and amounts seen here might be due to either the conformation or the amount of mutant U2 snRNP.

To test whether the reduced amount and migration of band 2 in the mutant extract was caused by the Cs U2 mutation and not by differences in the amount of mutant U2 snRNP or variability inherent in the assay, we assayed the wild type and mutant U2 snRNPs directly in a mock binding assay in which water was substituted for the oligo. The RNAs in the native gel of the mock assay were assayed by Northern analysis (Fig. 8C).

Probing for the other spliceosomal snRNAs in this experiment revealed three other differences between the mutant and wild type WCE, all of which involve the U1 snRNP. First, the bulk of mutant C62U U2 snRNP migrated more slowly during electrophoresis compared with the wild type at either temperature (left panel in Fig. 8C). Such a shift is indicative of a difference in U2 conformation between the wild type and mutant U2 snRNPs. Second, there was no decrease in the total amounts of mutant U2 snRNA in the reactions incubated at 15°C compared with 23°C. Third, there was a lower concentration of U2 snRNA in the mutant WCE compared with wild type WCE. As no reductions in U2 levels were previously observed in the C62U mutant in vivo (28), and all the spliceosomal snRNAs were lower in the mutant WCE prepared in this study (Fig. 8C), this lower mutant U2 concentration was introduced during extract preparation. As this lower concentration contributed to the lower amounts of mutant band 2 formed at 23°C, we could not assess any differences contributed by conformation at 23°C. Nevertheless, we could conclude that the reduction in oligo binding at 15°C compared with that at 23°C in the mutant extract was caused by the C62U mutation. Thus, the wild type U2 snRNP, which is predominantly in the stem-loop IIA conformation and more active for prespliceosome formation and splicing, bound the oligo more efficiently than the C62U mutant U2 which is mostly in the CCS form.

Probing for the other spliceosomal snRNAs in this experiment revealed three other differences between the mutant and wild type WCE, all of which involve the U1 snRNP. First, there
was a large reduction in form II of the U1 snRNP (Fig. 8C), and this form migrated more slowly in mutant versus wild type extract at both 15 and 23 °C. Second, both the mutant and wild type extracts accumulated form I (containing U1 and U5) at 23 °C, whereas only the wild type accumulated this band at 15 °C. Third, a form of U1 snRNP migrating between forms I and II was reduced in the mutant extract compared with wild type. This form may be the same as form III in other extracts (Fig. 2C) as it migrated with the same diffuse pattern, and we have noted variable migration of this form among different extracts. Although we do not understand the basis for these effects of the C62U mutation on the U1 snRNP, they do suggest an interaction between the U1 and U2 snRNPs.

**DISCUSSION**

Binding of pre-mRNA to the yeast U2 snRNP during prespliceosome formation requires ATP hydrolysis, the branch point region of the pre-mRNA including the highly conserved UACUAAC box, and several factors (1). Here we used a 17-nucleotide, 2'-OMe oligo encoding the UACUAAC box as a model in vitro assay for the binding of pre-mRNA to U2 in splicing extracts. The binding of 2'-OMe oligo to U2 was rapid, enhanced by ATP, and dependent on the integrity and conformation of the U2 snRNP. Thus, the binding of the 2'-OMe oligos to U2 snRNP relates in several aspects to prespliceosome formation on a pre-mRNA substrate. But as discussed below, we think that rather than exactly mimicking a pre-mRNA, 2'-OMe oligo binding is an indicator of the accessibility of U2 for pairing with pre-mRNA. Nonetheless, using this assay we have found that Prp5p activity was required for efficient binding of the oligo to the U2 snRNP. The prp5-1 mutation, which inhibited oligo binding, mapped to motif I, which constitutes part of the ATP binding and hydrolysis domain of the protein. Furthermore, Prp5p physically associated with the U2 snRNP in vitro. Our data indicate that the target of Prp5p is the U2 snRNP and suggest that the catalytic, ATPase activity of Prp5p is required to alter U2 snRNP conformation.

2'-OMe Oligo Binding as a Probe for U2 snRNP Conformation—Here we have shown that binding of the 2'-OMe oligo mU2-wt to form band 2 is specific to the U2 snRNP in three ways. The bound oligo co-migrated with the U2 snRNP in band 2 but not with the other spliceosomal snRNPs. Deoxyoligo-mediated RNase H cleavage of U2 RNA in the region that base pairs with the UACUAAC box abolished binding. Finally, reducing the number of possible base pairs between the oligo and U2 RNA from 16 to 6, as in the mU2-bp oligo, eliminated oligo binding. The inability of the mU2-bp oligo to form band 2 was somewhat surprising as this oligo still had an intact UACUAAC box but was nonetheless consistent with the binding of the mutant oligos mU2-4A and mU2-4A6C. These oligos had mutations in the UACUAAC box, but they would still form 15 bp with U2, and they bound as well as the wild type mU2-wt oligo. The same mutations in pre-mRNAs reduce spliceosome assembly by about 75% (21, 38, 69), but most pre-mRNAs usually have only the UACUAAC box and one or two flanking nts complementary to U2 RNA (70). Our data indicate that the UACUAAC box of an oligo was less important than its overall complementarity to U2, yet the small oligos can probe the accessibility of U2's BpIR for pairing with a UACUAAC box.

That the binding of the 2'-OMe oligo to U2 snRNP depends on the conformation of U2 is supported by the finding that the Cs C62U mutant form of U2 snRNP bound less oligo at 15 than at 23 °C. This finding directly correlates with previous results (28) that the mutant U2 formed the prespliceosome at a reduced rate at 15 °C. Furthermore, chemical probing showed previously (28) that most of the C62U mutant U2 is in the alternative RNA conformation, CCS, in vivo, consistent with the observation here that the bulk of the mutant U2 snRNP in WCE migrated more slowly than the wild type snRNP during native gel electrophoresis. Therefore, oligo binding is also indicative of the functional state of the U2 snRNP. This finding further underscores our assertion that oligo binding assays the accessibility of the U2 snRNP for pairing with the UACUAAC box. Additionally, we found that polyanions caused increased binding of a 2'-OMe oligo in the wild type U2 snRNP as well as increased mobility of the wild type snRNP during electrophoresis. These data suggest that there is protein, or a structure stabilized by protein, which normally occludes U2's BpIR. This protein may be removed by heparin or a competitor RNA or, as discussed below, by the action of Prp5p.

Our design of oligo mU2-wt was based in part on the sequence of a 25-nucleotide 2'-OMe oligo "b" found previously by Lamond et al. (36) to bind to the BpIR of the U2 in HeLa cell extracts. Although the two oligos bound similarly to U2, binding is stimulated by ATP more for oligo b than for mU2-wt. One possible explanation for this difference may lie in the sequences flanking UACUAAC. Oligo b could form 12, 5'-flanking bp extending into stem-loop IIA of U2, whereas oligo mU2-wt would form only 4 such base pairs. Two recent studies (71, 72) showed that nts 5' to the UACUAAC box confer ATP dependence to the binding of 2'-hydroxy (ribo-) oligos to U2 in HeLa extracts. Binding of an ~35-nt ribo-oligo was stimulated by ATP when the oligo had 6 or more upstream nts with 6 being the minimum number tested (72). Binding became strongly ATP-dependent when there were 18 or more upstream nts in either the ribo or deoxy form. Unlike oligos b and mU2-wt, however, the upstream region was not complementary to U2 snRNA and, in fact, could even be abasic. Furthermore, the ribo-oligos had a degenerate UACUAAC box sequence typical of mammalian pre-mRNAs as well as a polypyrimidine tract. We found that the level and ATP stimulation of mU2-dC binding equaled those of mU2-wt even though mU2-dC has five additional, noncomplementary 5' nts compared with mU2-wt. This suggests that the 5'-flanking sequences in the yeast system are not as important as in the mammalian one, but their role in the ATP effect in the yeast system needs further analysis.

Binding of the mU2-wt oligo did not depend on a functional U1 snRNP as ablation of the 5' end of U1 snRNA, which prevents U1 snRNP binding to the pre-mRNA and subsequent prespliceosome formation (59, 73), did not affect mU2-wt oligo binding. This findings as well as the lack of effect by UACUAAC box mutations on oligo binding indicates that prespliceosome formation on a pre-mRNA substrate requires more factors than does mU2-wt binding to U2. Two factors expected to be affected by UACUAAC mutations are the Bbp and Mud2 proteins. These proteins bind to the UACUAAC box and polypyrimidine tract of the pre-mRNA, respectively (17, 18, 74, 75). Binding of purified, recombinant Bbp to a small RNA is reduced from 20- to 100-fold (75) by the UACUAAC box mutations U4A and A6C, yet these same mutations had no effect on mU2-4A and mU2-6C oligo binding to U2 in cell extracts (Fig. 2). However, depletion of Bbp from either yeast (18) or human (76) cell extracts also has only mild effects on the kinetics of prespliceosome and spliceosome formation. Finally, the overall complementarity of the oligos such as mU2-4A, and the presence in WCE of Mud2p which binds cooperatively with Bbp (17), may override the effects of UACUAAC mutations on Bbp binding.

Prp5p Functions to Alter the U2 snRNP—The 2'-OMe oligo binding assay was also used to study Prp5p function. Previously we showed that heat inactivation of prp5-1 mutant extract in vitro inhibits prespliceosome formation (16). Here heat inactivation of this mutant extract resulted in two changes. It
reduced oligo binding to about one-third that of active extract, and it removed the ATP enhancement. Thus Prp5p is required for efficient mU2-wt binding to U2 snRNP and for ATP stimulation of this binding. However, the possibility that inactivation of the prp5-1 mutant protein reduced activity of another ATPase or ATP-binding protein cannot be ruled out. Nonetheless, in a similar study, complementing inactivated prp5-1 mutant extract with recombinant, purified Prp5p, restored ATP-stimulated, deoxyoligo RNase H degradation of U2 (25).

Although we have not shown here whether the stimulation by ATP is due to its binding or hydrolysis, the nature of the prp5-1 mutation suggests that the catalytic, ATPase activity of Prp5p is required for the stimulation. The mutation is predicted here to result in a Gly-293 → Asp substitution in motif I. Genetic (77, 78) and structural (79–81) studies of superfamily two (SF2) helicases similar to Prp5p indicate that motif I is important in ATP binding and hydrolysis. The Gly-293 residue of Prp5p is predicted to be in a similar position in the three-dimensional SF2 helicase structure as tyrosine 386 in Prp16p, another splicesomal DEAD-box protein (82). Substitution of this tyrosine with aspartate in Prp16p decreases the rate of ATP hydrolysis (83), so it is likely that the prp5-1 mutation alters ATP hydrolysis.

This study has also shown that Prp5p physically associates with the U2 snRNP in the absence of pre-mRNA or 2′-OmE oligo. This association is phylogenetically conserved as the recent purification and analysis of the human Prp5 protein also find Prp5p to be a U2 snRNP component. Furthermore, the ATPase activity of the Prp5p is stimulated by U2 snRNA (26). The combined results of our study and previous studies (16, 25, 26, 29) support the conclusion that Prp5p catalyzes a conformational change in the U2 snRNP which makes the BpIR of the U2 snRNP more accessible for pairing with the pre-mRNA during prespliceosome formation.

What is the target of the catalytic activity of Prp5p within the U2 snRNP? It is tempting to think that Prp5p acts as an RNase to remove Cus2p because removal of Cus2p by genetic deletion allows formation of a functional prespliceosome in the absence of ATP (84). However, this formation occurs at a reduced rate that can still be stimulated by ATP. Furthermore, it also still requires some function of Prp5p as inactivating prp5-1p in an extract without Cus2p prevents prespliceosome formation (84). Something else is being acted upon in an ATP- and Prp5p-dependent manner in the absence of Cus2p. If Prp5p is the catalyst, then it is acting on a target not completely abrogated by the loss of Cus2p. This target may be the U2 snRNA itself, another protein, or both U2 RNA and protein.

There are several proteins that are part of the U2 snRNP or required for prespliceosome formation which could be targets. Prp9p, an SF3 subunit, has been suggested previously (25) to be the target of Prp5p because SRU2 deoxyoligo-targeted degradation is stimulated by inactivation of the prp9 mutant protein. Indeed, any one of the several yeast SF3 subunits in addition to Prp9p is a likely target because of the genetic interactions of SF3 with Prp5p (16, 29, 33, 35, 85). Biochemical data also suggest that SF3 could be the target; the BpIR in purified human U2 snRNP is accessible to micrococcal nuclease in the absence but not the presence of SF3 (34). Two other proteins, Bbp and Mud2p, are released from the pre-mRNA during prespliceosome formation (18); however, this removal is likely catalyzed by another DEAD-box helicase, Sub2p (13). Interestingly the essential requirement for Sub2p in vivo can be bypassed by deleting MUD2, yet ATP is still required for prespliceosome formation in sub2 mud2 deletion mutant extracts (13). This suggests that the ATPase activity of the Prp5p is necessary for prespliceosome formation in the absence of Sub2p and Mud2p and is consistent with our observation that most ATP stimulation in oligo binding was removed during inactivation of prp5-1p.

One of three U2 snRNA structures is also a likely Prp5p target. Because the prp5-1 mutation is synthetically lethal with a specific subset of U2 mutations that alter stem-loop IIA (16, 29), a popular hypothesis is that Prp5p switches the two mutually exclusive, phylogenetically conserved conformations, stem-loop IIA and the competing CCS (Fig. 1). One such U2 mutation, C62U, causes the bulk of U2 RNA to assume the CCS form and, at low temperatures, imposes a rate-limiting step in prespliceosome formation (28). As shown here, it also reduces oligo binding at low temperatures. A simple explanation of these genetic and biochemical data is that normally Prp5p converts CCS to stem-loop IIA to activate the U2 snRNP for prespliceosome formation. As the C62U mutation destabilizes form IIA rather than hyperstabilizes CCS (28), the synthetic lethality of C62U with prp5-1 would be due to an excess of CCS exacerbating the putative lowered activity of mutant prp5-1p. An alternative explanation for the synthetic lethality is that C62U indirectly affects Prp5p activity because it directly alters interactions of SF3, Cus2p, or both with U2 RNA. In support of this idea, SF3b, a multimeric component of SF3, binds to the 5′ region of the human U2 (34), and yeast SF3b interacts genetically with Prp5p (33, 86). Additionally, some cys2 mutations suppress C62U by binding to an RNA (most likely U2), prevent accumulation of CCS, and interact genetically with mutations in SF3b or Prp5p (35). A third alternative, but not mutually exclusive, explanation for the synthetic lethality may lie in an interaction between the U1 and U2 snRNPs as the U2 C62U mutation also affects the migration and form of the U1 snRNP (Fig. 8C). This effect on U1 is probably protein-mediated as no direct contacts between the U1 and U2 RNAs have been detected in several studies (2), but a complex containing the U1 and U2 snRNPs in the absence of pre-mRNA has been observed (87).

The third possible RNA target for Prp5p as previously suggested (25) is a stem formed by the pairing of phylogenetically invariant nts 25–30 with nts 42–47 flanking either side of the BpIR of U2 (Fig. 1) (30). This possibility is supported by synthetic lethal interactions between mutations in some of these flanking nts and PRP5 (30). It is attractive because the unwinding of this stem in the prespliceosome would also free some of the U2 nts for subsequent pairing with U6. Some of these U2-U6 pairings are essential for eventual splicing catalysis (2). The use of the oligo binding assay should further delineate the target of the Prp5p within the U2 snRNP and the relationship of Prp5 with Cus2p, SF3, and U2 RNA structure.

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